Identification and Characterization of Novel Players in the Establishment of Receptor Polarity in Yeast

BY

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THESIS

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This thesis is dedicated to my family and friends
for all their unwavering support through the
years to help me complete my thesis

It always seems impossible until it’s done.

- Nelson Mandela
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List of abbreviations

µ                Micron
2µ               2 Micron
aF               MATa pheromone Factor
AS               Analog sensitive
BiFC            BiMolecular Fluorescent Complementation
CCR5            C-C Chemokine Receptor 5
CDC28           Cell Division Cycle 28
CEN             CENtromeric
CHO1            CHOline requiring 1
CTD             C-terminal domain
DCV1            Dependent on Cdc28 for cell Viability
DsRed           Discosoma sp. Red fluorescent protein
DTT             DiThioThreitol
ER              Endoplasmic Reticulum
ERG6            ERGosterol biosynthesis
FIG1            Factor-Induced Gene
FUS3            cell FUSion 3
GABA            Gamma-AminoButyric Acid
GABARS          Gamma-AminoButyric Acid Receptors
GAL             Galactose
GAP             GTPase Activating Protein
GEF             Guanine nucleotide Exchange Factor
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>Gα</td>
<td>G protein α subunit encoded by GPA1</td>
</tr>
<tr>
<td>Gβ</td>
<td>G protein β subunit encoded by STE4</td>
</tr>
<tr>
<td>Gγ</td>
<td>G protein γ subunit encoded by STE18</td>
</tr>
<tr>
<td>HACS</td>
<td>High Affinity Calcium Influx</td>
</tr>
<tr>
<td>INT</td>
<td>INTEGRATIVE</td>
</tr>
<tr>
<td>K-P</td>
<td>Pottasium phosphate</td>
</tr>
<tr>
<td>LACS</td>
<td>Low Affinity Calcium Influx</td>
</tr>
<tr>
<td>LACT-C2</td>
<td>C2 domain of Lactadherin</td>
</tr>
<tr>
<td>LEGI</td>
<td>Local Excitation Global Inhibition</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MAT</td>
<td>MATing type locus</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic Acid</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 Activated Kinase</td>
</tr>
<tr>
<td>PB</td>
<td>Processing bodies</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidyl Ethanolamine</td>
</tr>
<tr>
<td>PCL1</td>
<td>Pho85 CycLin 1</td>
</tr>
<tr>
<td>PCL2</td>
<td>Pho85 CycLin 1</td>
</tr>
<tr>
<td>PDE1</td>
<td>PhosphoDiEsterase 1</td>
</tr>
<tr>
<td>PH</td>
<td>Plekstrin Homology</td>
</tr>
<tr>
<td>Pho85</td>
<td>PHOSphate metabolism 85</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PH&lt;sup&gt;PLCδ&lt;/sup&gt;</td>
<td>PH domain of PCLδ protein</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidyl-Inositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidyl-inositol 3-Kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidyl-Inositol (4,5)-bisPhosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidyl-Inositol (3,4,5)-bisPhosphate</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>PS</td>
<td>PhosphatidylSerine</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphate and TENsin homologue</td>
</tr>
<tr>
<td>RAC</td>
<td>Subfamily of Rho GTPases</td>
</tr>
<tr>
<td>RAS</td>
<td>Rat Sarcoma superfamily of small GTPases</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulators of G-protein Signaling</td>
</tr>
<tr>
<td>RHO</td>
<td>Subfamily of Ras GTPases</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinases</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>STE2</td>
<td>STErile 2, <em>MATα</em> pheromone receptor</td>
</tr>
<tr>
<td>STE3</td>
<td>STErile 3, <em>MATα</em> pheromone receptor</td>
</tr>
<tr>
<td>VF</td>
<td>Venus Fragment</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich Syndrome Protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>Wasp-family Verpolin homologous protein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>YCK</td>
<td>Yeast Casein Kinase</td>
</tr>
</tbody>
</table>
ZO  Zona Occludens

αF  $MAT_α$ pheromone Factor

Δ  Deletion
Summary

All cells have the ability to sense and respond to their extra cellular environment. Many cells are able generate a directional response to an external chemical gradient. Directed cell growth is called chemotropism, and the related process, directed cell migration is called chemotaxis. Chemotropism and chemotaxis play important roles in various biological processes. These include embryonic development, neutrophil migration, cancer metastasis, and angiogenesis. Chemotropic and chemotactic cells exhibit a remarkable ability to interpret gradients and sense the direction of the source. The formation of mating projections (shmoos) by S. cerevisiae is a chemotropic response. In yeast, the two haploid mating types, MATa and MATα, sense the pheromone secreted by the opposite mating type, polarize their growth towards the closest mating partner, and fuse to form diploids. How do these cells sense direction? In MATa cells, the Ste2 pheromone receptor is the primary gradient sensor. Ste2 is uniformly distributed on the plasma membrane (PM) in vegetative cells, but upon ligand binding, it is rapidly internalized. It subsequently reappears as a polarized crescent that coincides with the incipient shmoo site. In mating mixtures, the newly emerged receptor crescents orient towards the closest mating partner. Although actin-dependent directed secretion stabilizes and amplifies receptor polarity, I have shown that receptor polarization precedes the polarization of actin cables and occurs in the absence of actin-directed secretion. In contrast, internalization of the receptor is essential for its polarization. In this thesis, I have investigated how receptor polarity is established and identified novel players in this process.

In a directed genetic screen, I found that deletion of the genes DCV1, PCL1, FIG1 or PDE1 conferred a significant defect in receptor polarization. Upon further characterization, I discovered only the DCV1 and PCL1 deletions affected receptor polarization upstream and
independent of actin-directed secretion. Unlike wild type (WT) cells, \textit{dcv1}\Delta and \textit{pcl1}\Delta cells were unable to establish a receptor polarization site that could be amplified independently of polymerized actin. These results suggested that Dcv1 and Pcl1 are required for the establishment of receptor polarity. Furthermore, \textit{dcv1}\Delta and \textit{pcl1}\Delta also conferred in defects in chemotropism. For these reasons, I further characterized the roles of Dcv1 and Pcl1 in the polarization of the receptor.

Dcv1 is predicted to be a membrane protein and belongs to the family of Claudins. Immuno-fluorescence (IF) microscopy in cells expressing Dcv1-HA suggested that the protein localizes uniformly to the PM in vegetative cells, but localizes away from the mating projection in pheromone-treated cells. Dcv1 tagged with CFP confirmed the IF results. Furthermore, Dcv1-CFP appears to localize as a ring at the base of incipient and early shmoos. This result is consistent with the observation that claudins function by acting as barriers and/or by forming membrane domains. \textit{dcv1}\Delta cells also exhibit abnormal localization of various lipids in the PM. These include phosphatidylinositol, phosphatidylserine and sterols. Lipidomic studies using mass spectrometry suggest differences in the composition of the PM in vegetatively-growing and pheromone-responding WT and \textit{dcv1}\Delta cells. Consistent with its effect on receptor polarization, \textit{dcv1}\Delta conferred a directional sensing defect in unilateral mating assays. In summary, our data strongly suggest that a gene of previously unknown function plays a role in receptor polarization and directional sensing. Dcv1 may regulate the polarization of the yeast pheromone receptor by altering the membrane lipid composition and/or forming a barrier on the PM that delineates the front and back of the cell.

I next characterized the role of Pcl1 in the polarization of the receptor. Pcl1 is one of the 10 cyclins for the cyclin-dependent kinase (CDK) Pho85. In contrast to the \textit{pcl1}\Delta phenotype,
over-expression of Pcl1 caused the receptor to polarize to a very narrow region in response to pheromone. Pcl1 over-expression also greatly increased sensitivity to pheromone as visualized by halo assays and frequency of shmoo formation. Furthermore, slight over-expression of Gβ in pcl1Δ cells caused a moderate increase in pheromone sensitivity. Given this Gβ-Pcl1 genetic interaction, and that Gβ is has a putative Pho85/Pcl1 phosphorylation motif, I hypothesized that Gβ is a substrate of Pho85/Pcl1. Consistent with this, Pcl1 interacted with Gβ in a bimolecular fluorescence complementation assay. I also showed that pcl1Δ conferred an orientation defect in mating assays, consistent with its effect on receptor polarization. In summary, our data strongly suggest that the cyclin Pcl1 and the CDK Pho85, previously implicated in cell polarization during vegetative growth, also play a role in GPCR polarization and directional sensing.

In conclusion, I propose that Dcv1 and Pcl1 promote receptor polarization by two distinct mechanisms. First, the claudin Dcv1 appears to contribute to the formation of PM domains or membrane rafts. Until recently, the formation of membrane domains and their role in cell polarity has been under appreciated. However, membrane rafts are emerging as an integral part of cell polarity and signaling in migrating cells. Although it is known that misregulation of claudins can lead to loss of cell-cell contact, loss of barrier formation, and metastasis of cancer cells, claudins have never been directly implicated in directional migration. To the best of our knowledge, our results implicate a claudin for the first time in both facilitating the polarization of a GPCR and chemotropism. Second, I have found a novel role for the cyclin Pcl1 and its kinase Pho85. Our work demonstrates that Gβ is a potential substrate of the Pho85/Pcl1 kinase complex. Phosphorylation of Gβ has been shown to be critical for receptor polarization and gradient sensing in yeast. Although Pho85 is known to play diverse roles in the regulation of
cellular responses to environmental stimuli such as nutrition, I propose that it is also important
for eliciting an appropriate response to pheromone stimulation.
I. Introduction

a. Significance of chemotaxis and chemotropism

Sensing extracellular environments is crucial to all cell types. Many cells are able to sense an external chemical gradient and generate responses such as directed growth and movement. Directed movement, also known as chemotaxis, is cell migration in response to an external chemical gradient. For example, neutrophils rely on chemotaxis to hunt for the bacterial prey and *Dictyostelium discoideum* amoebae chemotax to search for food or to aggregate into multi-cellular masses under starvation conditions [1]. In mammals, chemotaxis plays an important role in embryogenesis and it is critical in the development of tissues and organs, and the vascular and nervous systems. Chemotaxis is also known to play important roles in immune and inflammatory responses, and in metastasis of cancer cells [2].

A phenomenon related to chemotaxis is chemotropism, directed cell growth in response to a chemical gradient. Chemotropism is fundamental to cellular processes such as axon guidance, pollen tube formation, angiogenesis, and fungal infections. In order to form a functional nervous system, neuronal axons must connect with their targets [3]. In *Xenopus* spinal neurons, it has been shown that cAMP level determines polarity in response to specific neutrophins [4]. Many fungal species also exhibit chemotropism during mating. The availability of powerful genetic tools has made *Saccharomyces cerevisiae* mating the most extensively studied model for chemotropism and cell polarization in response to an external cue. The two haploid mating types of the yeast *S. cerevisiae* chemotrop to fuse and form diploid cells. Clearly, the two related phenomena, chemotaxis and chemotropism play important roles in numerous cellular processes that are fundamental to life and hence are of great interest to molecular and developmental biologists.
All eukaryotic chemotactic and chemotropic cells have to overcome a common set of challenges. They must be able to: i) accurately sense the gradient; ii) orient their axis of polarity towards the gradient source; iii) move (chemotaxis) or grow (chemotropism) up the gradient and toward their target; iv) continually track and respond to changes in gradient intensity and direction.

Both chemotroping and chemotaxing cells exhibit a remarkable ability to orient in a wide range of gradient slopes and ligand concentrations. Based on studies of model systems, it has been estimated that a 0.5-10% difference in the chemoattractant across the cell is sufficient to elicit a robust response [5]–[8]. However, it is not known how cells convert shallow external gradients into much steeper internal gradients. To sense direction, cells must be able to amplify the shallow external chemical gradient into a steep intracellular signaling gradient. As cells move up the chemical gradient, the absolute concentration of ligand increases several fold. Hence, the mechanisms that underlie directional sensing must respond to gradient slope rather than absolute ligand concentration.

b. **Models for directional sensing in chemotaxis**

Most studies of directional sensing during chemotaxis are of the neutrophil and *Dictyostelium* model systems. Neutrophils hunt for bacteria in the blood stream, whereas *Dictyostelium* perform a similar task in the soil in search of food. *Dictyostelium* also chemotax when the cells are starved and form multi-cellular aggregates [1]. Neutrophils and the amoebae *Dictyostelium* share many characteristics during their chemotactic response.
i. **Neutrophils and amoebae *Dictyostelium discoideum***

In most known models for chemotaxis, the chemical gradient is perceived by a G-protein coupled receptor (GPCR). In *Dictyostelium*, the cAMP signal is recognized by a seven transmembrane GPCR cAR-1 (cAMP receptor-1), which is coupled to a heterotrimeric G-protein, Ga2βγ. Neutrophils can respond to various chemoattractants, such as fMLP, C5a, and chemokines [9], [10]. In the best understood models of chemotactic cells, including neutrophils and *Dictyostelium*, the receptor and its G-protein remain uniformly distributed on the plasma membrane before and after stimulation [11]. Moreover, receptor occupancy and G-protein activation correlates with the external gradient. Hence the signal does not seem to be amplified at this level. On the other hand, phosphatidylinositol 3,4,5 phosphate (PIP3) forms steep intracellular gradients on the plasma membrane polarized on the up-gradient side of the cell [12], [13], and this is thought to mark the site for pseudopod formation. Both Neutrophils and Dictyostelium turn off the response by a process called adaptation, when they do not perceive a gradient or the when the receptor occupancy is held constant. Although most studies claim that the GPCR does not polarize, receptor polarization has been observed in some cell types. In Leukocytes, the chemokine receptors CCR2 and CCR5 are polarized to the leading edge and might act as a sensor mechanism for directed migration [14]. However, it is not yet fully understood how cells are able to sense the direction and accurately choose a growth site.

The binding of the receptor to its ligand activates the receptor and results in the disassociation and activation of the associated G-protein. When differentiated Dictyostelium are exposed to the ligand, cAMP, the receptor cAR1 is activated which results in the activation of Ga2 and Gβγ subunits [11], [15]. These events are followed by phosphorylation of cAR1 C-terminal tail, activation of the MAPK Erk2, and an influx of calcium [16], [17]. Other transient
responses have also been observed, including the activation of adenylyl cyclase ACA, the soluble guanylyl cyclase sGC, and the membrane bound guanylyl cyclase GCA. Upregulation of these enzymes results in production of cAMP and cGMP [18], [19]. Free Gβγ recruits the phosphotidyl inositol 3 kinase (PI3K), which catalyzes the phosphorylation of phosphotidyl inositol 4,5 phosphate (PIP2) to PIP3. Gβγ also activates the Rho GTPases, Cdc42 and Rac. PI3K activity has also been linked to activation of Rac and Cdc42 [20], [21]. Rac-GTP promotes the accumulation of PIP3 which in turn causes further activation of Rac-GTP, thus forming a positive feedback loop [22] that leads to the local concentration of PIP3. Furthermore, PIP3 also interacts with and activates Rac/Cdc42 effectors like WAVE and WASP that link upstream signals to actin polymerization. Polarized accumulation of active PIP3 leads to the formation of pseudopodia. A pseudopod is the growing or leading edge of chemotaxing cell. The pseudopod formation is thought to be mediated by activated Rac and dependent on actin. At the back of the cell, which is also known as the lagging edge, PIP3 activation is inhibited by the phosphatase and tensin homologue (PTEN) phosphatase, and the simultaneous delocalization of PTEN phosphatase at the leading edge of the cell is essential for PIP3 gradient formation [23], [24]. PIP2 is observed at the rear of the cell, where it may recruit important cytoskeletal elements that help define the frontness and backness of a chemotaxing cell, dictating where the cell should form a pseudopod and where it should retract.

**Mechanisms for gradient sensing:** Activation of receptor signaling and G-proteins, results in activation of the signaling mediated by the small GTPase Ras. Preventing Ras activation affects directional sensing [25]–[27]. Furthermore, Ras activation has been shown to be important for PI3K activation. PI3K is crucial for directional sensing, especially in shallow gradients [28], [29]. However, cells lacking PI3K can still respond quite well in steep gradients,
suggesting an alternate pathway for directional sensing in chemotaxing cells. It was later demonstrated that when both the PI3K and phospholipase A2 (PLA2) pathways were inhibited, the cells exhibited dramatic defects in directional sensing and chemotaxis. This suggested that the PLA2 was a component of the alternate pathway [29], [30].

Studies have shown that in the absence of f-actin, cells can polarize PI3K, PIP3, and PTEN [31], and sense direction [32]. Polarized growth is not a prerequisite for directional sensing. The most widely accepted directional sensing models usually include positive feedback loops wherein signaling molecules are selectively amplified at the leading edge. In neutrophils, for example, it has been shown that a Rac mediated PI3K accumulation is involved in a positive feedback loop. Furthermore a positive feedback loop has also been postulated between PIP3 accumulation and actin [33], [34]. Although it is widely accepted that positive feedbacks must be involved, the mechanisms that are required for initiating polarity are not yet clearly understood.

The cells may interpret the gradient by sensing differences in receptor occupancy over time (called temporal sensing) and/or over the length of the cell (called spatial sensing). In eukaryotic chemotaxis, the spatial mechanism for gradient sensing seems to be predominant: cells compare receptor occupancy over the cell surface and amplify the difference to choose a growth site. The most widely accepted model for gradient sensing involves a local activator and a global inhibitor, known as the local excitation global inhibition (LEGI) model. The LEGI model predicts a localized production or recruitment of a molecule in conjunction with a global degradation or inhibition of the same molecule which would result in the amplification of the intracellular gradient [35]–[37]. The kinetics between the activator and inhibitor differ, and the excitation is rapid relative to the inhibition [31], [32]. The steady-state level of either process must in turn be linked to receptor occupancy, which allows for the cells to respond to dynamic
gradients. In *Dictyostelium* or neutrophil cells responding to either uniform or a gradient of chemoattractant, LEGI model correlates well with the responses observed for the major proteins involved in signaling and polarization. There are many molecules that fit the description of an activator — e.g., PI3K and PIP3. However, the LEGI model predicts the presence of an inhibitor. Hence, numerous studies have focused on identifying one such inhibitor. Some studies propose PTEN as the inhibitor. Although there is strong evidence for the existence of an inhibitor, the identity of such molecule remains unknown.

In some cases, cells are also able to utilize a temporal mechanism for directional sensing in which the cell compares receptor occupancy over time. In this model, the cell extends pseudopods in random directions first. When a pseudopod is extended in the right direction it experiences positive forces that stabilizes the pseudopod and promotes the migration of the cell in that direction. However, negative forces are experienced when pseudopod extension is in the wrong direction and extinction of that pseudopod results. The advantage of a temporal mechanism is that relatively fewer molecules are sufficient for chemotaxis. Some bacterial species are known to use a temporal mechanism to chemotax [38], [39]. In eukaryotic chemotaxis, spatial mechanisms may play a role in gradient sensing rather than temporal mechanisms.

**Cell polarization:** Cell polarization occurs in two stages. First, a polarized localization of signaling molecules and proteins at the leading or lagging edge is observed. Second, the cell undergoes morphological changes such as extending a pseudopod, which is essential for chemotaxis and directional persistence. Cells are capable of polarizing the signaling molecules even when they can’t detect a gradient, although the site of polarization is random.
Many proteins have been demonstrated to polarize to the leading edge of a chemotaxing cell. For example, polarization of PI3K and PIP3 at the leading edge promotes the localization of PH domain containing effectors at the front. Actin and actin binding proteins also localize to the front. Certain other proteins are restricted to the lagging edge, like PTEN as described previously. The polarization of opposing activities, PI3K to the leading edge and PTEN to the lagging edge results in accumulation of PIP3 at the leading edge which is thought to facilitate actin polymerization by recruiting Dock180 [13], [40] and Myosin II. The actin binding protein cortexilin also localizes to the lagging edge. Myosin II is responsible for retracting the lagging edge and this process is regulated by Rho and calcium signaling. Myosin II localization at the lagging edge is especially important for inhibiting pseudopod formation at the back [41], [42]. p21 activated protein kinase A (PakA) also localizes to the lagging edge and prevents myosin II disassociation. The only known integral membrane protein to localize to the lagging edge of migrating Dictyostelium is ACA. It has been suggested that ACA results in extracellular cAMP secretion which acts as a signal for neighboring cells and promotes the collective head-to-tail migration of Dictyostelium cells [43]. Polarized distribution of these molecules to either the leading or lagging edge is critical for cell polarization and chemotaxis.

Apart from activating PI3K, Gβγ also activates the p21-activated kinase PAK1 [44] bound to PIXα. PIXα is a guanine nucleotide exchange factor (GEF) that activates Cdc42. Cdc42 in turn activates PAK1, thus forming a second positive feedback loop. Cdc42 and PAK1 polymerize actin cables towards the leading edge of the cell and also play a role in excluding PTEN from the leading edge. Even though polarization of actin is essential for maintenance of the polarized state and for cell migration in most eukaryotes, it is not essential for establishment of polarity and directional sensing [13], [45], [46]. Directional sensing depends on Gβγ through
activation of both the PI3K and PAK-Cdc42 pathways. It was recently demonstrated that anisotropy in the active αi and βγ subunits is sufficient to determine the direction of migration [47].

**Summary:** As outlined in the models described above, much is known about the polarization machinery. In contrast, relatively little is understood about how cells interpret external chemical gradients. Although the established models of chemotaxis provide some mechanistic insight into gradient sensing, they do not fully account for the rapid and accurate response of chemotaxing cells in very complex and dynamic gradients. Understanding how cells navigate in situ is a key challenge to the field.

ii. **Axon growth cone guidance**

During the development of nervous system in an embryo, neurons must extend their axons to reach their respective target areas to form synapses. This process is called pathfinding. Growth cone refers to the highly motile tip of a growing axon that facilitates axon guidance during pathfinding. It has long been known that the growth cone movement is guided by a variety of environmental cues such as netrins, semaphorins, slits, neutrophins, classical morphogens, neurotransmitters and extracellular matrix molecules (reviewed in [48], [49]), and GABA in vitro [50]. Axon growth cone guidance has characteristics common to both chemotaxis and chemotropism. It has been estimated that growth cones can interpret gradients as shallow as 0.1% across its surface [51]. They can extend the lamellipodia up the gradient in an actin-dependent manner and retract the filopodia in a microtubule-dependent manner with changing gradients. Much like neutrophils and *Dictyostelium*, growth cones also might employ a LEGI mechanism to steepen the external gradient [52]. Furthermore, similar to neutrophils and Dictyostelium, growth cones also adapt to a baseline concentration of guidance cues. However,
Unlike the rapidly changing gradients experienced by neutrophils and *Dictyostelium*, growth cones are presented with more stable gradients like the ones experienced by a chemotroping cell. Therefore, the response time is in minutes compared to a response time of seconds observed in neutrophils and *Dictyostelium*. Hence it is likely that some of the mechanisms employed in the decision making process are different. For example, neutrophils and *Dictyostelium* do not polarize the receptors but polarize signaling molecules during directional sensing. In contrast, in response to the chemo-attractant GABA, growth cones polarize GABA_A receptors (GABA_ARs) toward the up gradient in a microtubule and calcium dependent manner [53]. Receptor polarization is a property they share with chemotroping yeast cells.

The GABA_ARs are clustered into lipid rafts by a mechanism that requires microtubules and the polarized redistribution of the GABA_ARs promotes intracellular calcium gradients that are critical for growth cone guidance [54], [55]. Asymmetric receptor distribution could facilitate the asymmetric distribution or activation of other signaling proteins, which would in turn reinforce polarization of the receptor, resulting in a positive feedback loop that steepens the intracellular signaling gradient and contributes to directional sensing [53]. In stable gradients, growth cones may polarize the receptor during the directional sensing phase to produce a filtering mechanism that makes gradient amplification resistant to rapid external fluctuations and leads to accuracy during neuronal wiring [53], [56]. In Xenopus neurons, the receptor is rapidly and globally internalized when stimulated with a low-dose of Netrin-1 or Sema3A. Resensitization with a higher dose is achieved via receptor recycling to the membrane and local protein synthesis [57]. Furthermore, receptor endocytosis might act as a mechanism for global inhibition and downstream signaling is restricted only to a region where activated receptor concentration exceeds a minimal threshold.
Although growth cone motility is also driven by cytoskeletal dynamics and substrate adhesion, the mechanisms are slightly different from those used by neutrophils and *Dictyostelium*. The extension of a pseudopod followed by the retraction of the uropod is the driving force of motility in neutrophils and *Dictyostelium*. On the other hand, growth cones have specialized structures called filopodia, which are finger like projections from the pseudopod. In growth cones, the filopodium is the primary structure that encounters the gradient. The cytoskeletal dynamics underlying growth cone mobility are controlled by actin regulatory proteins such as Arp2/3, Fascin, N-WASP, Cofilin, profilin and myosin [58]. Growth cone movement is followed by the consolidation of the lagging edge into a stabilized axon. This event is regulated by myosin II-driven actin contraction at the lagging edge, stress fiber formation [59] and microtubule bundling by Doublecortin [60].

Contrary to what is observed in neutrophils and *Dictyostelium*, growth cones have an intrinsic basal front-back polarity. In the absence of a guidance cue, the growth cone moves along a straight path while stabilizing the axon behind it. However, in the presence of a guidance cue, these cells become highly polarized. Similar to what is observed in neutrophils and *Dictyostelium*, there is a set of proteins that localizes to the leading edge and a set that are at the lagging edge. These proteins are thought to define the front and the back of the cell. PI3K, Rac, Cdc42 and actin regulating proteins polarize to the leading edge. On the other hand, ROCK, Myosin II, PTEN and RhoA localize to the lagging edge. Furthermore, the microtubulin bundling protein doublecortin is also localized at the lagging edge. There is also evidence for asymmetric mRNA delivery and translation which has been shown to be important for growth cone guidance [61], [62]. It has also been shown that endocytosis and recycling of components from the
central zone of a growth cone to leading edge is critical for axon extension. This allows for the conversion of the rear of the growth cone into a stable axonal segment.

Growth cones can also develop a right-left polarity that results in a turn in response to a change in the gradient. The turning of growth cones is also a directional process. Local protein synthesis seems to be key for many growth cone events including growth cone turning [63]–[65]. Finally, a growth cone must collapse the side furthest from the turn to be able to complete the turn [59].

Similar to the gradients experienced by Dictyostelium, growth cones also encounter gradients of varying degree of steepness and over a long range. Although not many studies have compared growth cone guidance in shallow and steep gradients, it is likely that these cells employ a similar mechanism to neutrophils and Dictyostelium and many parallels can be drawn between these systems. However, how the cells convert the shallow external signal into a steep intracellular signal is still under investigation.

iii. *S. cerevisiae* mating pathway

Many fungal species produce unique diffusible peptides that act as chemoattractants during mating. *S. cerevisiae* can exist as either haploid or diploid cells. The haploid cells grow towards a mating partner to fuse and form diploids. There are two haploid mating types, *MATa* and *MATa*, both of which secrete a peptide pheromone which acts as a ligand for the GPCR expressed in the opposite mating type. *MATa* cells secrete a-factor (aF) which is bound by Ste3 expressed on the surface of *MATa*; *MATa* cells secrete alpha-factor (αF) which is the ligand for Ste2 expressed on the surface of *MATa* cells. The GPCR becomes activated upon binding to its ligand, which in turn leads to activation of the cognate heterotrimeric G proteins. Free Gβγ then transmits the signal to the nucleus via a MAP kinase (MAPK) pathway leading to activation of
mating-specific genes, pheromone-induced cell cycle arrest, polarized growth toward the mating partner, and cell fusion with the opposite mating type to form MATa/α diploids (Fig. 1).

Chemotropic *S. cerevisiae* and chemotactic cells both rely on GPCRs to bind chemoattractant, and like chemotactic cells, *S. cerevisiae* exhibits a remarkable ability to interpret shallow gradients. However, in *S. cerevisiae* GPCR and the G-protein polarize toward the source of pheromone upon gradient stimulation, unlike chemotactic cells, which generally maintain a uniform distribution of surface receptor.
**Figure 1: Chemotropism in *S. cerevisiae***

Haploid yeast exists as two mating types \(MAT^a\) and \(MAT^a\). They can reproduce asexually through budding or sexually through mating. The two mating types \(MAT^a\) and \(MAT^a\) constitutively secrete pheromones that act as a signal for cells of the opposite type. Pheromone binding to receptors expressed on the surface of opposite mating type initiates a series of intracellular events that result in activation of MAPK signaling, cell cycle arrest, and polarized growth toward the closest mating partner. The final steps are cell and nuclear fusion to form \(MAT^a/a\) diploids.
iv. **Signal transduction to the nucleus**

The yeast GPCR (Ste2) expressed by $MAT^a$ cells is activated upon binding to the pheromone ($\alpha F$) secreted by $MAT^a$ cells, which in turn leads to activation of the cognate heterotrimeric $G$ proteins (Fig. 2). The receptor promotes the exchange of GDP for GTP in $G^\alpha$ (Gpa1) resulting in the release of $G^\beta_\gamma$ (Ste4-Ste18) and phosphorylation of $G^\beta$. Free $G^\beta_\gamma$ activates the Ste20 PAK kinase (p21 activated kinase) which then transmits the signal to the nucleus via a MAP kinase cascade consisting of the scaffold protein (Ste5), MEKK (Ste11), MEK (Ste7), and MAPK (Fus3). The interaction between $G^\beta$ and Ste20 is equivalent to that observed in *Dictyostelium* between $G^\beta$ and PAK1. Activated Fus3 then shuttles into the nucleus where it phosphorylates Dig1 and Dig2 and prevents them from inhibiting the transcription factor Ste12, which then activates the mating-specific genes [66]–[68]. In the nucleus, Fus3 also phosphorylates Far1 which arrests the cell at G1 phase by inhibiting the cell division kinase Cdc28 [69]–[71].
Figure 2: Molecular model of pheromone response

In MATα cells, the signal is perceived by the Ste2, seven transmembrane domain receptor and its associated heterotrimeric G-protein (Gαβγ). Upon binding of pheromone to Ste2, the G-protein dissociates into its active GTP-bound α subunit and its free βγ dimer. The β subunit is rapidly phosphorylated on multiple sites and βγ initiates a MAPK signaling cascade that results in activation of the MAPK Fus3 which translocates to the nucleus, where it activates transcription of mating-specific genes and induces cell cycle arrest by activating Far1. Far1 complexes with Cdc24 and the complex is recruited to the membrane via Far1-Gβγ interaction which results in polarized growth.
MAPK cascade

Fus3

Ste12 (transcriptional activation)

Far1 (G1 arrest)

Nucleus
1. **Overview of heterotrimeric G-proteins**

   Ga has intrinsic GTPase activity which is accelerated by RGS proteins (regulators of G-protein signaling), which belong to the family of GAP (GTPase activating) proteins. Ga-GTP hydrolysis to Ga-GDP results in reconstitution of the heterotrimeric complex, and consequently, down-regulation of the pathway. It was thought that Ga and the RGS Sst2 only play a negative role in the pathway. However, it has been shown that Ga interaction with Fus3 is crucial for full pheromone-induced phosphorylation of Gβ and important for signal amplification at the chemotropic growth site [72], [73]. The heterotrimeric subunits localize to the PM and internal membranes in vegetative cells, and to the tip of the mating projection in pheromone-responding cells [72], [74], [75]. Ga and Gγ are post-translationally modified with lipid moieties that confer peripheral membrane localization on Gβγ [76], [77] and is required G-protein function [78]. The localization of Ga on the PM is not dependent on the receptor or Gβγ [76], [79]. However Gβγ requires Ga, but not the receptor, to localize on the membrane [76], [80].

2. **Cell polarization in response to pheromone**

   Much like chemotactic cells, yeast cells are also able to polarize in uniform ligand. When there is no pheromone gradient (isotropic conditions), the receptor and the cognate G protein are uniformly activated around the membrane. Hence, there is no internal gradient of the activated G-protein to establish the chemotropic shmoo. Nevertheless, the cells are still able to polarize. Shmooing occurs at the default bud site, which is marked by Bud1, and where the cell would have formed its next bud if not exposed to pheromone (Fig. 3A) [81], [82]. Bud1 localizes Cdc24-Bem1-PAK which in turn recruits and activates Cdc42, and the polarisome complex. Cdc24 is a Guanine nucleotide exchange factor (GEF) for Cdc42. Cdc42 is activated upon
exchange of GDP for GTP and promotes the localization of Bem1 to the site of polarized growth. Bem1 in turn recruits more Cdc42 to this site and this positive feedback loop results in the assembly of the polarisome complex. The polarisome consists of Bni1, Spa2, Bud6 and Pea2. Bni1 and Bnr1 are formins that nucleate actin cables thereby polarizing actin-dependent directed secretion. This results in the formation of a mating projection at the default site. This phenomenon is called default shmooing or default polarization.

In contrast, when the cell senses a pheromone gradient, free Gβγ acts as the positional determinant instead of Bud1. Free Gβγ recruits Cdc24 to the region of the most activated receptor through its interaction with phosphorylated Far1 (Fig. 3B) [81]. Furthermore, Gβγ also indirectly interacts with Bem1 through Ste5 and reinforces the localization of Bem1 to the site of polarized growth [83], [84]. Ga also recruits activated Fus3 to the PM [72]. Fus3 might then phosphorylate other substrates like Bni1 that are essential for polarized growth. Furthermore, the cortical recruitment of Bni1 and Cdc24 is dependent on Fus3 [85], [86]. Interestingly, Gβγ is thought to recruit another G-protein, active Rho1 [87] to the shmoo tip which in turn is known to bind Bni1. This might be a link between the two complexes. These mechanisms act together to establish polarity at the region sensing the highest pheromone concentration and overpower the bias to polarize growth at the default bud site. The delivery of new vesicles to this site in an actin-dependent manner results in the formation of a mating projection up the gradient which is also known as chemotropic shmooing.

Recent evidence has shown that apart from polarizing the proteins, like in growth cones, yeast cells also are capable of polarizing mRNAs. It was previously shown that mRNAs encoding polarity and secretion factors (POL) were enriched at the incipient bud site. Interestingly, in response to pheromone, the RNA binding protein Scp160 binds POL and mating
pathway mRNAs such as Fus3 mRNA and promotes the enrichment of these mRNAs in the shmoo tip in a Myo4 and cortical ER dependent manner. This enrichment is important for polarized growth and gradient sensing [88]. Furthermore, the mRNA of MFA2, which encodes aF, is bound by complex RNA processing structures called processing bodies (PBs). Small highly motile MFA2-PB’s are transported to the front of the cell where they could coalesce to form large PBs. The MFA2 mRNA is preferentially and locally translocated in the large PBs [89]. It was presumed that once the cells polarize, they could secrete the pheromone in a polarized or biased manner toward the partner, which would then strengthen the signal perceived by the chosen partner. Polarization of the distribution and translation of a-factor RNA at the mating projection is the first evidence for the existence of such a mechanism, supporting the idea that pheromone is preferentially secreted through the shmoo tip.
Figure 3: Complexes involved in polarization

Polarization can occur via two pathways involving overlapping components. (A) Budding/default polarization. During budding in yeast cells, the cortical marker Bud1 is localized to the incipient bud site and acts as a positional cue for other polarity proteins such as Cdc42, Cdc24, Bem1 and the polarisome. Actin cables are nucleated at this site resulting in bud formation. In the presence of isotropic pheromone, there is a strong bias to polarize and shmoo at this site. (B) Chemotropic polarization. In the presence of pheromone gradient, Gβγ recruits Far1-Cdc24 to the membrane to form the chemotropic complex. Many polarity proteins involved in budding are also involved in polarized growth during shmooing.
A. Budding/Default polarization

B. Chemotropic polarization
v. **Background and significance on the internalization and polarization of GPCR**

In vegetative cells, the receptor is uniformly distributed on the cell membrane and constitutively internalized [90]–[92]. Upon binding of \( \alpha F \), the Ste2 receptor expressed by \( MATa \) cells is activated and rapidly phosphorylated by the casein kinases - Yck1 and Yck2 [93]. This results in the ubiquitination [94] and global internalization of the receptor with a \( t_{avg} \) of approximately 8 minutes [95] from the membrane. In cells, stained with fluorescently tagged \( \alpha F \), the ligand induced receptor internalization from the membrane is not complete, contrary to what is observed in cells expressing Ste2-GFP. More importantly, although the receptor is globally internalized from the membrane, there is a region on the PM where the signal persists longer than the rest, suggesting asymmetric internalization of the receptor [96]. C-terminal truncation mutants of Ste2 lacking the last 105 amino acids have a fivefold increase in ligand binding sites on the surface. The mutant cells exhibit a tenfold increase in pheromone sensitivity and shmoo morphology defect [90]. Furthermore, the C-terminal cytoplasmic domain interacts with G\( \alpha \) to form a pre-activation complex [97], [98]. It has been proposed that receptor internalization and recycling is important for amplifying the signal and desensitization.

Similar to what happens in growth cones, the receptor is concentrated on the up-gradient side of the plasma membrane in pheromone-induced cells. The receptor has been known to localize to the mating projection. Early studies concluded that the receptor polarizes only when the cells begin to shmoo, likely as a consequence of directed secretion. [92], [99], [100]. However, recent evidence has demonstrated that, approximately 30 minutes after isotropic pheromone exposure, the receptor reappears as a polarized crescent on the membrane, prior to morphogenesis. In mating mixtures, the polarized receptor crescent orients towards the closest mating partner, and it does so prior to when morphological changes are observed [101].
Moreover, the receptor also tracks from the default site to the chemotropic site. This receptor movement is observed upstream of when active Cdc42 is redistributed to the chemotropic site [96]. Therefore, it is likely this polarized distribution is important for chemotropic growth and that it precedes the initiation of actin-cable dependent directed secretion.

vi. Gradient sensing and chemotropism

In *S. cerevisiae*, it has been estimated that a 0.5-1% difference in receptor occupancy across the cell in a pheromone gradient is sufficient to elicit a robust response and orientation towards the mating partner with high fidelity [8], [102], [103]. How do the cells interpret such shallow gradients and sense direction? It is widely accepted that, like neutrophils, yeast cells use a spatial mechanism to sense gradients. Over the years, multiple studies have been conducted to identify mutants that cause defects in chemotropism, but most of these are indirect assays. The gold standard methods for detecting defects in chemotropism are either the partner discrimination assay or the confusion assay. In the partner discrimination assay, *MATα* cells are tested for their ability to distinguish between αF-secreting and non-secreting *MATα* cells. Wild type cells will only mate with secretors, whereas chemotropic defective mutants will mate indiscriminately. In the confusion assay, the mating mixture is flooded with saturating pheromone, making it harder for the cells to detect a gradient. In this assay, wild type cells will be confused by the excess pheromone and their mating efficiency will be decreased. On the other hand, cells that are unable to detect pheromone gradients cannot be further compromised, and their already low mating efficiency will not be adversely affected by the excess pheromone. Although these tools have been crucial in elucidating the key players in chemotropism, these assays are indirect and the readout is far downstream of the gradient sensing and chemotropic mechanisms. However, it is evident from such studies that the receptor, Gβ, Gα, Sst2, Bar1 and
Far1 are necessary for efficient chemotropism [82], [104]–[106]. It has been long known that Bar1 (the protease secreted by MAT\textit{a} cells) is required for desensitization and degrading the αF pheromone in the event of an unsuccessful mating [107], [108]. However, recent work has revealed that Bar1 shapes the gradient sensed by the cells, and thereby facilitates mating [109]. Bar1 seems to play an especially important role in gradient sensing when the ligand concentration is high [103]. It has also been shown that some proteins that are necessary for polarized growth such as Spa2, Bni1, Fus1, Fus2, Pea2, Rvs161, Myo4 and tubulin are dispensable for chemotropism. This supports the idea that directional sensing and polarized growth are distinct cellular responses dependent on components that are unique to each. An assay using micropipette-generated pheromone gradients demonstrated that Bud1, Afr1 and Ste20 are not essential for chemotropism. On the other hand, the Gβγ-Far1-Cdc24 interaction that links the directional sensing machinery to the polarization machinery is required for chemotropism [110], [111]. When this link is broken, the cells can polarize via the default pathway only.

vii. Outstanding questions

Some of the outstanding questions in the field are: What are the key players in directional sensing? How are small differences in ligand concentration across the cell surface amplified while at the same time reducing stochastic noise? What are the first elements (protein or second messenger) that are substantially amplified within the cell in response to the gradient? Is there a global inhibitor, and if so, what is it? How are activation and inhibition signals spatially and temporally restricted? (reviewed in [112])
viii. **Goals of my project**

It is known that Ste2 is the most upstream component of the mating response pathway and is essential for sensing direction. Receptor polarization precedes polarization of actin cables and does not require actin-dependent directed secretion. On the other hand, internalization of the receptor is essential for its polarization [101]. Gβ can interact with Yck1 and locally inhibit phosphorylation of the receptor. This mechanism could lead to differential internalization of the receptor during the global internalization phase and contribute to the establishment of receptor polarity towards the up gradient. Differential receptor phosphorylation also promotes chemotropism. Receptor mutants that are defective in internalization [96] and Gβ mutants that cannot be phosphorylated exhibit defects in chemotropism [113]. In mating mixtures, the receptor orients toward the closest mating partner prior to when morphogenesis is observed [101]. Moreover Ste2 polarization also precedes polarization of Gic2 which is a reporter for active Cdc42 [96]. Thus Ste2 polarization is one of the earliest (if not the earliest) observable polarity event in mating cells. Taken together, these results strongly suggest that the proper polarization of the receptor is crucial for marking the growth site and direction sensing. Therefore, I am interested in understanding the players and mechanisms that contribute to the establishment of receptor polarity during the pheromone response.
II. General materials and methods

a. Strains and media

The genotypes of yeast and bacterial strain used in this study are listed in Table I and II respectively. Standard molecular and microbiological techniques were used to generate bacterial and yeast strains.

b. Yeast culture and pheromone treatment

All integrative strains were grown in YPD medium whereas strains requiring plasmid selection were grown in synthetic media lacking the nutrients indicated. For most experiments, overnight and diluted back the following morning and the cells were allowed to reach log-phase. Cells were treated with 120 nM of pheromone unless otherwise indicated. All strains expressing a Gal inducible plasmid, were grown to log-phase in synthetic medium containing 2% sucrose with the omission of nutrients necessary for plasmid selection. Expression from Gal promoter was induced with 2% galactose for 3 hours. Cells were then treated with pheromone if applicable.

c. Fluorescence microscopy

All live cell fluorescence microscopy was performed with actively growing log-phase cultures. 200 µl aliquots were taken every 15 minutes, pelleted at 4000Xg and resuspended in appropriate volume of 1X PBS. The cells were spotted onto slides and imaged within 1-5 minutes. All cells expressing GFP tagged proteins were imaged under the FITC filter using Axioscop 2 microscope (Carl Zeiss) and images were acquired using the Axiovision software unless otherwise indicated. Images were analyzed using Photoshop CS5.1 (Adobe systems) and/or ImageJ (National institutes of health). Proteins tagged with DsRed or mCherry or Probes conjugated with Alexafluor 594 were imaged using the Texas Red filter.
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III. Directed genetic screen to identify novel players in the establishment of receptor polarity

a. Introduction

Yeast cells exist as three distinct cell types with different developmental capabilities. There are two haploid mating types and a diploid. All three cell types reproduce mitotically by budding, which is also known as vegetative growth. When they sense a mating partner of the opposite type, the haploid mating types *MATα* and *MATα* activate the mating pathway, undergo chemotropism, and fuse to form diploids. When starved for nitrogen, the *MATα/MATα* diploids undergo meiosis and produce four haploid spores, two *MATα* and two *MATa*. The Mendelian segregation of cell-type identity indicates that mating type is determined by a single gene, now called the *MAT* locus. The mating-type specific phenotypes are controlled by expression of one of the two non-homologous alleles, *MATα* and *MATα*. Yeast strains have silent copies of *MATα* and *MATα* in the heterochromatin locus *HMLα* and *HMLα*, respectively, and hence, are considered to be homothallic. In nature, the homothallic yeast strains are capable of switching between *MATα* and *MATα* identity by replacing the respective allele in *MAT* locus from the *HML* locus by site-specific recombination. However, all lab strains are mutated for the HO endonuclease which is required for site-specific recombination, so that mating type switching occurs when an exogenous copy of HO is expressed.

There are unique sets of genes expressed in each cell type that are controlled by a combination of DNA binding proteins and transcription factors. Haploid-specific genes are expressed in both *MATα* or *MATα* haploids but are repressed in diploids. Some genes are expressed in *MATα* or *MATα* cells only. These are called a-specific and α-specific genes, respectively. Similarly, some genes are diploid-specific, expressed only in *MATα/MATα* diploids.
The $MATa$ locus encodes the two proteins $Mat\alpha 1$ and $Mat\alpha 2$. When the constitutively expressed transcription factor Mcm1 complexes with $Mat\alpha 1$, $\alpha$-specific genes such as $STE3$ and genes encoding $\alpha$-factor are activated [121]–[123]. On the other hand, when Mcm1 is bound by $Mat\alpha 2$, they repress $\alpha$-specific genes such as $STE2$ and genes encoding $\alpha$-factor (Fig. 4A). In contrast, in $MATa$ cells, the homodimeric Mcm1 activates $a$-specific genes. $\alpha$-specific genes are not activated due to the absence of $Mat\alpha 1$ (Fig 4B). The $MATa$ locus encodes $MATa1$ and $MATa2$. Although $Mat\alpha 2$ is highly conserved among multiple fungal species, its function is unknown. $Mat\alpha 1$ is not required for expression of $a$-specific genes; it is required for repression of haploid-specific genes in diploids. The $a1-\alpha 2$ heterodimer represses all haploid specific genes in diploids (Fig. 4C). Furthermore, a subset of haploid-specific genes is pheromone induced. In diploid and vegetatively growing haploid cells, Dig1 and Dig2 prevent Ste12 from activating pheromone-induced genes. In the presence of pheromone, Fus3 phosphorylates Ste12, Dig1 and Dig2, thereby relieving repression of pheromone-induced gene expression (reviewed in [124]).

If the $MAT$ locus is deleted, $a/a$ diploids behave like $MATa$ cells i.e., they are able to shmoo in response to $\alpha$F and successfully mate with $MATa$ cells [125]. It was also shown that if haploid-specific genes are artificially activated in a $MATa/\alpha$ diploid cell by knocking out $DIG1$ and $DIG2$, these cells also behave like $MATa$ cells and mate with $MATa$ cells [126]. These double mutant diploids ectopically express many $a$-specific genes including $STE2$ (the gene encoding the pheromone receptor in $MATa$ cells), which was attributed to defective $Mat\alpha 2$ unable to repress haploid specific genes. Interestingly, when $STE2-GFP$ is ectopically expressed from a heterologous promoter in $MATa/\alpha$ diploids, it localizes to the plasma membrane and internalizes in response to pheromone treatment (Suchkov and Stone, unpublished data). However, in the absence the expression of all other haploid-specific genes, pheromone does not
induce receptor polarization in these cells. Addition of pheromone to diploids expressing \textit{STE2-GFP} induces receptor internalization, but not its polarization. Taken together, all these observations, strongly suggested that at least some of the haploid specific-genes must be key components of polarization of the receptor, which contributes to mating. Hence, in this chapter I performed a genetic screen to identify potential haploid-specific genes that are necessary for establishment of receptor polarity.

From the screen, I identified four haploid-specific genes, \textit{DCV1}, \textit{PCL1}, \textit{FIG1} and \textit{PDE1}, which contribute to polarization of the receptor. Further characterization revealed that only Dcv1 and Pcl1 were required for the polarization of receptor upstream and independent of actin-directed secretion. Dcv1 and Pcl1 were also required for efficient chemotropism. In summary, I have devised a screen and a set of experiments that can be used to identify novel players in receptor polarization and gradient sensing.
Figure 4: Regulation of cell-type specific gene expression

Interplay between the transcription factor Mcm1 and DNA binding proteins Matα1, Matα2 and Matα1 to regulate expression of a-specific, α-specific, and haploid-specific genes in (A) MATa, (B) MATα and (C) MATa/α diploid cell type.
A) a-specific genes

B) α-specific genes

C) haploid-specific genes

Transcription activated

Transcription repressed
b. **Materials and methods**

i. **Molecular and microbiological techniques:**

All stains used in this chapter were derived from BY4741 (*MAT*a his3D1 leu2D0 met15D0 ura3D0) which is isogenic control strain from the Yeast haploid knock out collection. All deletion strains used in this chapter were taken from the Yeast haploid knock out collection. All strains expressing Ste2-GFP were generated by integrating HpaI digested LHP1921 into respective strains by transformation. The pRS316/yDsRed-Sec4 (MSB17) plasmid was the kind gift of Dr. Nava Segev’s lab from University of Illinois at Chicago. MSB17 was transformed into MSY128, MSY143, MSY139, MSY147, and MSY161 to generate MSY204, MSY206, MSY337, MSY339 and MSY208 respectively. To generate *MAT*a strains, pGAL-HO (MSB19) was transformed into MSY128, MSY143, MSY139, MSY147, and MSY161.

ii. **Mating type switching**

The MSY128, MSY143, MSY139, MSY147, and MSY161 strains transformed with MSB19 were induced with 2% Galactose to activate the expression of HO endonuclease. *MAT*a cells were selected by plating them on YPD plates containing 2µg/ml αF. The resultant colonies were tested for their ability to mate with *MAT*a strains and MSY184, MSY186, MSY187, MSY190, and MSY191 were generated.

iii. **Cell cycle synchronization by centrifugal elutriation**

Yeast cultures were synchronized as described in [101]. Briefly, Cells were grown in IL of synthetic medium contain 2% sucrose and 0.1% dextrose at 30°C until an optical density of
$A_{600} = 1$ was reached. Cultures were sonicated (Branson digital sonifier) for 30 seconds X 2 at 40% power and loaded onto the elutriation chamber for Beckman JE 5.0 elutriation rotor in a Beckman J-6 M/E centrifuge (Beckman coulter, Fullerton, CA). Cells were eluted using the synthetic media they were grown in by reducing the RPM in decrements of 100 until small newly budded cells were collected in a 50ml conical tube. Elutriation efficiency was calculated by counting the percentage of unbudded cells in the elution. Cells were concentrated down to an O.D of $A_{600} = 1$.

iv. **FM4-64 staining**

Cells were grown to log-phase in YPD medium. 1ml of cells were pelleted down at 5000Xg and suspended in 50µl of fresh YPD contain 1µl of FM4-64 (Sigma) stock solution (1.6µM in DMSO). 3µl were spotted on a slide immediately imaged under the texas-red filter. Aliquots were images every 5 minutes until 30 minutes after staining.

v. **F-actin staining**

F-actin staining was performed as described in [127] using Alexa Fluor 594 Phalloidin (Invitrogen). In brief, 500µl aliquots of log-phase cells treated with pheromone were harvested at the indicated time points and fixed in 4% Formaldehyde for 10 minutes at room temperature. Cells were harvested and washed in 1X PBS 2 times. Cells were resuspended in 25µl of 1X PBS containing 1.5µM of Alexa Fluor 594 Phalloidin and incubated in the dark at 4°C for 30 minutes. Cells were washed with 1X PBS three times. Cells were imaged using the texas-red filter.

vi. **ConA Staining**

MSY147 cells were grown to log phase. 2ml of culture was concentrated into 500µl of media. ConA was added to a final concentration of 10µg/ml. Cells were incubated in the dark for
30 minutes and washed 3X with YPD. Cells were resuspended in 500 µl of fresh YPD, treated with pheromone and images were acquired every 15 minutes.

vii. LatrunculinA treatment

Cells were treated with LatA (Enzo Life Sciences) as described in [96]. In brief, Log-phase cells were treated with 120 nM of αF and 200 µM of LatA was added 15 minutes later. Images were acquired every 15 minutes using the FITC filter.

viii. Measurement of orientation defect in mating mixtures

The respective MATa and MATα strains were grown to log-phase. 3X10⁶ cells from each culture were taken and pelleted at 5000Xg. 100µl of supernatant was collected and saved. Rest of the supernatant was discarded and cells were resuspended in their respective supernatants. MATa and MATα were mixed together and sonicated for 6 seconds at 17% power. The mixture was immediately plated onto YPD plates and zygotes were allowed to develop at 30°C. DIC images were acquired after 2, 3 and 4 hours. Fusion angles were measured using ImageJ software.

ix. Quantifications

The crescent size quantification was performed by tracing a line through the periphery of the cells using ImageJ. Gray values were acquired and percentage of the membrane that was at least 20% brighter than the mean of the dimmer half was calculated. The polarity indices were calculated by obtaining the gray values in the same way and run through an excel program to calculate the ratio of mean signal intensity from the brightest 1/3rd of the membrane divided by the mean signal intensity on the rest of the PM.
c. Results

i. Identification of candidates for the directed genetic screen

In order to identify novel proteins that are required for the establishment of receptor polarity, I performed a directed genetic screen. Yeast has about 6000 genes, too many to test in my microscope-based assay. There is small a set of genes that are only expressed, or which are negligibly expressed, in haploid cells. Because only haploid cells chemotrop, I reasoned that at least some of the components required for gradient-induced receptor polarization to the chemotropic growth site must be haploid specific. Although it was known that certain genes are expressed in a haploid-specific pattern, a database of such genes was not available. Therefore, I compiled a list of haploid-specific genes based on literature. Table 3A lists the genes that were known to be haploid-specific genes [128], and Table 3B lists genes that were predicted to be haploid-specific. I also compiled a third set of genes that are expressed at three times higher levels in haploids than in diploids (Table 3C) [129]. Finally, a small set of genes defined as ploidy-regulated genes, whose expression in haploids was at least 1 standard deviation higher than the \textit{MATa/\alpha} diploids was also included (Table 3D) [130]. Altogether there were 106 genes that were considered for screening. I further narrowed the list based on what was known about each gene. Essential genes were not included; preferred genes included those whose expression is regulated by pheromone, and genes known to encode proteins with relevant functions (e.g., cell polarity) or localization (e.g., sites of polarized growth). Based on these criteria, I picked 21 candidate genes to screen (Table 4). For each candidate gene, the respective deletion strain was taken from the yeast deletion library [114]. The \textit{STE2} receptor in each deletion strain was tagged with GFP \textit{in situ} and the resulting strains were treated with pheromone and followed over time to look for changes in receptor polarization, such as, either a defect or delay in polarization (Fig. 5).
Table 3: List of Haploid-specific genes

A. Known haploid-specific genes

<table>
<thead>
<tr>
<th>A</th>
<th>HO</th>
<th>NJE1</th>
<th>FUS3</th>
<th>STE4</th>
<th>STE18</th>
<th>GPA1</th>
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<tr>
<td>MET31</td>
<td>YGL052W</td>
<td>FAR1</td>
<td>AXL1</td>
<td>SGA1</td>
<td>EST1</td>
<td>FAS1</td>
<td></td>
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<td>DCV1</td>
<td>KAR1</td>
<td>YGL193C</td>
<td>FMP39</td>
<td>AMN1</td>
<td>MATα1</td>
<td>PRM5</td>
<td>YLR159W</td>
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<td>YPS6</td>
<td>BUD3</td>
<td>GUP2</td>
<td>ICS3</td>
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<td>CNN1</td>
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<td>FUS1</td>
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<td>MSB2</td>
<td>YJL202C</td>
<td>PDE1</td>
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<tr>
<td>UGA2</td>
<td>RME1</td>
<td>STB2</td>
<td>YBR051W</td>
<td>YPL0256</td>
<td>EMP46</td>
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<td></td>
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</tbody>
</table>

B. Predicted haploid-specific genes

<table>
<thead>
<tr>
<th>B</th>
<th>URB1</th>
<th>PRM8</th>
<th>PRM9</th>
<th>YKL162C</th>
<th>CDC25</th>
<th>ALD6</th>
<th>YBR028C</th>
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<tr>
<td>SAP155</td>
<td>YMR269W</td>
<td>TRK1</td>
<td>THR4</td>
<td>ILV3</td>
<td>PYC2</td>
<td></td>
<td></td>
<td></td>
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</tbody>
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C. Genes that have at least 3-fold higher expression in haploid/diploid:

<table>
<thead>
<tr>
<th>C</th>
<th>SST2</th>
<th>BAR1</th>
<th>STE6</th>
<th>STE2</th>
<th>AXL1</th>
<th>ASG7</th>
<th>YDR210W</th>
<th>PRM4</th>
<th>HSP26</th>
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<tr>
<td>RME1</td>
<td>AGA2</td>
<td>TMA23</td>
<td>TEC1</td>
<td>YIL067C</td>
<td>AFR1</td>
<td>YDR306C</td>
<td>UTH1</td>
<td>IMD1</td>
<td></td>
</tr>
<tr>
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<td>YIL055C</td>
<td>HOR7</td>
<td>SIC1</td>
<td>ORC5</td>
<td>SIM1</td>
<td>MET17</td>
<td>SCW10</td>
<td>STP1</td>
<td></td>
</tr>
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<td>YOR052C</td>
<td>RCR2</td>
<td>HAP4</td>
<td>CWP1</td>
<td>YLR049C</td>
<td>HXT7</td>
<td>STE12</td>
<td>FIG1</td>
<td></td>
</tr>
<tr>
<td>HXT2</td>
<td>PDR15</td>
<td>YLR256W</td>
<td>HSP26</td>
<td>SCW4</td>
<td>YMR12W-A</td>
<td>DIG1</td>
<td>IMD1</td>
<td>STP1</td>
<td></td>
</tr>
</tbody>
</table>

D. Genes are that are expressed at least a standard deviation higher in haploid/diploid:

<table>
<thead>
<tr>
<th>D</th>
<th>FLO11</th>
<th>PCL1</th>
<th>PRY2</th>
<th>GIC2</th>
<th>CLN1</th>
</tr>
</thead>
</table>

*aThe genes selected for screening are highlighted in green.*
Figure 5: **Pipeline for directed genetic screen**

106 haploid-specific genes were narrowed to 21 candidates for the screen. For each candidate, the receptor (*STE2*) in the respective deletion strain was tagged with GFP *in situ*. WT and deletion cells were treated with pheromone and imaged every 15 minutes using FITC filter to detect changes in polarization of the receptor. Genes highlighted in green (*DCV1* and *PCL1*) were found to affect receptor polarity upstream of actin-directed secretion. These were further characterized in chapter IV and V. Genes highlighted in red (*FIG1* and *PDE1*) either affected actin polarization or affected receptor polarization downstream of actin-directed secretion. These genes were not further characterized.
Final hit: Dcv1, Pcl1, Pde1, Fig1
Deletion of DCV1, PCL1, FIG1 and PDE1 confer a receptor polarization defect

In vegetatively growing WT cells, the receptor is uniformly distributed on the PM. Upon binding pheromone, it is rapidly and globally internalized from the membrane, before reappearing as a polarized crescent 30 - 45 minutes after pheromone treatment (Fig. 6A). I screened the deletion strains for either a delay or defect in receptor polarization during this time period. The screen revealed that deletion of DCV1, PCL1, FIG1 and PDE1 causes a defect in receptor polarization (Fig. 6A), as indicated by receptor crescent size at discrete times after pheromone treatment. Crescent size was defined as the percentage of the PM that was at least 20% brighter than the average value for the rest of the PM. For quantification of this parameter, cells were synchronized in G1 by centrifugal elutriation. In WT cells, the crescent size was about 1/3rd the of the cell circumference. In dcv1Δ, pcl1Δ and fig1Δ cells, there was at least a 33% increase in crescent size at all time points, including the earliest time point at which receptor crescents are observed (Fig. 6B). However, the defect appeared later and was less pronounced in pde1Δ cells: Polarization of the receptor at 30 minutes was comparable to WT and only a 20% increase in crescent size was observed 45 minutes after pheromone treatment (Fig. 6B).

Shmooring WT cells exhibit a characteristic mating projection morphology that is pointed and narrow at the growing end. In contrast, all four mutant cells exhibited a broad shmoo phenotype: Their mating projections did not narrow at the growing end. The fig1Δ conferred an especially dramatic broad shmoo phenotype such that the back and front of shmooing fig1Δ cells were indistinguishable on the basis of morphology. In fact, the receptor often appeared to polarize to the back of the cell. To determine whether fig1Δ somehow unlinked receptor polarization from the growth site, I used the dye Concavalin A (ConA), which stains the cell wall. Log-phase cells were stained with ConA, washed and refreshed with new medium, treated
with pheromone, and imaged for both ConA staining and Ste2-GFP localization over time. In this assay, the old cell wall is fluorescently labeled with the dye, whereas regions containing new cell wall (i.e., where the cell is growing) are unlabeled. This experiment revealed that Ste2-GFP polarized to the growing end of the cell, as expected, but that surprisingly, the growing (front) end was broader than the back. To our knowledge, this is a unique phenotype, perhaps due to an unusual defect in actin polarization.
Figure 6: Receptor polarization in WT and deletion strains

(A) Receptor was depolarized in the deletion mutants. Representative images of WT, *dcv1Δ, pcl1Δ, pde1Δ*, and *fig1Δ* cells expressing Ste2-GFP. G1-synchronized daughter cells were isolated by centrifugal elutriation and treated with pheromone. Images were acquired at the indicated time points using FITC filter. Yellow line marks the start and end of a crescent. (B) Quantification of mean receptor crescent size. Cells were traced using the segmented line tool in ImageJ, grey values were derived for each pixel, and crescent size was calculated as a percentage of the cell circumference. Mean crescent size for each time point was plotted on the graph for WT and deletion mutants. (*p* ≤ 0.0001; 26 ≤ n ≤ 46).
A  Ste2-GFP

WT

dcv1Δ

pcl1Δ

fig1Δ

pde1Δ

Mean crescent size (%)

0'  15'  30'  45'  60'  75'  90'

B

Time after αF (min)

Mean crescent size (%)
Figure 7: **ConA staining in fig1Δ cells**

Shmoo morphology defects in fig1Δ cells. Representative images of WT and fig1Δ cells expressing Ste2-GFP were stained with ConA at the indicated time points. Multi-dimensional images were acquired at the indicated time points with FITC, TRITC and DIC filters.
iii. **Deletion mutants are not defective in general endocytosis**

We have previously shown that the global internalization of the receptor is required for its polarization [101]. It is therefore conceivable that a defect in general endocytosis could affect the polarization of the receptor. Hence it was important to verify that *dcv1Δ, pcl1Δ, fig1Δ* and *pde1Δ* cells do not have a general endocytosis defect. I stained the WT and deletion strains with FM-464, a dye used to track PM to vacuolar internalization. *dcv1Δ, pcl1Δ* and *pde1Δ* internalized the dye with comparable dynamics to WT (Fig. 8A). However, *fig1Δ* appeared to internalize the dye at a slightly faster rate (Fig. 8A & 8B). Nevertheless, these results suggested that the four deletion strains did not have a general endocytosis defect.
Figure 8: **FM4-64 internalization dynamics in WT and deletion strains**

(A) Endocytosis of FM4-64 dye in WT and deletion strains. Representative images of log-phase WT, *dcv1Δ, pcl1Δ, pde1Δ*, and *fig1Δ* cells stained with FM4-64. Images were acquired at the indicated time points using the TRITC filter to follow membrane to vacuolar internalization. (B) Dynamics of FM4-64 internalization. Cells were scored as having either plasma membrane (PM, blue bars), intracellular (Orange bars), or vacuolar (Grey bars) signal for each time point and plotted as a percentage of total for WT and deletion strains. *n* = 8-10 for each time point.
iv. **Relevance of actin-dependent directed secretion to the receptor depolarization mutant phenotypes**

We have previously shown that the establishment of receptor polarity is upstream and independent of actin-cable dependent directed secretion [101]. However, actin-dependent secretion is required for the maintenance of receptor polarity. Therefore, it is possible that a depolarized receptor phenotype could result from an actin-based failure to maintain polarity, rather than a failure to establish it. I performed a number of experiments to distinguish these possibilities.

1. **In dcv1Δ, pcl1Δ, and pde1Δ cells, actin polarization dynamics are comparable to WT**

   I first asked whether polarization of actin cables is normal in the deletion strains by staining the G1-synchronized cells with Alexafluor-phalloidin. We found that polarized actin cables are apparent in the majority of WT cells 45 minutes after pheromone treatment (Fig. 9A), 15 minutes after the emergence of receptor crescents. This is consistent with our previous observation that establishment of receptor polarity is upstream of actin-directed secretion [101]. Whereas the dynamics of actin polarization were comparable in dcv1Δ, pcl1Δ, pde1Δ, and WT cells, fig1Δ appeared to confer a defect in actin-polarization (Fig. 9A). To quantify these results, I characterized the cells as either having disorganized cables, normally polarized cables, broadly polarized cables or more than one axis of polarity (Fig. 9B). This analysis indicated that although actin cable polarization occurred with normal kinetics in dcv1Δ and pcl1Δ cells, a small subset of these mutant cells (13-25%) had broadly polarized cables that corresponded with a broader mating projection. Because the receptor ultimately determines where actin cables are nucleated, we hypothesize that the broad polarized growth results from the initially depolarized receptor. However, from these results alone, it is also possible that broadly polarized actin cables
lead to a defect in receptor polarity. The $pde1\Delta$ cells exhibited a distinct phenotype: actin cable polarization was comparable to WT early in the time course, but broadened at the later time points, consistent with our previous result that polarization of the receptor in $pde1\Delta$ cells is normal prior to morphogenesis but becomes depolarized at the onset of polarized growth. Lastly, $fig1\Delta$ cells exhibited a dramatic defect in narrowly polarizing actin cables. A subset of $fig1\Delta$ cells also had more than one axis of polarized actin cables, a phenotype that was never observed in WT or any of the other deletion strains. In summary, $dcv1\Delta$ and $pcl1\Delta$ cells exhibited actin polarization dynamics that are comparable to WT; $pde1\Delta$ cells started out with polarization dynamics comparable to WT but later show a progressive defect that correlated with the late onset of receptor polarization; $fig1\Delta$ cells appeared to have a pronounced defect in actin polarization.
Figure 9: **Polarization of actin in WT and deletion strains**

(A) Actin polarization dynamics is comparable in WT and deletion strains. Representative images of WT, *dcv1Δ, pcl1Δ, pde1Δ, and fig1Δ*. G1-synchronized daughter cells were stained with Alexafluor-phalloidin and imaged at the indicated time points to stain for polymerized actin. (B) Quantification of actin cable polarity. Deletion cells had higher percentage of cells with broadly polarized actin cables. Cells were scored according to the degree of actin cable polarity, as indicated. The small images (bottom) are representative images for the indicated categories, disorganized (yellow bars), narrowly polarized (orange bars), broadly polarized (brown), or more than one polarized axis (green).
2. **The defect in receptor polarization in dcv1Δ, pcl1Δ and fig1Δ is detectable before the initiation of actin-cable directed secretion**

Although phalloidin staining provides information on how well actin is polarized, it does not allow us to assess vesicle delivery, which if defective, could contribute to the receptor polarity phenotypes. Moreover, phalloidin staining is not a live-cell assay, as it requires fixation. Therefore, to simultaneously study directed secretion and receptor polarization in the same cell, yDsRed-Sec4 was expressed along with Ste2-GFP. SEC4 encodes an essential GTPase that is required for the last step of vesicle fusion to the plasma membrane during exocytic secretion [131], [132] and was used here as a marker for actin-cable dependent secretion. Log-phase WT and deletion strains that were co-expressing Ste2-GFP and yDsRed-Sec4 were treated with pheromone and multi-dimensional images were acquired using the GFP and Texas-red filter. In WT cells, Sec4 polarized as a tight spot at the tip of the mating projection and this was observed concomitant with morphogenesis; occasionally, we could see a Sec4 crescent on the membrane prior to morphogenesis (Fig. 10A). Importantly, 82% of the WT cells had formed polarized receptor crescents 30 minutes after pheromone treatment, while only 28% had polarized Sec4 (either as a crescent or a tight spot) by that time point. Most notably, the polarization of the receptor was always observed prior to when Sec4 polarization was detected in WT cells; the converse was never observed. This result is consistent with our previous data suggesting that polarization of the receptor occurs upstream of actin-cable directed secretion. The polarization of Sec4 in dcv1Δ and pcl1Δ was comparable to WT. More importantly the defect in receptor polarity was apparent before Sec4 polarized in dcv1Δ and pcl1Δ cells, suggesting that the problem in polarizing the receptor occurs upstream of actin-directed secretion (Fig. 10B & C).
In contrast to dcv1Δ and pcl1Δ cells, the polarization of Sec4 in pde1Δ and fig1Δ cells was notably different from WT. In pde1Δ cells, the Sec4 polarization dynamics was comparable to WT until about 60 minutes after pheromone treatment. However, the majority of the mutant cells had depolarized Sec4 by 75 minutes: Sec4 localized diffusely to the entire mating projection instead of in a tightly polarized punctum at the tip of the mating projection. By 90 minutes, the majority of the cells had no detectable polarized Sec4 (Fig. 10D). These results are consistent with our original observation that receptor depolarization in pde1Δ cells is only seen after the initiation of polarized growth. Taken together, these data suggest that the defect in receptor polarization in pde1Δ cells is likely downstream of actin-cable directed secretion.

The polarization of Sec4 in fig1Δ cells was almost undetectable, manifesting only as very broad crescents in actively shmooing cells. The characteristic tight localization to the tip of the mating projection was not seen in the fig1Δ strain (Fig. 10E). This observation correlates with the extremely broad shmoos formed by fig1Δ cells. It is important to note the polarization of Sec4 in vegetatively growing fig1Δ cells appeared normal. Together with the results of the phalloidin-staining experiment, these results suggest that fig1Δ confers defects in actin polarization and actin-cable directed secretion.
Figure 10: **Polarization of Sec4 in WT and deletion strains**

Polarization of Sec4 is downstream of receptor polarization in WT or receptor polarity defect in deletion strains. Representative images of WT (A), *dcv1Δ* (B), *pcl1Δ* (C), *pde1Δ* (D), and *fig1Δ* (E) cells co-expressing Ste2-GFP and yDsRed-Sec4. Log-phase cells were treated with pheromone and multi-dimensional images were acquired at the indicated time points using FITC, TRITC and DIC filters. For A-E, First row - Ste2-GFP, middle row – yDsRed-Sec4, bottom row – merge.
v. *dcv1Δ, pcl1Δ and fig1Δ cells are unable to polarize the receptor in the absence of polymerized actin*

As a final way to characterize the candidate deletion stains and to distinguish the actin-dependent and actin-independent processes in the cell, the drug Latrunculin A (LatA) was used to de-polymerize actin. We have previously shown that WT cells that are concurrently treated with LatA and αF fail to polarize the receptor [101]. This is not surprising because global internalization of the receptor is essential for its polarization, and internalization requires polymerized actin. Therefore, cells that are concomitantly treated with LatA and αF neither internalize nor polarize the receptor. However, if LatA is added after the global internalization of the receptor (i.e., 15 minutes after pheromone treatment), the treated cells are able to robustly polarize the receptor (Fig. 11A). Cells deprived of polymerized actin after global receptor internalization establish receptor polarity just as well as untreated cells, as measured by polarity index (PI) (Fig. 11B). PI is calculated by dividing the mean signal intensity of the brightest 1/3rd of the membrane by the mean signal intensity of the rest of the PM. A cell with uniform receptor distribution would have a P.I value close to 1.0. Cells with polarized receptor crescents have a higher P.I, which is directly related to the degree of receptor polarity. Since actin is required for the maintenance and amplification of receptor polarity, the established polarity is lost over time in the absence of polymerized actin. How do cells polarize the receptor even in the absence of polymerized actin? Sagot et al proposed that in the absence of polymerized actin, vesicles are uniformly secreted to the membrane but can dock and fuse in a biased manner to a pre-existing polarity site [133]. This suggests that a polarity site is established within the first 15 minutes after pheromone treatment, during the global internalization phase, and that this site can later be amplified independently of f-actin. I reasoned that, if any of the candidate gene deletions
prevented polarity establishment during the global receptor internalization phase (the first 15 minutes of pheromone stimulation), then I would expect to see a defect in the polarization of the receptor in cells treated with LatA at the end of this period. Unlike WT cells, dcv1Δ and pcl1Δ cells were completely unable to polarize the receptor in this experiment. Although the receptor reappears on the membrane, it does not polarize, and the difference in P.I values at each time point is extremely statistically significant. fig1Δ cells exhibited a moderate defect in the polarization of the receptor. The difference in P.I when compared to WT cells is not quite statistically significant at 30 minutes, but is significant at all time points after that. In contrast, pde1Δ cells were able to polarize the receptor comparable to WT cells in the absence of f-actin following global receptor internalization (Fig. 12A). This observation is consistent with my previous finding that pde1Δ only results in only a moderate increase in crescent size and the defect manifests concomitant with morphogenesis and not earlier. WT and pde1Δ cells have comparable P.I values at all time points. These results indicate that dcv1Δ and pcl1Δ cells are unable to establish a polarization site during the global receptor internalization phase of the response, fig1Δ cells are moderately defective in this process, and pde1Δ cells are able to establish a polarization site very well with in the first 15 minutes.
**Figure 11: Receptor polarization in LatA treated WT cells**

A) WT cells treated with LatA, 15 minutes after pheromone, polarize the receptor in the absence of polymerized actin. Representative images of G1-synchronized WT cells expressing Ste2-GFP treated with LatA at 0’ concomitant with pheromone treatment or 15’ after pheromone treatment.

B) Polarization of receptor in WT cells treated with LatA 15 minutes after pheromone is comparable to untreated cells. Bar graphs represent polarity index (P.I) for LatA untreated WT cells (blue bars) or WT cells treated with LatA 15’ after pheromone (red bars). P.I was calculated at indicated time points by taking the ratio of mean signal intensity in the brightest 1/3rd of the membrane to the mean signal intensity in the entire membrane. 

\( n = 45 \) for each time point from 3 trials.
Figure 12: Receptor polarization in LatA treated WT and deletion strains

A) dcv1Δ, pcl1Δ or fig1Δ cells treated with LatA are unable to polarize the receptor. Representative images of G1-synchronized WT, dcv1Δ, pcl1Δ, fig1Δ, pde1Δ cells expressing Ste2-GFP treated with LatA 15’ after pheromone treatment. Images were acquired at the indicated time points using FITC filter B) Quantification of P.I show the extent of receptor polarization defect in deletion strains. Bar graphs represent mean P.I values for the indicated time points for WT (light blue), dcv1Δ (orange), pcl1Δ (grey), fig1Δ (dark blue), and pde1Δ (yellow) cells. *p ≤ 0.0002; $p > 0.0015 ≤ 0.0081; ^p = 0.0178; n = 45 for each time point from 3 trials.
vi. *dcv1Δ, pcl1Δ* and *fig1Δ* cells exhibit a defect in orienting towards the mating partner

The receptor is the most upstream component of the pheromone response pathway and the primary gradient sensor. It is known that certain receptor mutant alleles such as a C-terminal truncation mutant or internalization-defective mutant [96] confer defects in chemotropism. We hypothesized that proper establishment of receptor polarity is crucial for directional sensing. Since deletion of *DCV1, PCL1, FIG1* or *PDE1* caused a defect in receptor polarization, I asked whether the deletion strains exhibited a defect in orienting toward their mating partners by examining the zygotes formed in unilateral and bilateral mating assays. In unilateral mating assays, only one of the mating partner is mutant, whereas in bilateral mating assays, both mating partners are mutants. If the two mating partners are able to orient accurately, they fuse to form a straight zygote with a fusion angle of 0°. However, if the two mating partner are defective in the initial orientation, they form angled zygotes and have fusion angles higher than 0°. The more severe the defect in initial orientation, the greater the fusion angle (Fig. 13A). The mean fusion angle for the control mating (WT X WT) was about 5-7°. *pcl1Δ* cells exhibited significantly higher fusion angles compared to WT in unilateral and bilateral mating assays. *fig1Δ* cells had a slight increase in fusion angles in the unilateral mating assay when *MATα* was the mutant which was nearly statistically significant. Unilateral mating assays with *fig1Δ MATα* cells had a statistically significant increase in fusion angles compared to WT. I also observed a reduction in the number of zygotes in the *fig1Δ* bilateral assay, which is consistent with previous reports that Fig1 is required for efficient mating and cell fusion [134], [135]. The *dcv1Δ* cells exhibited unique and surprising phenotype: An orientation defect was only observed in the *MATα dcv1Δ* unilateral cross, and not for the reciprocal unilateral cross or the bilateral mating. Lastly, *pde1Δ* cells had fusion angles comparable to WT in both unilateral and bilateral mating assays; they did
not exhibit an orientation defect in the mating assays (Fig. 13B). Given that \textit{pde1Δ} caused only a moderate defect in receptor polarization, and that the cells were able to establish polarity in the absence of f-actin, this result was not surprising.
Figure 13: Mating assays for detecting defects in orientation

A) Illustration of fusion angle measurement for straight and angled zygotes. Representative images for straight and angled zygotes. Zygote angles were measured from base of one cell to the other and subtracted from 180° to calculate fusion angle. B) The deletion strains had varying defects in orienting toward a mating partner. Quantification of fusion angles for WT and deletion cells. Mating mixtures were set up such that both mating types were WT (control, blue) or only one mating type was mutant (Unilateral, mutant MATa – red, mutant MATa – green) or both mating type were mutant (bilateral, purple) * $p \leq 0.0001$; **$p > 0.067 < 0.087$; $n = 133-298$ from 3 biological repeats.
A

Straight zygote

Angled zygote

B

![Bar chart showing fusion angles for different genotypes: dcv1Δ, pef1Δ, fig1Δ, pde1Δ. The chart includes control and different types of MAT (MATa, MATα). Significant differences are indicated by * and **.](image-url)
d. **Discussion**

Uniformly distributed receptor in vegetative cells becomes polarized in pheromone-responding cells. Even though it has long been known that receptor polarizes in response to pheromone, the significance and mechanisms underlying receptor polarization were poorly understood. It was previously thought that the receptor polarization was a consequence of actin-cable directed secretion. However, the cells polarize the receptor upstream and independent of this process. On the other hand, receptor internalization is essential for its polarization. Very little is known about the mechanisms and proteins necessary for initial receptor polarization, prior to directed secretion. It has been reported that the receptor is differentially internalized from the membrane due in part to protection from phosphorylated free Gβ, thereby generating receptor and G-protein polarity on the up-gradient side. The cells expressing only an unphosphorylatable species of Gβ, are still able to polarize the receptor and chemotrop, albeit defectively [96]. Hence there must be other mechanisms that contribute to receptor polarization.

The directed screen of haploid-specific genes described here was designed to identify and characterize novel players in the establishment of receptor polarity. The deletion of four genes out of 21 screened conferred varying defects in receptor polarization of directional sensing as discussed below.

i. **Dcv1 and Pcl1 are required for the establishment of receptor polarity.**

The deletion of the Yeast claudin *DCV1* or the cyclin *PCL1* results in depolarization of the receptor and defects in chemotropism without affecting actin-directed secretion. Strikingly, cells lacking Dcv1 or Pcl1 are unable to establish a polarization site when actin polarization is blocked following global receptor internalization, and consequently, cannot form receptor crescents, as do wild type cells. Moreover, when stimulated with natural gradients in mating
mixtures, *dcv1Δ* are unable to polarize the receptor. These results suggest that Dcv1 and Pcl1 are essential for establishment of receptor polarity. In contrast, mutant cells treated with isotropic pheromone whose f-actin is unperturbed do form receptor crescents, albeit significantly broader (i.e., depolarized) ones. It is likely that the receptor polarization seen in *dcv1Δ* and *pcl1Δ* cells under these conditions is partially due to rescue from actin-cable directed secretion. Cells treated with isotropic pheromone shmoo at the default polarity site, where positional cues mark the next bud. Once a shmoo site is established, actin-cable directed secretion delivers nascent receptor to it. The targeted stream of receptor to the incipient shmoo site could partially rescue receptor polarization.

Dcv1 is predicted to be a membrane protein. It belongs to the PMP-22/EMP/MP20/claudin superfamily [136], but has no reported function. Claudins are often involved in forming membrane barriers, organizing membrane domains, and/or facilitating the trafficking of other membrane proteins. The Dcv1 transcript is rapidly repressed 70-fold in response to pheromone, but recovers to its initial level in 30 minutes. Pcl1 is one of 10 cyclins that partner with the cyclin-dependent kinase (CDK) Pho85. Although the Pho85/Pcl1 complex has many known substrates which have been implicated in cell polarity during vegetative growth, a potential role in polarization during mating and chemotropism is novel. For these reasons, Dcv1 and Pcl1 were considered to be the best candidates to regulate the establishment of receptor polarity. My investigations of Dcv1 and Pcl1 are discussed in detail in chapters IV and V, respectively.
ii. **Fig1 is required for receptor polarization but is also necessary for actin polarization, directed secretion, and cell morphogenesis**

Eukaryotic cells use calcium (Ca\(^{2+}\)) ions to signal information about their environment and to regulate diverse biological processes such as transcription, cell shape, hormone secretion, neuro-transmitter release, and membrane fusion. Two calcium influx pathways are activated during mating in *S. cerevisiae*, the high (HACS) and the low (LACS) affinity calcium influx system. LACS is the prevailing system for calcium influx in rich media, whereas HACS is the predominant system in minimal media [135]. Elevated calcium levels in pheromone responding cells coincides with morphogenesis [137]. It was previously thought that regulation of calcium levels was only essential for maintaining the viability of unmated cells over prolonged pheromone exposure and late stages of mating. It was later demonstrated that the regulation of Ca\(^{2+}\) levels is also important for polarized morphogenesis and cell fusion [135]. Fig1 is a transmembrane protein that has a known function in the mating pathway during cell fusion [134] and it regulates the LACS system [135]. Interestingly, *fig1* mutants have previously been reported to have mating projections with much broader tips than WT [134] which is consistent with my observations in the *fig1Δ* background. Mating projection formation and cell fusion are events that occur from the middle to the end of the mating process. However, my data suggest that Fig1 plays an earlier role in the pheromone response — the establishment of receptor polarity — as *fig1Δ* cells exhibit a moderate defect in their ability to polarize the receptor in the absence of polymerized actin. It is interesting to note that like Dcv1, Fig1 also belongs to the PMP-22/EMP/MP20/claudin superfamily [136]. I noticed that in *fig1Δ* cells, the receptor appeared to be localized to the back of the cell, away from the mating projection. This made us wonder whether *fig1Δ* caused the region of receptor polarization to become uncoupled from
where the cell was growing. Upon further investigation, however, it was evident that the receptor was polarizing to the growing end, which was broader than the back of the cell. Although numerous defects in cell morphogenesis during mating has been previously reported — e.g., rounded mating projection tips, peanut shmoos, and small multiple mating projections — the inverted shmoo shape (shmoos broader at the front, narrower at the front) is unique to fig1Δ mutants.

It is also interesting that fig1Δ conferred defects in actin polarization, as evidenced by the following. First, the actin cap was more spread out as compared to the tightly localized cap seen in WT cells, consistent with the extraordinarily broad mating projections seen in fig1Δ cells. Second, fig1Δ cells frequently form more than one axis of polarity. Third, Sec4 was weakly polarized, again indicating a defect in actin-cable directed secretion. What is Fig1’s role in establishment of receptor polarity? And how is a protein thought to play a role in the final step of mating also playing a role early in the pathway? It is possible that LACS functions much earlier than currently known. Perhaps an intracellular calcium gradient is required for polarity establishment and chemotropism. It was recently reported that a selective or local influx of calcium through a calcium transporter at the back of migrating cells generates a front to rear increasing Ca^{2+} gradient. This intracellular Ca^{2+} gradient was also found to be critical for maintaining front-rear polarity by inhibiting spontaneous lamellipodia formation at the back [138]. Although these observations and questions are very intriguing, it was beyond the scope of my project to further characterize the role of Fig1 in the mating response. Because my goal was to identify novel players in the establishment of receptor polarity, and because fig1 mutants clearly affect actin, I decided to not pursue the investigation of Fig1.
iii. **Pde1 is likely to be involved in the maintenance rather than the establishment of receptor polarity**

Pde1 is a low-affinity cyclic adenosine monophosphate (cAMP) phosphodiesterase (PDE). PDEs decompose intracellular secondary messengers such as cAMP or cGMP that play critical roles in various biological processes such as nutrient sensing. In *S. cerevisiae*, like pheromone sensing, nutrient or glucose sensing also occurs via GPCR signaling. Addition of glucose to cells grown on non-fermentable carbon source triggers an immediate but transient increase in cAMP levels. The GPCR encoded by *GPR1* binds to glucose which results in the activation of its cognate Gα Gpa2, which in turn leads to activation of adenylate cyclase through a MAPK cascade. Many components such as Ste20, Ste11 and Ste7 are common to both pathways. *pde1* null mutants accumulate cAMP in a agonist-induced manner such as glucose addition [139]. I observed that *pde1Δ* cells exhibit a moderate defect in receptor polarity but does not affect establishment of receptor polarity, suggesting a role for Pde1 for polarity maintenance rather than establishment. *PDE1* is induced in response to pheromone and interestingly Pde1 transcript levels are about 3 fold higher about 45 minutes after pheromone treatment [140] which coincides with the earliest observed receptor polarization defect in the deletion cells. *pde1Δ* cells also seem to orient well toward their mating partners as demonstrated by the mating assay. My results therefore indicate a minor role for Pde1 in the maintenance of receptor polarity. It is not however clear how or why a PDE would be involved in the pheromone response pathway. In humans, PDE inhibition by drugs has been shown to inhibit T-cell chemotaxis [141] and activated lymphocytes [142]. Perhaps, there is an unknown role for the second messenger cAMP in yeast chemotropism. It is also possible that sensing glucose levels is somehow interlinked with the cells ability to respond to pheromone. Although these observations are very interesting, it
deviates from the core of my project which is to identify novel players in the establishment of receptor polarity and yeast chemotropism. My results suggest a role for Pde1 in receptor polarity maintenance but not establishment. Hence the role of Pde1 in the pheromone response pathway will not be further investigated in this thesis.
IV. The yeast claudin Dcv1 facilitates the polarization of the receptor by affecting the PM lipid composition and distribution

a. Introduction

The formation of specialized cellular sheets that demarcate different structures is crucial to maintenance in all multicellular organisms. For example, epithelial cells and endothelial cells line the cavities and surfaces of all blood vessels and organs throughout the body. These sheets are composed of cells that are tightly linked together in order to maintain their structural integrity. The cells form an impermeable barrier; exchange of solutes is prevented between them. When two epithelial cells join together to form an impermeable barrier via the apical complexes, they are said to form tight junctions. The epithelial cells must maintain the apical basal polarity at all times to protect the structural integrity of the organs they line. Tight junctions demarcate the apical and basal portions of the membrane and act as a barrier that controls diffusion of proteins and lipids between these PM domains.

Claudins are a family of proteins that were originally identified as an integral component of tight junctions in all vertebrates. Claudins are required for the barrier function in tight junctions and also regulate membrane permeability [143]. They are also important for maintaining the apical-basal polarity of epithelial cells. Along with Occludin, another tight junction protein, claudins form tight-junction strands that are necessary for barrier function. Claudins are 4-pass integral membrane proteins roughly ranging from 22-27 Kda in size. The diversity in barrier function and permeability of tight junctions across various tissues is thought to depend in part on the which specific claudin is expressed in particular tissues (reviewed in [144]). Furthermore, abnormalities in claudin expression lead to barrier malfunctions that have been implicated in numerous human diseases. These include many types of cancer, asthma,
pathogenesis of viruses and bacteria, and non-syndromic recessive deafness. For example, Claudin-7 is normally expressed in bronchial epithelial cells, but lung cancer cells were found to have either poorly expressed or mislocalized claudin. Engineered expression of Claudin-7 in such cells suppressed cell migration and invasion, possibly by inhibiting the upregulation of ERK/MAPK pathway [145].

Two models have been proposed to explain how claudins modulate barrier function - a protein model and a lipid model. In the protein model, integral membrane proteins form linear arrays with the lipid bilayer, thereby forming a protein barrier. In the lipid model, cylindrical lipid micelles form within the PM, and these underlie barrier function. Most claudin research supports the protein model, as tight junction-specific proteins such as claudins and occludins are required to maintain barrier function. However, this does not rule out the possibility that the formation of tight junction strands depend on a specific lipid composition. Super resolution microscopy experiments have revealed gaps between claudin molecules on the membrane and this observation does not support a model based solely on protein aggregation [146]. It has also been established that the apical membrane of epithelial cells is enriched for glycerosphingolipids and sphingomyelin, which are polarized to the outer leaflet. Phospholipids also polarize between the apical and basal domains. PIP3 is enriched in the basal and lateral domains, whereas PIP2 is enriched exclusively in the apical domain. The PTEN phosphatase localizes to the apical domain and is required for the polarized distribution of PIP3 and PIP2 [147]. PTEN localization also seems critical for CDC42 polarization to the apical domain, and to promote establishment of epithelial cell polarity.

The polarized segregation of lipids in the epithelial PM between the apical and basal domains was one of the first lines of evidence for existence of lipid rafts. Lipid rafts are
10-200 nm membrane microdomains on the plasma membrane that are heterogeneous, dynamic, and enriched for specific lipids, especially cholesterol and sphingolipids. Lipid rafts have been associated with compartmentalization of the PM, actin rearrangements, and PM receptor-mediated signaling events (reviewed in [148]). Lipid rafts have also been implicated in cell polarization, endocytosis, downstream signal transduction, migration, and cytoskeletal tethering. During T-cell lymphocyte migration, lipid rafts are asymmetrically distributed. Association of proteins to the rafts is thought to be critical for T-cell polarization and migration [149]. Lipid rafts have also been implicated in polarizing membrane proteins during cell migration. In chemotaxing T-cells, rafts are found at the leading and lagging edges. These structures are termed leading-edge rafts and lagging-edge rafts. Of relevance to the study of chemotropism and chemotaxis, the localization of the chemokine receptor CCR5 and PI3K to lipid rafts is dependent on chemoattractant-induced cytoskeletal polarization and membrane cholesterol. Depletion of membrane cholesterol abrogates polarized distribution of these proteins and cell migration. Furthermore, these cholesterol-depleted cells were also unable to asymmetrically recruit PH-domain containing proteins to the PM [150], [151]. Taken together, these results suggest that lipid rafts act as an organizing platform for polarizing structural and signaling proteins during gradient sensing and cell polarization [150].

Although it is now widely accepted that lipid rafts are pivotal for many biological processes, very little is understood about how such microdomains are generated and regulated. Cholesterol and cytoskeletal elements are required for formation of membrane lipid rafts. At tight junctions, claudins are recruited to the cytoplasmic interface via interaction of their C-terminal tails with the junction-specific protein, ZO-1/2/3 (Zona Occludens). Ectopic expression of claudins in L-fibroblasts lacking endogenous claudins, results in formation of tight junction-
like strands between the juxtaposed membranes of adjacent cells. The claudins within these stands are not static, suggesting lipid movement in these strands [152]. Lingaraju et al. have proposed a model in which interaction between tight junction proteins and lipids results in the formation of a unique lipid-protein structure that promotes paracellular permeability and contributes to barrier function in tight junctions. In this model, ZO-1/ZO-2 recruit claudins to nascent tight junctions. The claudins then organize specialized lipid domains within the junctions. It is possible that the special claudin-lipid structures recruit additional tight junction-specific proteins to facilitate the formation of tight junction strands [153].

Mating yeast cells polarize their growth in order to chemotrop towards their partners. This requires polarized delivery of vesicular cargo to the growth site via actin cables which results in both PM and cell wall expansion to form the mating projection. It has long been known that proteins involved in the mating and cell polarization — such as the pheromone receptor, G proteins, Cdc42, and Spa2 — are enriched at the cortex of the mating projection. More recently, it has been discovered that certain PM lipids are also enriched in the mating projection. Documented examples include ergosterol [154], and signaling phospholipids such as phosphatidylserine (PS) and phosphatidylinositol-4,5-bisphosphate (PIP2) [155][117]. PIP2 membrane anisotropy is required for Ste5 localization to the shmoo tip and MAPK activation [156]. PS anisotropy is required for the polarization and activation of Cdc42 and for cell polarity in both budding and pheromone-responding cells [117]. There is also evidence for an overall change in the lipid bilayer that alters membrane fluidity: the PM of the mating projection is less fluid compared to the rest of the PM and sphingolipids are required for this structural specialization to develop in response to pheromone [157]. Furthermore, ergosterol and sphingolipids are required to polarize proteins to the tip of the mating projection, and for
efficient pheromone signaling and mating [154], [158]. Optimal GPCR signal transduction is also dependent on ergosterol composition [159]. These observations led to the conclusions that membrane microdomains are generated in yeast cells responding to pheromone and that these domains are required for the generation and maintenance of polarity during mating and cell fusion. Despite advances in identifying membrane domains as integral to cell polarization, not much is known about how they are generated or maintained, and what proteins contribute to their formation.

In this chapter, I discuss my discovery that the claudin superfamily member Dcv1 is required for receptor polarization in mating cells, and for the proper distribution of ergosterol, PS and PI. My data also implicate Dcv1 in the maintenance of cell integrity, which correlates with its effects on the lipid composition of the PM. Moreover, I confirmed the predicted PM localization of Dcv1 by live-cell imaging and immunostaining. Remarkably, Dcv1 and the pheromone receptor are inversely localized in shmooing cells, with the receptor on the surface of the mating projection and Dcv1 at the back of the cell. Altering specific lipid levels in the cell also affects receptor polarization, consistent with the idea that lipid domains promote receptor polarization. Mating cells lacking Dcv1 also exhibit delays in stable Fus1 polarization and cell fusion. In summary, these observations support a role for Dcv1 in organizing mating-specific membrane domains essential for the polarization of the receptor and other mating functions.

b. Materials and methods

i. Yeast and bacterial strain construction

The Dcv1 over-expression plasmids MSB20 and MSB45 were transformed into MSY128 strain to generate MSY198 and MSY213 respectively. The CFP region was amplified from pBS5 using the forward primer – GATCGTCGACATGTCTAAAGGTGAAG and Reverse primer –
ATGCGGATCCTTTGTACAATTCCATCCATACCATGGG. The PCR product and Ycplac33 were digested with BamH1 and SalI and ligated to generate the MSB26 construct. The promoter (upstream 400 bases) and first 360 bases of DCV1 ORF were amplified using ATGCGGATC CTTTGTAATAATCCATCCATACCATGGG and GCATAAGCTTTCTTGAGATCGGCGGT TCG. The PCR product and MSB26 were digested with HindIII and SalI and ligated together to generate MSB72. Finally, the primes GCATAAGCTTTCTTGAGATCGGCGGT TCG and GATCGAGCTCGGTCTGTGGAATGTTTTGTC were used to amplify DCV1 ORF bases from 361 to the stop and 300 bases downstream of the ORF. The PCR product and MSB72 were digested with BamH1 and SstI and the products were ligated together to generate MSB79 (DCV1[120]-CFP). MSB79 was transformed into MSY116 to generate MSY275. The mCherry fragment was amplified using GCACCTCCCCTTGTTATTATTATTATTATTTTTTCAAGTTCGAC GGTGGAAGTGGTGGAGGTAGTGGTGAATGGTGAGCAAGGGCGAGGAGGATAAC and GCACCTCCCCTTGTTATTATTATTATTATTTTTTCAGGTCGACCGGTGGAAGTGGTGA GGTAGTGGTGGAATGGTGGAGCAAGGGCGAGGAGGATAAC. The PCR product was digested with BamH1 and SalI. MSB79 digested with BamH1 and SalI to excise the CFP fragment and the vector was gel purified and ligated to the digested mCherry PCR garment to generate MSB100 (DCV1[120]-mCherry). MSB100 was transformed into MSY116 to generate MSY345. The divergent Gal/Gal10 promoter from MSB4 was subcloned into Yiplac211 using BamH1 and EcoR1 to generate MSB67. The Lact-C2 domain was amplified from MSB56 using the GCAGACGGATCCGCAACATTCCATCCATCCATCCATGGG and GCAGACCAAGCTTC TAACAGCCCAGCAGCTCCACTCG. The PCR product and MSB67 were digested with BamH1 and HindIII and the products were ligated together to generate MSB68 (GAL-LACT-C2-GFP). MSB68 was transformed into MSY101 and MSY116 to generate PS reporter strains.
MSY347 and MSY348 respectively. MSB57 (2X-PH-GFP) was transformed into MSY101 and MSY116 to generate PIP2 reporter strains MSY349 and MSY340 respectively. The plasmid 379 (GFP-FUS1) was transformed into MSY101 and MSY116 to generate MSY353 and MSY354 respectively.

ii. **Testing the sensitivity to cell integrity drugs**

Cells were grown to log-phase in either YPD medium for deletion strains or synthetic medium lacking uracil containing either 2% dextrose or 2% galactose for over-expression strains. The drugs Congo red (Sigma; 100µg/ml), SDS (Sigma; 0.001%), Caffeine (Sigma; 12mM) or Hygromycin B (50µg/ml) were added to the plates at the indicated final concentrations. Spot assays were performed by performing 10-fold serial dilutions with a starting concentration of 3 X 10^6 cells. The plates were incubated at 30°C for two overnights to allow for the spots to develop. Control plates had no drug added.

iii. **Immunofluorescence staining**

Cultures of MSY198 and MSY213 were grown to log-phase in synthetic media containing 2% sucrose. Gene expression was induced with 2% galactose for 3 hours. Cells were treated pheromone and 500µl aliquots were obtained every 15 minutes for immune-staining. Cells were fixed in 4% formaldehyde at room temperature for 15 minutes. Cells were pelleted at 5000Xg and resuspended in 300µl of 100mM of Potassium phosphate buffer; pH – 6.4 (K-P). Cells were pelleted again and resuspended in 300µl of K-P buffer containing 1.2M sorbitol. Cells were pelleted and resuspended in 300µl K-P buffer containing 1.2M sorbitol and 10mM DTT. 100units of lyticase enzyme was added and cells were incubated for 1 hour at 30°C. Cells were pelleted and resuspended in 1ml of K-P buffer containing 1.2M sorbitol and the step was repeated. Cells were resuspended in 500µl of blocking buffer (5% BSA and 0.1% tween-20 in
1X PBS) and incubated at room temperature for 1 hour. The blocking buffer was pipetted out and mouse anti-HA antibody was added to final concentration of 1:1000. The cells were incubated overnight at 4°C. The antibody was pipetted out and the cells were washed with 1X PBS 3 times. Secondary antibody anti-mouse-FITC in blocking buffer was added to a final concentration of 1:200. Slides were incubated in the dark for 1 hour and washed once with 1X PBS. The buffer was aspirated and the cells were resuspended in mounting medium containing DAPI. Cells were stored in the dark at -20°C until imaged using the FITC filter.

iv. **Filipin staining**

Filipin staining procedure was modified from the protocol described in [160]. Briefly, log-phase cells MSY101 and MSY116 cells were treated with pheromone and 200µl aliquots were taken every 15 minutes for filipin staining. The cells were pelleted at 4000Xg and resuspended in 100µl of 1X PBS containing 4µl of Filipin (Sigma) stock solution (5mg/ml in ethanol). Cells were directly spotted onto the slide and imaged live within 1-5 minutes. Images were acquired using the Zeiss observer Z.1. 25µm optical sections of cells were taken, deconvolved using Zeiss Zen software and sum projection were made in ImageJ.

v. **Lipid profiling of plasma membrane**

The PM from MSY101 and MSY116 vegetative and pheromone-treated cells were isolated as described in [161] with the following modifications. The protease inhibitor cocktail III (EMD Milipore) was used at a final concentration of 1:100. Cells were disrupted by mechanical lysis using beadbeater-16 instrument (BioSpec Products) with fifteen 20 second pulses. At the density gradient step, 0.5ml of resuspended solution from the pellet formed at 22,000 x g were loaded onto 2ml Sucrose step gradients (1.1 M/1.65 M/2.25 M) and centrifuged
at 80,000 x g for 20 hours. 0.5ml fractions from the bottom of the tube were collected using _____ pump equipped with a long syringe.

Purity of plasma membrane was determined by probing for other organelle markers such as Pma1 (PM), HSP60 (Mitochondria), Nup53 (Nucleus), Vph1 (Vacuolar) and Dpm1 (ER). The primary antibodies were used at a concentration of 1:5000, 1:4000, 1:4000, 1:1000 and 1:4000 respectively. All primary antibodies were purchased from AbCam. Anti-mouse secondary antibody conjugated with HRP (Jackson ImmunoResearch) was used at concentration of 1:10,000 against all primary antibodies except anti-Hsp60. Anti-Goat secondary antibody conjugated to HRP was used at concentration of 1:10,000 against anti-HSP60.

Phospholipids were extracted from the purified PM as described in [162]. In brief, 70µg of protein equivalent volume was made up to a final volume of 500µl with water. Prior to extraction, the samples were spiked with the following lipid standards PS 17:0-14:1 (39 picomoles), PA 17:0-14:1 (23 picomoles), PI 17:0-14:1 (50 picomoles), PC 17:0-14:1 (47 picomoles), PE 17:0-14:1 (47 picomoles). 990µl of 17:1 Chloroform/Methanol (v/v) was added, samples were mixed by brief vortexing and incubated on ice for 2 hours. The lower organic phase was collected in a glass vial and labeled as 17:1 phase. 990µl of 2:1 Chloroform/Methanol (v/v) was added to what was left, vortexted briefly and incubated on ice for 2 hours. The lower organic phase was extracted and transferred to a fresh glass vial labelled as 2:1 phase. The samples were vacuum evaporated and resuspended in 1:2 Chloroform/Methanol (v/v). The post extraction lipid standards PC 17:0-17:0 (47 picomoles) and PE 17:0-17:0 (47 picomoles) were added to the 17:1 phase. The standards PA 17:0-17:0 (23 picomoles), PS 17:0-17:0 (39 picomoles), PC 17:0-20:4 (50 picomoles) were added to the 2:1 phase.
Sterol extraction was performed using 70% Isooctane/30% Ethyl acetate. 70µg of protein equivalent volume was made up to a final volume of 500µl with water. The samples were spiked with Cholesterol D7 (57picomoles). 1ml of Isooctane/ethyl acetate (7:3) was added to the samples and incubated on ice for 30minutes. The upper aqueous phase was extracted and the above step was repeated two more times. The samples were vacuum evaporated and resuspended in 1:2 (Chloroform/Methanol).

All internal standards were purchased from Avanti polar lipids. The samples were sent to Wayne State University lipidomics core (This study was supported in part by National Center for Research Resources, National Institutes of Health Grant S10RR027926) for shotgun analysis and the lipids were analyzed by the lipid profiler.

vi. **Time lapse imaging of mating mixtures**

Time lapse analysis of zygote formation in mating mixtures was performed precisely as described in [96]. Images were acquired every 10 minutes from 15-1 fields using Deltavision Elite microscope (GE Healthcare Biosciences) with a 60X oil immersion SoftWorx. All images were processed in the ImageJ and the center slice was chosen for representative images.

c. **Results**

i. **Dcv1 affects cell integrity**

Expression of *DCVI* transcripts is tightly regulated in response to pheromone. Immediately after pheromone treatment, *DCVI* transcript levels are repressed about 80-fold within 10 minutes, but recover to steady-state levels over the next 30 minutes. Is the acute repression of Dcv1 essential for proper receptor polarization? Because *dcv1Δ* conferred a defect in the polarization of the receptor, I wondered if over-expression of Dcv1 would adversely affect this process. To test this, I expressed Dcv1 (Gal-Dcv1-HA) under the control of the inducible
galactose (GAL1) promoter on a 2µ high-copy plasmid in cells expressing Ste2-GFP. Receptor polarization was followed over time in pheromone-induced cells. To the limit of sensitivity of our measurement, the receptor polarized normally when Dcv1 was over-expressed (Fig. 14). This result suggested that the immediate repression of Dcv1 transcript levels following pheromone induction was not necessary for receptor polarization. However, while assaying the effect of excess Dcv1 on receptor polarity, I noticed that cells over-expressing Dcv1 were abnormally prone to lyse. Perhaps this could explain the lack of receptor polarization defect in cells over-expressing Dcv1. If over-expression of Dcv1 affected cell integrity, the cells could have been under selective pressure to maintain a low copy number. Also since cells over-expressing Dcv1 lysed more readily, we were probably biasing our experiments by looking at only intact cells that comparatively had a low expression of Dcv1.

To confirm lysis phenotype, I used the trypan blue exclusion assay of cell viability. Trypan blue is actively excluded from healthy and intact cells, but stains dead cells that have lost membrane integrity: The cytoplasm of dead cells becomes blue, whereas viable cells remain clear. Cultured cells over-expressing Dcv1 showed a five-fold increase in the percentage of trypan blue positive cells compared to control cells (Fig. 15A).

Next I tested the sensitivity of cells overexpressing Dcv1 to various drugs that affect cell integrity by performing spot assays on plates containing caffeine, Hygromycin B, Congo red and SDS. These compounds all perturb cell integrity but they do so by different mechanisms. Caffeine is a purine analog that has pleiotropic effects on the cell, including loss of cell integrity. In high doses, caffeine causes cell death. Sensitivity to Hygromycin B has been associated with glycosylation defects [163]. Congo red directly interferes with cell wall synthesis [164]. Lastly, the detergent SDS affects membrane stability [165]. Over-expression of Dcv1 slightly increased
sensitivity to Congo red, Hygromycin B, and SDS (Fig. 15B). Taken together, these results demonstrate that over-expression of Dcv1 adversely affects cell integrity.

To determine whether loss of Dcv1 also affects cell integrity, I tested the sensitivity of \textit{dcv1}\textsuperscript{Δ} cells to the same panel of drugs. \textit{dcv1}\textsuperscript{Δ} conferred hypersensitivity to Congo red and a slight increase in sensitivity to Caffeine (Fig. 16). This demonstrates that both excess Dcv1 and the absence of Dcv1 results adversely affect cell integrity.
Figure 14: **Overexpression of Dcv1 does not affect the polarization of the receptor**

Representative images of log-phase *MATa* cells expressing Ste2-GFP and either empty vector (control, top line) or Gal1-Dcv1-HA (experimental, bottom two lines) were grown with or without the inducer galactose (2%), as indicated, for 3hr, then treated with pheromone. Images were acquired at the indicated time points using FITC filter.
Figure 15: **Effects of Dcv1 overexpression on cell viability**

(A) *dcv1*Δ cells had a five-fold increase in trypan blue staining. Log-phase control cells and isogenic cells transformed with Gal-Dcv1-HA were induced with 2% galactose for 3 hours. The trypan blue exclusion assay was performed by staining with 0.2% trypan blue for 15-minute at room temperature. The bar graph represents the percentage of trypan blue positive cells. (n = 900 over three biological trials; p = 0.0002) (B) Cells over-expressing Dcv1 are slightly sensitive to certain drugs that affect cell wall integrity to varying degrees. Log-phase control cells and isogenic cells transformed with Gal-Dcv1-HA were grown in the presence of 2% galactose for 3 hours. Sensitivity to the drugs were tested by spotting 10-fold serial dilutions onto plates with the indicated sugar (2% galactose or dextrose) and the indicated drugs (Caffeine (12mM), Congo red (100µg/ml), Hygromycin (50µg/ml), or SDS (0.001%)). The plates were incubated at 30°C for two overnights to allow for colonies to develop. Control cells were transformed with the empty Gal-HA vector.
Figure 16: Effects of \textit{dcv1Δ} on cell integrity

WT and \textit{dcv1Δ} cells were grown to log phase in YPD medium and sensitivity to the drugs were tested as previously described. 10-fold serial dilutions were spotted onto YPD plates containing the indicated drugs (Caffeine (12mM), Congo red (100µg/ml), Hygromycin (50µg/ml), SDS (0.001%)) and incubated at 30°C for two overnights to allow for colonies to develop.
WT

dcv1Δ

WT

dcv1Δ

WT

dcv1Δ

WT

dcv1Δ

1 10^{-1} 10^{-2} 10^{-3} 10^{-4}

+ Caffeine

+ Congo red

+ Hygromycin B

+ SDS
ii. **Dcv1 localizes to the plasma membrane and away from the mating projection**

As a member of the claudin family, Dcv1 is predicted to be a membrane protein. However, at the outset of my project, its intracellular localization pattern had never been studied. To ask where Dcv1 localizes in the cell, I first performed immuno-fluorescence microscopy in cells expressing Gal-Dcv1-HA. Dcv1-HA appeared to be uniformly distributed in the PM or at the cortex of vegetative cells (Fig. 17A). Remarkably, Dcv1 was localized away from the mating projection in cells responding to pheromone, i.e., on the back of the cell. This localization pattern, which we define as Dcv1 anisotropy, was observed concurrent with morphogenesis (Fig. 17A). As the receptor polarized to the mating projection, Dcv1 appeared to inversely localize.

To visualize Dcv1 in live cells at near endogenous levels, I made a series of GFP- and CFP-tagged DCV1 constructs. In cells expressing an N-terminally tagged GFP-DCV1 reporter, the signal was diffuse throughout the cell, perhaps because Dcv1 contains an N-terminal signal peptide predicted to be cleaved prior to insertion in the membrane. Tagging the C-terminus of Dcv1 with GFP also failed to yield a useful reporter. The Dcv1-GFP signal was intracellular and punctate. This is also not surprising, as claudin family members have been reported to require a free C-terminal tail for proper localization and protein-protein interaction [166]. Thus, I engineered DCV1 clones that were internally tagged at one of the three predicted loops. Among these, cells expressing DCV1 internally tagged with CFP at the predicted intracellular loop 2, which begins at amino acid residue 120 (DCV1[120]CFP), exhibited PM fluorescence. Cell expressing Dcv1[120]CFP corroborated the immunofluorescence results which suggested that the protein localizes uniformly to the PM in vegetative cells and away from the mating projection in cells responding to pheromone (Fig. 17B). Remarkably, Dcv1 was found to localize as a ring to
the base of incipient shmoos in a very few cells (n = 4). That the ring signal at the base of
shmoos was observed rarely may be due to one or more of the following factors: 1) CFP is very
weak fluorophore; 2) Our yeast strains exhibit some PM auto fluorescence when observed with
the CFP filter; 3) Dcv1 is not an abundant protein. These problems might be solved by tagging
Dcv1 with a different fluorophore. Therefore, I changed the Dcv1 loop 2 tag from CFP to
mCherry. I chose mCherry so that I would have the option of studying the localization of both
Ste2-GFP and Dcv1-mCherry in the same cell.

Consistent with the two previous Dcv1 localization experiments, cells expressing
Dcv1\textsubscript{[120]} mCherry and Ste2-GFP also localized Dcv1 uniformly to the PM in vegetative cells
and to the back of cells responding to pheromone. Co-expression of the two reporters revealed
that in pheromone-induced cells, polarized receptor crescents formed before Dcv1 localized
away from the mating projection. Dcv1 localization to the back of the cell was observed
concurrent with morphogenesis (Fig. 17C). These results suggest that Dcv1 redistribution to the
back of the cell occurs after Ste2 polarization. It should be noted, however, that the data might be
misleading due to an extremely low Dcv1\textsubscript{[120]} mCherry signal. In addition, I did not observe the
transient ring localization at the incipient shmoo site which I had previously seen in Dcv1-CFP
expressing cells, albeit rarely. This could be due to a low signal, or because the ring localization
does not persist long enough to be seen in the imaging experiment I performed. Time-lapse
imaging of these cells might clarify this point.

Together, these localization experiments clearly demonstrate that Dcv1 localizes to the
PM, as predicted, and is dramatically redistributed away from the mating projection in cells
responding to pheromone, consistent with a role for Dcv1 in forming and/or maintaining PM
barriers.
Figure 17: **Dcv1 mixes with the receptor in the PM of vegetative cells but localizes away from the receptor in cells responding to pheromone**

(A) Immunostaining show that Dcv1 localizes uniformly to the PM of vegetatively growing cells and to the rear of a cell responding to pheromone. Representative images of immune-stained log-phase cells expressing Dcv1-HA. Cells were induced with 2% galactose for 2 hours, pheromone-induced, immuno-stained using anti-HA antibody at the indicated time points and images were acquired using the TRITC filter. (B) Live imaging of cells expressing Dcv1[120]CFP recapitulate immunostaining results. Representative images of log phase cells expressing Dcv1 internally tagged with CFP at residue 120. Cells were treated with pheromone and imaged at the indicated time points using CFP filter. Z-stacks of cells that were 0.25 µm apart were acquired and sum projection were generated using ImageJ. (C) Receptor polarization is observed prior to Dcv1 localization to the rear. Representative images of log-phase cells expressing Ste2-GFP and Dcv1 internally tagged with mCherry at residue 120 (Dcv1[120]mCherry). Cells were treated with pheromone and multi-dimensional images were acquired at the indicated time points using FITC, TRITC and DIC filters.
Deletion of *DCV1* affects the distribution and composition of plasma membrane lipids

Recent work in yeast has demonstrated that PM lipids such as PIP2, PS, and sterols are polarized in cells responding to pheromone, resulting in the formation of distinct membrane domains. The polarization of these lipids is essential for signal transduction, cell polarization and mating functions. However, little is known about how these domains are generated or maintained. Is Dcv1 involved in the formation or maintenance of these membrane domains? To address this possibility, I asked whether Dcv1 is required for polarization of PIP2, PS, or sterols.

I monitored sterol localization in live cells by staining the cells with the dye filipin. Sterol localization was comparable in vegetative WT and *dcv1Δ* cells. However, a profound difference was observed in WT and *dcv1Δ* cells responding to pheromone, especially prior to morphogenesis. More than half of the WT cells polarized sterols to a single, well-focused spot on the PM before the initiation of shmooing (morphogenesis). In contrast, 70% of the *dcv1Δ* cells were unable to polarize sterols to single spot prior to morphogenesis (Fig. 18A and B). Rather, sterols appeared as multiple polarized spots around the PM of the mutant cells until the onset of shmooing, after which the sterol spots coalesced to a single spot at the tip of the mating projection, as in WT cells. Vesicles delivered to the growth site via actin-cable directed secretion likely include sterols, which could account for the rescue of the depolarized sterol phenotype. These results suggest that in the absence of Dcv1, cells are unable to polarize sterols on the PM prior to the start of polarized growth.

I next looked at the localization of PIP2 by expressing the GFP-2xPH-PLCδ reporter in WT and *dcv1Δ* cells. In WT cells responding to pheromone, PIP2 appeared to be localized away from the mating projection — i.e., to the back of shmooing cells. Recall that this localization pattern is similar to what’s observed for Dcv1. In cells lacking Dcv1, this localization pattern
was not apparent; the reporter was localized uniformly on the membrane (Fig. 18C). Garrenton et al. reported that PIP2 was localized to the tip of the mating projection [156]. This contradicts what I observed, PIP2 localized to the back of the cell. However, Fairn et al. did not observe shmoo tip enrichment; they reported that PIP2 was uniformly localized to the PM in cells responding to pheromone [117]. I wondered whether the difference in PIP2 localization pattern might be due to strain differences, or to slight differences in the reporter and its promoter. Therefore, I expressed the reporter in two unrelated yeast strains, but noted difference in localization pattern. I also expressed another PIP2 reporter under the control of inducible Gal1 promoter and monitored its localization in cells responding to pheromone. (Gal-GFP-PH$^{PLC\delta}$-PH$^{PLC\delta}$-GFP). The PIP2 localization pattern was similar to what I initially observed, and thus, independent of the particular reporter (data not shown). At this writing, we do not know why PIP2 localization is described differently by labs using the same method. Nevertheless, it is clear from my experiments that PIP2 localization is considerably different in WT and dcv1Δ cells.

To monitor the localization of PS, I expressed the Lact-C2-GFP reporter in WT and dcv1Δ cells, and acquired images before and after pheromone treatment. Consistent with published observations [117], PS polarized to sites of polarized growth (e.g., the bud neck and presumptive bud site) in vegetative cells. After pheromone-treatment the PS reporter was markedly enriched in the mating projection of WT cells. Since the overall fluorescence signal was quite weak in dcv1Δ cells, I was only able to detect a slight concentration of the PS reporter at sites of polarized growth during the vegetative cell cycle. In dcv1Δ cells responding to pheromone, however, polarization of the reporter to the mating projection was almost undetectable, which may have been due in part to the weak signal in these cells (Fig. 18D). Unexpectedly, dcv1Δ cells expressing the Lact-C2-GFP reporter lysed very readily. Because
Lact-C2-GFP is slightly toxic to WT cells, I wondered whether the reporter, which was expressed from a high-copy plasmid under the control of a strong constitutive promoter (ADH1), was creating a synthetic toxicity in combination with deletion of DCV1. In this case, there would be a strong selection for low copy number of the reporter in dcvlΔ cells, which would explain the very low Lact-C2-GFP signal in the mutants.

To mitigate the potential problems of reporter-induced toxicity and variable reporter copy number, I created a plasmid with Lact-C2-GFP under the control of the GAL1 promoter, and integrated it into the GAL1 locus of both WT and dcvlΔ cells. I then repeated the PS localization study in cells induced to express the reporter for only 3 hours. Consistent with my previous observation, PS was enriched at sites of polarized growth during vegetative growth in WT cells, and to the mating projection in WT cells responding to pheromone. In dcvlΔ cells, however, I was unable to see a PM signal even though the cytoplasmic signal of the reporter was comparable to that in WT cells (Fig. 18E). There are a number of ways that dcvlΔ could confer this phenotype: 1) Low level PS synthesis; 2) Poor delivery of PS to the PM; 3) PS is flipped to the outer leaflet of the PM. In healthy yeast cells, majority of the PS is localized to the inner leaflet, but when cells subjected to certain stresses or are undergoing autophagy, PS gets flipped to the outer leaflet. As the reporter is cytoplasmic, it cannot bind to PS on the outer leaflet.

Taken together, the reporter and staining results show that distribution of sterols and certain phospholipids on the PM is altered in dcvlΔ cells.
Figure 18: Cells lacking Dcv1 have altered PM lipid distribution

(A) dcvlΔ cells have aberrant sterol polarization prior to morphogenesis. Representative images of G1-synchronized WT and dcvlΔ cells, treated with pheromone, and stained with the sterol reporter filipin at the indicated time points. Z-stacks of cells that were 0.25 µm apart were acquired using DAPI filter and the images were deconvolved using Zen axiovision software. Sum projections were then generated using ImageJ. (B) Quantification of sterol polarization show that dcvlΔ cells have significantly higher percentage of cells with more than one polarized punctum. Percentage of cells with polarized sterols was calculated by grouping them as either having one polarized patch (Blue) or multiple patches (Red) at the indicated time points (C) Unlike WT cells, PIP2 localization is uniform in dcvlΔ cells. Representative images of WT and dcvlΔ cells expressing the PIP2 reporter (GFP-2xPH-PLCδ), imaged at the indicated time points after pheromone treatment using FITC filter. (D) PS was highly enriched at the mating projection of WT but not in dcvlΔ cells. Representative images of WT and dcvlΔ cells expressing the PS reporter (GFP-Lact-C2), imaged at the indicated time points after pheromone treatment using FITC filter. (E) Representative images of WT and dcvlΔ cells in log-phase, expressing the integrative PS reporter (Gal-GFP-Lact-C2). Log phase cells were grown in the presence of 2% galactose for 3 hours to induce the expression of the PS reporter, treated with the pheromone and imaged at the indicated time points.
Figure A: Images of WT and dcv/Δ cells at various time points (0' to 75').

Figure B: Bar graph showing the percentage of cells over time after a treatment.

Figure C: Images of WT and dcv/Δ cells at various time points (0' to 120').

Figure D: Images showing the distribution of cells at various time points (0' to 120').

Figure E: Images showing the distribution of cells at various time points (0' to 120').
3. **Dcv1 also affects PM lipid composition**

Because *dcv1Δ* affects the PM distribution of ergosterols and certain phospholipids, I wondered whether Dcv1, affects PM lipid composition. To analyze of many lipid classes at the same time, we took advantage of MS lipidomics. I purified the PM from WT and *dcv1Δ* cells that were either growing vegetatively or which had been treated with pheromone for 90 minutes. The purity of the resulting PM fractions was estimated by western blotting for PM, mitochondrial, nuclear, vacuolar and ER markers (Fig. 19). The western blot data showed that the PM purification was largely successful, with very little contamination from other membranes. However, all PM fractions from all samples contained the ER marker. It may be that cortical ER membranes cannot be entirely separated from PM in the purification procedure I employed. In all samples, fraction 1 had the least contamination from other membrane markers and hence was selected for further steps.

Phospholipids were recovered from the membrane preparations by a 2-step differential extraction. Sterols were extracted in a separate procedure that included chemical acetylation, which is necessary for efficient detection of sterols by MS. The samples were analyzed by electron-spray ionization (ESI) MS. The absolute amounts were compared to identify differences between the vegetative and pheromone-induced samples, as well as differences between the WT and mutant cells (Fig. 20).

In WT cells, pheromone-induced an increase in PI levels. *dcv1Δ* cells had lower PI levels in vegetative cells. In pheromone-induced *dcv1Δ* cells, half of the PI species increased to WT levels, whereas pheromone dependent induction was not observed in the other PI species (Fig. 21A). PA and PE levels were similar WT and *dcv1Δ* cells. In response to pheromone, PE levels were reduced in both WT and *dcv1Δ* cells (Fig. 21 B and C). PC levels were also reduced in
pheromone-treated WT cells. However, levels of PC species in \textit{dcv1\Delta} cells showed varied responses. While some of the PC species were reduced as in WT cells, other species remained the same (Fig. 21 D). Of the two PS species that were detected, one increased in response to pheromone, whereas the second was reduced in WT cells. In contrast, the absolute levels of these PS species differed in \textit{dcv1\Delta} cells, and their levels were less responsive to pheromone. (Fig. 21E). Although preliminary, these results suggest that the phospholipid content of certain phospholipid classes or certain species of phospholipids are significantly different in WT and \textit{dcv1\Delta} cells. A difference was observed in either absolute concentration (≥ 2-fold), or pheromone-dependent change in levels (increased or decreased), or both. We are currently waiting for the MS analysis of sterols from latest trial of this experiment. In an earlier trial, however, we observed a reduction in ergosterol levels in response to pheromone in WT cells. While the absolute levels of sterols were clearly lower in \textit{dcv1\Delta} cells, their pheromone-induced reduction was comparable to that in WT cells (Fig. 21F). In summary, \textit{dcv1\Delta} likely affects the absolute levels of PM ergosterol and some phospholipids, as well as the pheromone regulation of some PM lipid species.
Figure 19: Purification of PM from yeast

PM preparations were made from log-phase WT and \textit{dcv1Δ} cells or WT and \textit{dcv1Δ} cells that were induced with pheromone for 90 minutes. The PM fractions (1, 2 or 3) from each sample were immunoblotted against the indicated organelle markers to determine the purity of each fraction. * indicates an unloaded lane; some material spilled over from adjacent lanes. PM marker – Pma1; Mitochondrial marker – Hsp60; Nuclear marker – Nup53; Vacuolar marker – Vph1; ER marker – Dpm1.
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Figure 20: **Schematic overview of lipid extraction from yeast PM.**

Yeast PM preparations were spiked with internal standards. Phospholipids were recovered by 2-step differential lipid extraction designed to yield polar and apolar lipids in distinct phases. Sterols were extracted in a separate step involving acetylation. The lipid extract phases were analyzed by ESI-MS.
Isolation of PM from yeast samples

Spike with internal standards

Apolar lipid extraction with 17:1 Chloroform/Methanol

Sterol extraction with 7:3 Isooctane/Ethyl acetate

Polar lipid extraction with 2:1 Chloroform/Methanol

17:1 phase extract (PC, PE)

2:1 phase extract (PA, PI, PS)

Semi-quantitative shot gun analysis
Figure 21: Lipid profiling of WT and *dcv1Δ* cells

Lipid analysis of yeast PM from vegetative and pheromone-induced WT and *dcv1Δ* cells. Bar graphs represent absolute amount of each lipid species in picomoles or ng as indicated (A) PC (B) PE (C) PA (D) PI (E) PS (A-E) - Blue – WT (vegetative); Orange – WT + αF; Gray – *dcv1Δ* (vegetative); Yellow – *dcv1Δ* + αF. (F) Sterols
iv. **Altering lipid composition affects the polarization of the receptor**

Since Dcv1 affects receptor polarization and PM lipids, I wondered whether directly altering lipid levels by mutation would affect receptor polarization. Because Sterols, PS and PIP2, are known to have important functions in the mating pathway, I focused on genes that regulate their levels — *ERG6*, *CHO1*, and *IRS4* — to determine whether these PM constituents play a role in receptor polarization. Ergosterol is critical for mating functions such as signal transduction, Ste5 polarization to the tip of mating projection, and cell fusion. The Erg6 enzyme is in the ergosterol biosynthetic pathway, and has been reported to affect sterol distribution on the PM [167]. Cho1 is the phosphatidylserine synthase, and has been shown to be required for Cdc42 polarization during the pheromone response and for efficient mating [117]. Irs4 down regulates PIP2 levels [168], and some irs4 mutant allele has been shown to have a detrimental genetic interaction with Dcv1 in a high-throughput screen [169].

The receptor in the *ERG6*, *CHO1* and *IRS4* deletion strains was tagged with GFP *in situ*. The resulting strains were treated with pheromone and followed over time to monitor polarization of the receptor. The *cho1Δ* cells displayed a low level of receptor on the membrane, and in response to pheromone, the receptor concentrated as a spot at the tip of the mating projection, rather than forming a crescent spanning the growth site. Moreover, the *cho1Δ* cells exhibited an abnormal shmoo morphology — very short and narrowly polarized (Fig. 21). Although this phenotype is interesting, *cho1Δ* cells did not phenocopy *dcv1Δ* cells. On the other hand, *erg6Δ* cells appeared to broadly polarize the receptor when compared to WT cells, similar to what was observed in *dcv1Δ* cells. These results need to quantified and repeated to determine whether *erg6Δ* confers a significant defect in receptor polarization. Lastly, *irs4Δ* cells appeared
to polarize the receptor normally. Although these results are preliminary, they suggest that manipulating specific lipids (e.g., ergosterol and PS) can affect receptor polarization.
Figure 22: Mutants defective in lipid synthesis exhibit defects in receptor polarization

Representative images of WT, *erg6Δ*, *irs4Δ*, and *cho1Δ* cells expressing Ste2-GFP. Log-phase cells were treated with pheromone and images were acquired at the indicated time points using FITC filter. Last panel - representative DIC images for *cho1Δ* cells are shown for the indicated time points.
v. The receptor is completely unable to polarize in \textit{dcv1Δ} bilateral mating mixtures

In chapter II, I measured zygote fusion angles in \textit{dcv1Δ} unilateral and bilateral mating mixtures to determine whether \textit{dcv1Δ} confers a defect in gradient sensing, and thus in orientation towards the nearest potential mating partner. The results of this endpoint assay were perplexing: a defect was observed in the unilateral cross in which the mutant was \textit{MATα}, but not in the unilateral crosses in which the mutant was \textit{MATα}, or in the bilateral crosses, in which both partners were mutant (Fig. 13B). To better understand the nature of this unique phenotype — and in particular, to explain why two mutant cells (which form straight zygotes) are seemingly better able to orient toward one another than are \textit{dcv1Δ MATα} and \textit{WT MATα} cells (which form angled zygotes) — we employed time-lapse DIC and fluorescent imaging of mating mixtures. We reasoned that the ability to watch the orientation and fusion of mating pairs develop over time, and the ability to distinguish \textit{MATα} and \textit{MATα} cells using fluorescent markers, would tell us much more about how \textit{dcv1Δ} affects the mating process than the “snap shot” zygote formation assay used previously.

The time-lapse mating assays were set up as follows: The \textit{WT MATα} and \textit{dcv1Δ MATα} cells expressed Ste2-GFP, allowing us to track localization of the receptor in the \textit{MATα} partner. The \textit{WT MATα} and \textit{dcv1Δ MATα} cells expressed RFP-Bud1, which served both to identify the \textit{MATα} partners, and to distinguish pre-zygotes from zygotes (i.e., RFP-Bud1 served an indicator of cell fusion). Control (\textit{WT X WT}) and bilateral (\textit{dcv1Δ X dcv1Δ}) mating mixtures were set up on agarose pads and selected fields were imaged every 10 minutes for 4 hours.

As before, partners in the bilateral mating mixtures formed zygotes with near 0° fusion angles. Nevertheless, the time-lapse experiments revealed a number of defects in the bilateral crosses, which could combine to give the appearance of a rescued orientation defect by allowing
illegitimate fusion. In contrast to the WT X WT mating, $dcv1\Delta \text{MAT}a$ cells in the bilateral cross completely failed to polarize their receptor (Fig. 23A), were significantly delayed in cell fusion (Fig. 23B), and took significantly longer to progress from pre-zygotes to zygotes (Fig. 23C).

Partners are said to be in the pre-zygote stage when they have contacted one another and the cell walls are apposed, but cell wall degradation has not yet advanced sufficiently to allow mixing of their membranes (See red asterisk in Fig 23A). In the bilateral mating mixtures, there was also a significant reduction in the number of zygotes formed. To quantify this, I estimated the number of expected zygotes by scoring eligible mating pairs, defined as cells of opposite mating type initially positioned less than 1µm away from one another. The total number of observed zygotes was divided by the number of potential zygotes to give a measure of overall mating efficiency. Mating efficiency in the control cross was about 90% as compared to about 56% in the mutant cross ($p = 0.0082$ by a chi-square analysis; $n = 57$). Unlike in the wild type cross, many eligible $dcv1\Delta$ partners were typically unresponsive to one another, and mating was only rarely observed between partners that were not already in contact. Together, these data indicate that although $dcv1\Delta$ mating partners form straight zygotes, and therefore appear to interpret natural pheromone gradients as well as wild type cells, their apparent normality belies pronounced defects in the underlying mechanisms. We hypothesize that $dcv1\Delta$ cells in the bilateral mating mixtures do not, in fact, sense the gradient efficiently, but rather, they take advantage of an already existing contact site and depolarized fusion zones to form straight zygotes. This hypothesis is explored further in the next section.
Figure 23: *dcv1Δ* bilateral mating mixtures exhibit complete depolarization of receptor and a fusion delay

Mating mixtures were set up on agarose pads using log-phase cells. WT X WT cross was set up between WT *MATα* cells expressing Ste2-GFP and WT *MATα* cells expressing Bud1-RFP. *dcv1Δ* bilateral cross was set up between *dcv1Δ MATα* cells expressing Ste2-GFP and *dcv1Δ MATα* cells expressing Bud1-RFP. Multi-dimensional, time lapse, z-stack (0.25 μm sections) images were acquired every 10 minutes using the FITC, TRITC and DIC filters. (A) Receptor does not polarize in *dcv1Δ* bilateral mating mixtures. Representative images were selected from the indicated time points. The red asterisk indicates a pre-zygote and white asterisk indicates a zygote. (B) *dcv1Δ* bilateral mating mixtures exhibit a delay in zygote formation. Bar graph representing the average time to fusion in minutes (31 ≤ n ≥ 44; p = 0.0001; significance was determined by chi-square analysis) Box represent the range for 50% of the values and whiskers represent the rest of the values. (C) Mating efficiency is significantly reduced in *dcv1Δ* bilateral mating mixtures. Bar graph representing the percentage of cells that took 10, 20 or 30 minutes to progress from pre-zygote (PZ) to zygote (Z) formation (31 ≤ n ≥ 43; p = 0.0027; significance was determined by chi-square analysis).
vi. **Polarization of the fusion marker Fus1 is delayed in dcvlΔ bilateral mating mixtures**

We have recently shown that MATa cells expressing Ste27XR, a mutant form of the α-factor receptor that cannot be polarized, are moderately defective in gradient sensing and orientation, and consequently, they form angled zygotes. The ability of dcvlΔ MATa X dcvlΔ MATa cells to form straight zygotes is especially puzzling given that they also fail to polarize the receptor. Our working hypothesis is as follows. Although dcvlΔ MATa cells, like Ste27XR MATa cells, cannot redistribute the receptor in response to gradient stimulation, their residual ability to orient toward potential mating partners is supported by differential phosphorylation of the receptor and the resulting concentration of active, unphosphorylated receptor at the chemotropic growth site. Unlike Ste27XR cells, however, dcvlΔ cells fail to properly polarize other chemotropic elements in addition to the receptor. Whereas WT cells generate a tightly focused fusion zone highly enriched in ergosterols and fusion-essential proteins such Fus1, Fus2, and Prm1, dcvlΔ cells are unable to do so. The fusion competent zone in dcvlΔ cells is depolarized, allowing fusion to occur over a much larger area of the cell surface. The residual ability of dcvlΔ cells to orient towards potential partners, combined with their expanded fusion zone, allows them to form straight zygotes with any partner initially in direct contact.

As a first test of this hypothesis, we studied the localization of Fus1-GFP in WTxWT and dcvlΔ bilateral mating mixtures using time-lapse DIC and fluorescent imaging as described above. Fus1 is an enzyme that localizes to the mating projection and is required for cell fusion [157]. As shown in Fig. 24A, WT MATa cells formed a polarized Fus1-GFP crescent shortly after being mixed with WT MATa cells. In some cases, the Fus1-GFP crescent appeared to track local pheromone gradients; it moved between potential mating partners. In most cells (86%), however, the crescent stably oriented toward the eventual mating partner within the first hour of
interaction (Fig. 24B). At the onset of polarized growth, the Fus1-GFP crescent condensed to a tight cap on the tip of the mating projection. The transition from crescent to cap took an average of 30 minutes in WT cells. In *dcv1Δ MATa* cells challenged with *dcv1Δ MATα* cells, on the other hand, the dynamics of Fus1-GFP localization were dramatically different. Although Fus1 crescents appeared at a similar time in the mutant as in the WT cells, a high percentage of mutant cells did not initially orient their Fus1 crescent toward their eventual mating partner (Fig. 24B). Often, the Fus1 crescent wandered between two nearby *MATα* cells, and the point of stable orientation toward the eventual partner was significantly delayed (Fig. 24C). Once stable crescents were established, however, they progressed to caps significantly faster than in WT cells (mean ± SEM in minutes = 30.48 ± 4.65 for WT; 15.38 ± 3.15 for *dcv1Δ MATα*; p = 0.0006; n = 21-26), perhaps because other essential factors continued to accumulate at the fusion site during the initial delay in stable Fus1 polarization.

The observation that *dcv1Δ* confers a defect in the polarization of Fus1 as well as compromising polarization of the receptor, sterols, and PM phospholipids, is consistent with the idea that the fusion zone is expanded in *dcv1Δ* cells, allowing straight zygote formation even though gradient tracking is not fully functional. However, additional experiments will be required to confirm or refute this hypothesis.
Figure 24: Fus1 polarization dynamics in the \textit{dcv1Δ} bilateral mating mixtures

(A) Polarization of Fus1 in control and bilateral mating mixtures. Representative images of the control mating and the bilateral mating mixtures expressing Fus1-GFP. Mating mixtures were set up on agarose pads using log-phase cells. WT X WT cross was set up between WT \textit{MATα} cells expressing Fus1-GFP and WT \textit{MATα} cells expressing Bud1-RFP. \textit{dcv1Δ} bilateral cross was set up between \textit{dcv1Δ MATα} cells expressing Fus1-GFP and \textit{dcv1Δ MATα} cells expressing Bud1-RFP. Multi-dimensional, Z-stack (0.25 µm sections), time lapse images were acquired every 10 minutes using FITC, TRITC and DIC filters. Representative images were selected from the indicated time points. (B) Fus1 wanders more in \textit{dcv1Δ} bilateral mating mixtures. Bar graph representing the percentage of cells that stabilize Fus1 crescent at a site different from the site of initial appearance (21 ≤ n ≥ 26; p = 0.002) (C) \textit{dcv1Δ} bilateral mating mixtures take longer to stabilize Fus1 crescent toward the mating partner. Scatter plot represent time taken in minutes to stably oriented Fus1 crescents. Box represent the range for 50% of the values and whiskers represent the rest of the values. (21 ≤ n ≥ 26; p = 0.09; chi-square analysis)
d. **Discussion**

Yeast cells polarize their growth during both the vegetative cell cycle (when they bud) and mating (when they shmoo). However, there are important differences in how growth is regulated during these processes. Bud emergence occurs just after the mother cell transitions from G1 to S. Actin cables direct vesicular traffic to the tip of the nascent bud, driving polarized apical growth. When the mother cell reaches G2, vesicular delivery changes from polarized to uniform (the apical to isotropic switch), causing the daughter to expand symmetrically. During mating, on the other hand, the cell must maintain apical growth at the shmoo tip while making small adjustments in the position of vesicle delivery in response to changes in the pheromone gradient. It is critical that the expansion of the PM and cell wall are precisely coordinated to prevent the cell from lysing as the mating projection rapidly elongates. Tight spatio-temporal regulation of polarized delivery is also essential as the shmooing cell prepares to fuse with its partner. Cell fusion requires highly localized degradation of the cell wall while cell wall integrity is maintained outside the small region of cell-cell contact if lysis is to be avoided. There is growing evidence that certain lipid constituents of the PM are more robustly polarized during shmoo formation than during bud emergence. This reinforces the idea that organization of the PM into ordered domains plays a role in shmoo formation and other mating functions. The discovery that the localization of key polarity and signaling proteins such as Cdc42 and Ste5 depends on polarized PM lipid domains adds further support to this paradigm. Nevertheless, little is known about how these domains are generated and maintained.
i. **Dcv1 might regulate receptor polarization by altering membrane lipid composition and/or acting as a barrier**

In this study, I found that the claudin-like protein Dcv1, previously of unknown function, is critical for polarization of the receptor and to maintain appropriate distribution and composition of PM lipids. My data demonstrate that Dcv1 localizes to the PM, as predicted. The inverse localization of the receptor and Dcv1 is consistent with the idea that Dcv1 plays a barrier role in the PM, analogous to other claudins. Cells lacking Dcv1 are completely unable to polarize the receptor when exposed to natural pheromone gradients, as might be expected if Dcv1 provides a barrier function.

Dcv1 could function as a barrier in two ways. The concentration of Dcv1 away from the mating projection PM might itself be sufficient to demarcate front and back domains. For mammalian claudins, protein enrichment was thought to be the key to forming a barrier until recent evidence suggested that claudins and lipids are both required for barrier function (reviewed in [153]). On the other hand, Dcv1 could contribute to the formation of membrane domains, differing in protein and/or lipid content, that attract some proteins and repel others, and thereby promote the polarization of structure and function. This hypothesis is supported by our finding that cells lacking Dcv1 have altered PM lipid composition and distribution. The *Candida albicans* claudin homolog, Sur7, has also been implicated in PM organization [170].

The most pronounced effect of *dcv1Δ* on phospholipid distribution that we have observed thus far is the abrogation of pheromone-induced sterol polarization prior to morphogenesis. The sterol polarization observed after isotropically-treated cells begin to shmoo is most likely due to delivery of new sterol-rich membrane vesicles to the default growth site. Sterol polarization plays vital roles in mating-specific signal transduction and recruitment of fusion proteins to the
Cells devoid of sterols arrest as pre-zygotes, confirming the importance of PM ergosterol in cell fusion. The cell fusion delay observed in \textit{dcv1}\(\Delta\) bilateral mating mixtures could be due to defect in sterol polarization.

I first observed a loss of cell integrity when Dcv1 was over-expressed. The increased sensitivity of Dcv1 over-expressing and \textit{dcv1}\(\Delta\) cells to SDS, Caffeine, Hygromycin B and Congo red confirmed a connection between Dcv1 and cell integrity. With the exception of SDS, these compounds are generally used to tease out defects in cell wall integrity. However, a PM defect could be exacerbated by a chemical insult to the cell wall or by perturbation of the cell wall integrity pathway. The greatly enhanced toxicity of the PS reporter when expressed in \textit{dcv1}\(\Delta\) cells is also consistent with a loss of cell integrity. Together with our live-cell reporter and mass spec lipidomic analyses, these results indicate that Dcv1 impacts PM lipid stoichiometry and distribution, and that absent these functions, cell integrity is compromised.

The delays in cell fusion and Fus1 crescent stabilization that we observed in \textit{dcv1}\(\Delta\) bilateral mating mixtures also suggest a role for Dcv1 in the establishment/maintenance of polarity that is more general than a function that affects the receptor alone. The most economical explanation, given our current data, is that Dcv1 affects PM domains, which in turn, affect polarized mating functions.

\textbf{ii. Our model}

Based on the results presented here and the established roles of mammalian claudins, I propose a model in which Dcv1 facilitates the polarization of the receptor and other polarity proteins involved in mating by establishing and/or maintaining PM lipid domains (Fig. 25). In vegetative cells, Dcv1 and the receptor are uniformly distributed on the membrane. Upon activation of the receptor, the two proteins segregate away from one another by an unknown
mechanism. The receptor polarizes as a crescent at the incipient chemotropic growth site, while Dcv1 localizes to the boundary that demarcates this region from the back of the cell. Through a series of unknown steps, Dcv1 promotes the reorganization of membrane lipids into distinct domains that distinguish and separate the front of the cell from the back. For example, Dcv1 might facilitate the polarization of sterols to the front of the cell by inhibiting sterol synthesis at the back, much like PTEN localization to the back of chemotaxing cells helps generate PIP2 polarization. In mature shmoos, the receptor and Dcv1 are inversely localized to the growing mating projection and to back of the cell, respectively. Although this model is necessarily speculative, we imagine that the concentration of Dcv1 around the neck of incipient shmoos and to the back of mature shmoos contributes to the formation and/or maintenance of membrane domains that are crucial for the polarization of mating-specific functions from the beginning to end of the mating process: the receptor that determines the direction of the response, proteins that focus and amplify the spatial signal (e.g., Ste5), and proteins required for cell fusion (e.g., Fus1). This model raises a lot of immediately testable questions that will be discussed in the future direction section.
Figure 25: Proposed model for Dcv1’s role in receptor polarization and maintenance of membrane domains

In vegetative cells, Dcv1 and the receptor are uniform on the membrane. However, upon activation of the receptor, Dcv1 localizes to the boundary of the growth site, while the receptor polarizes as a crescent at the growth site. In mature shmoos, the receptor and Dcv1 are inversely localized to the growing projection and to back of the cell, respectively. Once localized to the back, perhaps then Dcv1 would recruit enzymes to alter the PM lipid composition at the back, thereby reinforcing receptor polarization to the front. Polarization of active receptor at the growth site could in turn promote Dcv1 localization to the back. This would trigger a positive feedback loop, that would promote the establishment and/or maintenance of membrane domains that define the front and the back of a cell and promote polarization of the receptor and other polarity proteins involved in the mating pathway to the mating projection.
Inactive receptor $\xrightarrow{\alpha F}$ Activated receptor $\xleftarrow{\text{Dcv1}}$
e. **Future directions**

i. **What promotes the localization of Dcv1 to the back?**

One of our key observation is that Dcv1 and the receptor inversely localize to each other in cells responding to pheromone. This raises the following questions about Dcv1 anisotropy. 1) Is it dependent on activation and/or polarization of the receptor? 2) Is it dependent on actin-directed secretion. Examining Dcv1 localization in cells over-expressing Gβ, which causes autonomous shmooing, will allow us to address whether receptor activation is necessary for Dcv1 anisotropy. Examining Dcv1 localization in cells expressing Ste2\(^{7XR}\) (internalization defective receptor) will allow us to address whether receptor polarization is necessary for Dcv1 anisotropy. Since receptor internalization is essential for its polarization, cells expressing Ste2\(^{7XR}\) do not polarize the receptor. Lastly, since Dcv1 anisotropy is observed concurrent with morphogenesis, it raises the question if it is dependent on actin directed secretion. LatA can be used to depolymerize all actin and assay whether Dcv1 anisotropy occurs.

ii. **Is sterol polarization aberrant in dcv1Δ cells in mating mixtures**

My results demonstrate that sterol polarization is aberrant in dcv1Δ cells responding to isotropic pheromone and this is evident prior to morphogenesis. Furthermore, dcv1Δ bilateral mating mixtures exhibit a fusion delay and sterols are essential for cell fusion. Hence, observing the localization of sterols in mating mixtures in WT and dcv1Δ cells will shed insight into whether the cell fusion delay observed in bilateral mating mixtures is due to depolarized receptor. To address this question, time-lapse mating experiments can be performed with \(MATα\) and \(MATα\) cells pre-stained with filipin. However, since filipin is easily bleached this can be a technical caveat. To overcome this issue, mating mixtures could be set up in a microfluidic device with a constant supply of filipin along with media. Alternatively, an in-cell reporter for
sterol could be generated based on sterol binding domains found in Oxysterol binding proteins. Theoretically, such a domain fused to GFP would report the localization of sterols in live cells negating the need for staining with filipin.

iii. **Is Dcv1 required for ordered membrane domains?**

Although the differences in lipid distribution in *dcv1Δ* cells are suggestive of defective membrane domains, these data do not directly correlate with lipid ordering. Laurdan is a dye whose emission peak is a function of its lipid environment. This dye has been used to show that the lipids are more condensed in the yeast mating projection relative to the rest of the PM [157]. I will use laurdan to determine whether the overall lipid order of the PM is affected by deletion of *DCV1* in either vegetative or pheromone-treated cells.

iv. **Identify interacting partners**

As with any protein, a lot can be learned about its function by identifying its molecular interactions. The following two methods can be used to identify Dcv1 interactors.

1. **Perform a MYTH (Membrane yeast two hybrid screen)**

Conventional yeast two-hybrid screens are not suitable for integral membrane proteins, which cannot be easily localized to the nucleus due to their hydrophobic nature. If membrane proteins can be forced into the nucleus, they are likely to be misfolded. Hence, I performed a conventional yeast two-hybrid screen by using just the predicted loops and the C-terminal domain (CTD) as bait. The screen did not yield any good candidate interactors (Appendix I). A split ubiquitin membrane two-hybrid (MYTH) system can be used to circumvent this disadvantage.
In the MYTH system, the bait protein is fused to the C-terminal domain of ubiquitin (Cub) and the transcription factor LexA-VP16. For this screen, Dcv1 would be internally tagged with Cub at residue 120, which is the same position I inserted CFP and mCherry in what proved to be functional Dcv1 reporters. The prey cDNA library is tagged with the N-terminal domain of ubiquitin (NubG). If the bait and prey interact, the ubiquitin is reconstituted. This results in the cleavage and release of LexA-VP16, which then translocates to the nucleus where it activates reporter gene expression.

2. **Capture Dcv1-HA on affinity beads and perform MS analysis to identify candidate Dcv1 interactors.**

   Another approach is to pull down HA-tagged Dcv1 from vegetative and pheromone-treated cells, and identify potential interactors by mass spec. I used Dcv1-HA to perform the immunofluorescence experiments described in this thesis, and it localized to the PM. Because Dcv1-HA is tagged on its C-terminus, which has been shown to be important for claudin interactions, it might be better to do the pull-down/mass spec experiment using an internally tagged version of Dcv1 as the bait.

v. **Identify Dcv1 domains that are essential for receptor polarization or lipid distribution**

   Cells lacking Dcv1 exhibit defects in receptor polarization and the distribution of PM lipids. What domains of Dcv1 are necessary for these functions. As previously mentioned, the C-terminal tail of most claudins has been shown to be important for its localization, protein interactions, and barrier function. It is therefore of interest to determine whether a C-terminal tail truncation mutant of Dcv1 would also affect receptor polarization and/or lipid distribution. Furthermore, a pBLAST search revealed that Dcv1 loop 3 is 30% identical and 51% similar to the oxysterol binding domain of Osh3. A DCV1 deletion mutant lacking loop3 could be used to
ask whether receptor polarization and lipid distribution depends on this domain. One caveat of this approach is that the deletion of loop 3 could cause Dcv1 to misfold or mislocalize, in which case the experiment would be uninformative. Thus, all Dcv1 deletion clones should be tagged with a fluorescent protein to determine whether they localize normally.
V. The cyclin Pcl1 for the CDK Pho85 may facilitate the polarization of the receptor via phosphorylation of Gβ

a. Introduction

Cyclin-dependent kinases (CDK) are proline-directed, serine/threonine kinases that were first identified as kinases that regulate cell cycle. CDKs are ubiquitously found in all eukaryotes and their function in cell cycle is evolutionarily conserved. However, CDK’s are now known to play roles in variety of cellular processes such as regulating transcription, mRNA processing and nerve cell differentiation. CDKs require a regulatory subunits called cyclins for their activity and the catalytic subunit of CDK must associate with the cyclins. The activity and expression of cyclins are regulated by cell cycle, hence their name.

Most CDK’s associate with multiple cyclins allowing them to phosphorylate different targets and differentially respond to numerous intracellular and extracellular cues. The CDK’s that bind to multiple cyclins usually play prominent roles in signaling pathways that regulate cell division, cell differentiation, and transcription. In the budding yeast, the CDKs Cdc28 and Pho85 are examples of this group. There are some CDK’s that associate with only one cyclin and these are often involved in regulating only transcription. Owing to their pleiotropic functions, deregulation of CDK’s expression and activation has been implicated in numerous diseases such as malignant transformation, neurodegenerative disease, cardiovascular diseases and cancer. The atypical mammalian CDK, Cdk5 is the only known mammalian CDK to play a role in chemotaxis. Cdk5 has diverse function in the neurons of developing nervous systems which include neuronal migration, neurite outgrowth, axon guidance and synapse development.

The budding yeast has 6 CDKs and 22 cyclins. The CDK Cdc28 is an essential kinase that is required for control of cell cycle progression. On the other hand, Pho85 is non-essential,
but plays diverse roles in regulating cellular responses to nutrients and cell cycle progression. Cdc28 and Pho85 pair with 9 and 10 different cyclins respectively. The other 4 yeast CDKs are involved in regulation of transcription. The CDK Pho85 and its cyclins Pcl1 and Pcl2 are required for cell cycle progression when the Cdc28 cyclins Cln1 and Cln2 are absent, suggesting a role in regulating cell cycle progression. Pho85 and Cdc28 share 51% sequence similarity and many common targets. A quadruple mutant lacking \textit{PCL1, PCL2, CLN1,} and \textit{CLN2} is synthetic lethal, suggesting at least one overlapping essential function. Pho85 regulates G1 progression, cell polarity, actin cytoskeleton, gene expression phosphate and glycogen metabolism and signaling changes in the environment [171], [172]. Pho85/Pcl1 and Pho85/Pcl2 have been implicated to play a role in cell polarity and morphogenesis, as some of the known targets are septin components such. Consistent with a role in morphogenesis Pcl1 and Pcl2 localize to sites of polarized growth and genetically interact with Cdc42 [120].

There are many known targets of Pho85 kinase. The protein targets play role in various cellular processes such as endocytosis and exocytosis (Rvs167), maintaining bud neck integrity and septin organization (Bni4), regulation of glycogen (Glc8) and phosphate (Pho4) metabolism, regulation of cell cycle through transcription (Whi5) and signal transduction during cell proliferation in response to nutrients (Rim15). Since Pho85 regulates variety of processes through post translational modification of the targets, how substrate specificity is achieved temporally and spatially has been the focus of many studies. It has been proposed that substrate specificity is controlled by the CDK and the cyclin it’s paired with [173]. For example, the mammalian Cdk2 has been shown to have differential targets based on the cyclin it is paired with [174]. Likewise in yeast, cyclins have implicated to play a role in determining substrate
specificity for both Cdc28 [175] and Pho85 [176]. Some data suggest that cyclins have substrate docking motifs that could be part of the cyclin box and C-terminus of cyclins [175], [177].

Pho85 has a mammalian functional homologue Cdk5 [178] and they share 56% amino acid sequence identity. Expression of Cdk5 in yeast, complements the *pho85* null phenotypes and expression of Pho85 in insect cells results in phosphorylation of Cdk5 substrates. Unlike other CDKs, Cdk5 is not expressed in proliferative cells. Instead, Cdk5 has been shown to play a critical role in cell polarization during neuronal migration, neurite outgrowth and growth cone guidance [179]. Cdk5 is thought to phosphorylate the Rho GTPase, Rac and the Pak1 kinase, which are both involved in regulation of actin cytoskeleton [180].

Although there are numerous identified targets for Pho85, there are no known targets involved directly in the mating pathway. In a large-scale analysis for phosphorylation sites by specific yeast kinases, Pho85/Pcl1 was predicted to phosphorylate Gβ (Ste4) at the residue S335. It has long been known that Gβ is rapidly phosphorylated at multiple sites in response to pheromone. Phosphorylation at S335 is required for full phosphorylation of Gβ [181]. Phosphorylation of Gβ has been shown to be crucial for its pheromone induced localization to the mating projection and localization of other polarity proteins. Phosphorylation of Gβ is also critical for stabilizing the chemotropic shmoo growth. Furthermore, Gβ phosphorylation is also important for orientation and re-orientation in pheromone gradients. [113]. Lastly, phosphorylation of Gβ was also shown to be important for chemotropism and proper polarization of the pheromone receptor in yeast [96]. Certain kinases have been implicated to phosphorylate Gβ. Deletion of Fus3 results in hypo-phosphorylation of Gβ and hence it has been suggested to phosphorylate Gβ. The yeast casein kinases, Yck1/Yck2 is also thought to phosphorylate Gβ. Gβ and Yck1 exhibit genetic and physical interaction [96]. Since Gβ is phosphorylated at multiple
sites and serine to alanine mutation at specific sites results in 4 different phospho species, it raises a couple of questions such as; Does Gβ phosphorylation at multiple sites occur sequentially or concurrently? And whether multiple kinases could be responsible for Gβ phosphorylation. Considering that, Gβ is still partially phosphorylated in the absence of Fus3 and the casein kinases prefer their targets to be pre-phosphorylated, it is likely that there are more kinases that phosphorylate Gβ. Hence, in this chapter I addressed whether Gβ is a target of the Pho85/Pcl1 complex.

My results demonstrate that only Pcl1, but not its closely related cyclin Pcl2 is required for proper receptor polarization. In contrast to PCL1 deletion, over-expression of Pcl1 causes the receptor to hyper polarize to a very narrow region on the membrane. Pcl1 localizes to sites of polarized growth during mating, similar to its localization pattern in budding cells. Pcl1 localizes as a ring to the tip of emergent shmoos and later to the neck of mature shmoos. Lastly, Gβ and Pcl1 exhibit genetic and physical interaction. Taken together, these data suggest a role of Pho85/Pcl1 in the mating pathway via regulating phosphorylation of Gβ.

b. **Material and methods**

i. **Yeast and bacterial strain construction**

The primers GGCTAGTTACCTATTTAGTGGAAAGAGATCTATTGTTC CCGATGATAAAGCTGTCAAC and CGCAGGCCAGGCATATTTTCGACATTTAAAAT CACGATTTGCCTCTCAAGAAATAGC were used to amplify URA3 from Ycplac33. The 5’ end of the primers were homologous to approximately 300 base pairs upstream and downstream of PCL2 respectively. Transformation of the PCR product into MSY161 resulted in the transplacement of PCL2 gene with URA3 gene resulting in a deletion of PCL2 and generated MSY188 (pctlΔ pcl2Δ) strain. The plasmids MSB37 (Gal-GST-Pcl1) and MSB39 (Gal-GST-
Pho85) were transformed into MSY128 to generate MSY210 and MSY212 strains respectively. RDB121 (Ste4-GFP) was transformed into MSY101 and MSY155 to generate MSY214 and MSY215 respectively. MSY242 was generated by transforming the plasmids MSB49 (p416-VF1-Pcl1) and p415-VF2 into MSY240 strain. Transformation of MSY240 strain with AiB201 (p415-Ste4-VF2) and MSB49 resulted in MSY244. The plasmids p415-VF1 and AiB201 were transformed into MSY240 to generate MSY246. pJM40 (Pcl1-GFP) was transformed into MSY155 to generate MSY316.

All GFP localization assays, over-expression assays with galactose and pheromone treatment were performed as outlined in general materials and methods.

ii. Pheromone halo assays

Top agar was made from synthetic medium containing 0.5% agar and was maintained at 50°C to prevent solidification. 10⁵ log-phase cells were added to the top agar, vortexed briefly and added onto the respective synthetic medium containing plate agar. The top agar was allowed to solidify and dry. 5µg or 10µg of pheromone was spotted onto the center of the plate and cells were incubated at 30°C for 2 overnights. The halo diameters were measured using a ruler. Each halo diameter was measured three times from different points and the average was taken as the halo size.

iii. Split Venus bimolecular fluorescence complementation assay

Over-expression of Gβ results in permanent cell cycle arrest. Hence the BiFC plasmids p415-VF2, p416-VF1, p415-VF2-Ste4, p416-Pcl1-VF1 were expressed under a strain where the mating pathway is only conditionally activated by the induction of GAL-Ste7. MSY242, MSY244 and MSY246 were grown to log-phase in synthetic medium lacking uracil, leucine and tryptophan to select for all plasmids. The cells were treated with 2% galactose for 3 hours and
induced with 30nM of pheromone. Aliquots were acquired every 30 minutes, concentrated with 1X PBS and imaged on DeltaVision Elite microscope equipped with a 60X oil immersion objective. 400µM sections of cells were taken and deconvolved using SoftWorx. Sum projections were made in ImageJ and analyzed for reconstitution of BIFC signal.

c. Results

i. Deletion of *PCL2*, a cyclin closely related to Pcl1 does not affect receptor polarization

Pcl2 is another cyclin for Pho85 which is known to have redundant functions with Pcl1 and the two cyclins belong to the same subfamily [182]. Their activity and expression peak during G1 phase [182], [183]. The two cyclins also have similar localization pattern in the cell. Both Pcl1 and Pcl2 localize to sites of polarized growth during budding to the presumptive bud site [120]. Pcl1 and Pcl2 are required for phosphorylation of key regulators of polarized growth [184], [120] septin ring assembly [185] and morphogenesis [186] in G1. Since Pcl1 and Pcl2 had common properties and function, I tested whether deletion of Pcl2 would also affect receptor polarization. I deleted *PCL2* from *pcl1Δ* cells expressing *STE2-GFP* and generated a *pcl1Δ pcl2Δ* strain expressing *STE2-GFP*. Polarization of the receptor in *pcl2Δ* was comparable to WT. Also *pcl1Δ pcl2Δ* double deletion did not exacerbate the *pcl1Δ* phenotype (Fig. 26). These results indicate that the role of PCl1 in receptor polarity establishment is unique.
**Figure 26: Pcl2 is not required for receptor polarization**

(A) Receptor polarization is not affected in *pcl2Δ* cells. Representative images of WT, *pcl1Δ*, *pcl2Δ*, and *pcl1Δ pcl2Δ* cells expressing Ste2-GFP were acquired at the indicated time points after pheromone treatment using FITC filter. (B) Line graph represent crescent size quantification. Crescent size was measured as a percentage of the cell surface using ImageJ.
ii. **Over expression of Pcl1 but not Pho85 causes the receptor to polarize to a very narrow region on the plasma membrane and increases pheromone sensitivity**

The expression of cyclins, is very tightly controlled and is only active during a very specific stage of the cell cycle. For example, Pcl1 is expressed and is active only during late G1 phase [182]. What happens when the cyclic expression of Pcl1 is taken away? So I next tested whether over-expression of Pcl1 under the control of the GAL promoter would affect receptor polarization. Vegetative cells over-expressing Pcl1 had very faint receptor localization on the PM. Remarkably, pheromone-responding cells over-expressing Pcl1 exhibit the opposite phenotype of pcl1Δ cells (Fig. 27). Over-expression of Pcl1 caused the receptor to hyper polarize to a narrow region on the PM. Under isotropic pheromone conditions, often a receptor polarization defect can be partially rescued due to secretion of newly synthesized receptor to the default polarity site. Even after the cells form mature shmoos the receptor remains localized to a very narrow region. Not surprisingly, over-expression of the kinase Pho85 did not alter receptor polarization as Pho85 is about 10 times more abundant than Pcl1 and is constitutively expressed.

It is worthy of noting that over-expression of Pcl1 increased the pheromone sensitivity of cells. I first performed pheromone halo assays and measured the diameter of halo. Increase in pheromone sensitivity directly correlates to the halo size. I observed a significant increase in halo size in cells over-expressing pheromone (Fig 28A). I also tested the same by a more sensitive assay by directly looking at pheromone responding cells under the microscope and scored the percentage of vegetative cells, single shmoos, double shmoos, adapted or elongated cells. At every time point, there were a higher percentage of cells that were shmooin in cells over-expressing Pcl1 compared to control cells. Pcl1 over-expressing cells also formed double shmoos more frequently. Furthermore, Pcl1 over-expressing cells also took longer to adapt i.e. to turn off
the mating pathway. These observations show that over-expression of Pcl1 increases sensitivity to pheromone.
Figure 27: Overexpression of Pcl1 causes the receptor to hyperpolarize
Representative images of cells expressing Ste2-GFP and Gal Pcl1 or Gal-Pho85. The log-phase cells were either grown in the presence of 2% Galactose to induce protein expression (top two rows) or in the absence of galactose (bottom two rows) as controls. Cells were treated with pheromone and images were acquired at the indicated time points using FITC filter.
Figure 28: Pcl1 overexpression increases pheromone sensitivity

(A) Cells over-expressing Pcl1 exhibit an increased sensitivity to pheromone halo assays. Bar graph represent the halo diameters for Control and cells expressing Gal-Pcl1. Log-phase control cells and cells expressing Gal-Pcl1 were grown in the presence of 2% galactose for 3 hours and $10^5$ cells were plated onto selective media containing galactose. Pheromone was spotted onto the center of the plate as indicated (5 µg or 10 µg). Plates were incubated in 30°C for 2 overnights and to allow for colonies to develop. Halo diameters were measured in centimeters. Mean of three trial were taken and error bars represent standard error of mean.

(B) Cells over-expressing Pcl1 form frequent multiple projections and take longer to adapt. Bar graph represent responsiveness of control and cells over-expressing Pcl1 to pheromone. Log-phase Control and Gal-Pcl1 cells were grown in the presence of 2% galactose for 3 hours, treated with pheromone. The cells were categorized as bud, 1 shmoo, 2 shmoo, adapted or elongated for each time point. n = 300 X 3 biological trials.
A

![Bar graph showing Halo diameter (cm) for different Pheromone concentrations and treatments.](image)

B

![Bar graph showing Cells in the indicated category (%) for different time points and treatments.](image)
iii. **Pcl1 interacts with Gβ**

It is long been known that Gβ is rapidly phosphorylated in response to pheromone [187]. Gβ phosphorylation is critical for efficient chemotropism [113] and establishment of receptor polarity [96]. Gβ is phosphorylated on multiple residues in response to pheromone [96] and most likely has more than one kinase that phosphorylates it. Fus3 and Ycks are required for full phosphorylation of Gβ [96], [181]. Deletion of *FUS3*, results in a hypo-phosphorylation of Gβ [181] and not a complete loss suggesting another kinase activity. Casein kinases also prefers their substrate to be previously phosphorylated [188], [189]. It is likely that there are other kinases that phosphorylate Gβ. Pho85/Pcl1 has been predicted to phosphorylate Gβ [190]. The substrate specificity of a CDK is dependent on the cyclin it’s complexed with [173], [191]. There is evidence to support the notion that, cyclins also have a substrate docking motif [191][174]. Hence, I tested whether Pcl1 and Gβ interact.

1. **Gβ localization is not affected in pcl1Δ cells**

Protein phosphorylation could be critical for appropriate subcellular localization for certain proteins. Gβ polarizes to the mating projection and a non-phosphorylatable form of Gβ polarizes later and less stably than Gβ [113]. Hence, I attempted to visualize GFP-Gβ localization in *pcl1Δ* cells. As expected Gβ was polarized to the mating projection in WT cells (Fig. 28A). *pcl1Δ* cells appeared to polarize Gβ to the mating projection comparable to WT. This was not entirely surprising because as mentioned previously Gβ is likely to be phosphorylated by multiple kinases. Therefore, deletion of *PCL1* might not completely abrogate Gβ phosphorylation.
Figure 29: Localization of Gβ in pcl1Δ cells

Representative images of WT or pcl1Δ cells expressing GFP-Ste4. Log-phase WT and pcl1Δ cells expressing Ste4-GFP were treated with pheromone and imaged at the indicated time points using FITC filter.
2. **Slight over-expression of Gβ in pcl1Δ cells causes the cells to autonomously shmoo and increases pheromone sensitivity**

I observed a unique phenotype that, pcl1Δ cells expressing GFP-Gβ frequently formed shmoos (14%) in the absence of pheromone termed as autonomous shmoos. (Fig. 30A). This is only very rarely (2%) observed in WT cells expressing GFP-Gβ. When Gβ is over-expressed it causes autonomous activation of the mating pathway, cell cycle arrest and shmoo formation. In this experiment, although GFP-Gβ was being expressed under the control of its own promoter, it was being expressed from a yeast centromeric (2-5 copies/cell) vector meaning it was slightly over-expressed. Although WT cells are able to suppress the phenotype of this slight increase in Gβ levels, pcl1Δ cells are unable to do so.

Since slight over-expression of Gβ in pcl1Δ cells, caused autonomous shmooring, I wondered whether it would also cause an increase in sensitivity to pheromone. To test this, I performed pheromone halo assays using WT, WT cells expressing GFP-Gβ, pcl1Δ and pcl1Δ cells expressing GFP-Gβ. WT and WT cells expressing GFP-Gβ had comparable halo sizes. However, pcl1Δ cells had a moderate increase in halo size which was further increased in pcl1Δ cells expressing GFP-Gβ (Fig. 30B). Unlike WT cells, pcl1Δ cells are unable to suppress phenotypes associated with the slight over-expression of Gβ. This is evidence for Pcl1 and Gβ genetic interaction which possibly stems from a physical interaction.
Figure 30: Evidence for Gβ and Pcl1 genetic interaction

(A) Slight over-expression of Gβ in pcl1Δ cells causes autonomous shmooing. Representative images of cells autonomously shmooing in the absence of pheromone in pcl1Δ cells expressing GFP-Ste4. Images were acquired using the FITC filter. (B) Slight over-expression of Gβ in pcl1Δ cells also increases pheromone sensitivity in halo assays. Bar graphs represent the halo diameters for WT, pcl1Δ, and pcl1Δ + GFP-Ste4 cells in centimeters. 5µg of pheromone was spotted onto plates containing 10^5 cells from the respective strain. Cells were allowed to grow for two overnights at 30°C and halo diameters were measured. Error bars represent standard error of mean from three trials.
A

GFP-Ste4

B

<table>
<thead>
<tr>
<th>Halo diameter (CM)</th>
<th>WT+STE4-GFP</th>
<th>pcl1Δ</th>
<th>pcl1Δ + Ste4-GFP</th>
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<tr>
<td></td>
<td>2.1</td>
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<td>2.4</td>
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Error bars indicate standard deviation.
3. **Gβ and Pcl1 physically interact in a bi-molecular fluorescence complementation assay (BiFC)**

In order to determine whether Pcl1 and Gβ physically interact, I took advantage of the split-venus BiFC assay. (Remy et al., 2004). The Venus Fragment 1 (VF1) was fused to the N-terminus of Pcl1 and Venus Fragment 2 (VF2) was fused to the C-terminus of Gβ. The constructs were constitutively expressed under the ADH1 promoter and from a multi-copy plasmid. Recall that constitutive overexpression of Gβ is lethal. Therefore, I expressed these constructs in ste7Δ::KanMX YCplac22/GAL1-STE7 background, which allows for conditional activation of the mating pathway only in the presence of Galactose. If Gβ and Pcl1 physically interacts, this would bring the two halves of Venus proximal, resulting in the reconstitution of the fluorophore and a BiFC signal would be visualized. BiFC can also be used to assay the subcellular localization where the two proteins interact. A BiFC signal was detectable on the plasma membrane of pheromone-treated cells at the shmoo tip or shmoo neck in cell expressing VF1-Pcl1 and Gβ-VF2 (Fig. 31). However, this signal was only observed in approximately 1-2% of the population. A mating specific signal was not detectable in control cells expressing only either VF1-Pcl1+VF-2 or VF1+Gβ-VF2 (data not shown). However, all three cell types had inconsistent membrane signal. Since both Gβ and Pcl1 localize to sites of polarized growth and get delivered to these sites, false positives could be observed. These membrane signals could be consequence of that.

The lethality of Gβ over-expression can be rescued by an interacting partner that sequesters the excess Gβ [87]. While performing the BiFC assay, I observed that cells expressing VF1-Pcl1 and Gβ-VF2 had fewer autonomous shmoos than cells expressing Gβ-VF2 alone. I quantified this by growing the cells in the presence of Galactose overnight and inducing the
mating pathway. The percentage of autonomously shmooing cells while expressing both VF1-Pcl1 and Gβ-VF2 was reduced to about 32% from 48% in cells expressing Gβ-VF2 alone. Furthermore, over-expression of Pcl1 causes a morphogenetic defect and the cells are more oblong or elongated and the newly formed buds also appear elongated. Co-expression of VF1-Pcl1 and Gβ-VF2, also suppressed this phenotype in half (data not shown). Taken together, all these results strongly suggest that Pcl1 and Gβ interact.
Figure 31:  **Gβ and Pcl1 exhibit interaction in a BiFC assay**

Representative images of cells expressing Gβ-VF2 and VF1-Pcl1. Log-phase cells expressing the BiFC constructs, were grown in the presence of 2% galactose for 3 hours and treated with pheromone and images were acquired at the indicated time points using FITC filter. Top row - BiFC signal, yellow arrows point to reconstitution of BiFC signal; Bottom row – DIC images.
iv. **Pcl1 localizes to sites of polarized growth during mating**

Pcl1 has been shown to localize to sites of polarized growth during budding [120]. However, its localization pattern has never been visualized in cells responding to pheromone. If Pcl1 also localized to sites of polarized growth during budding, it would be further suggestive of a role in the mating pathway as well. Hence, pheromone-induced cells expressing GFP-Pcl1 were imaged to observe localization of Pcl1. Pcl1 was found to localize to the tip of mating projection in newly formed shmoos and was later localized to the shmoo neck in mature shmoos. Since, Gβ also localizes to the mating projection, Pcl1 and Gβ appear to have a similar localization pattern in the cells. This supports the hypothesis that Gβ and Pcl1 interact.
Figure 32:  **Pcl1 localization in cells responding to pheromone.**

Representative images of cells expressing GFP-Pcl1. Log-phase cells expressing GFP-Pcl1 were treated with pheromone and images were acquired at the indicated time points using FITC filter. Top row- DIC; Bottom row- GFP-Pcl1. White arrows point to polarized Pcl1 signal.
d. **Discussion**

Cyclin dependent kinases and cyclins were originally identified as essential for regulation of cell polarity events during cell cycle. It is now known that CDKs and cyclins are a large family of proteins that regulate diverse cellular events such as transcription and nutrition sensing. In *S. cerevisiae*, cell cycle is primarily regulated by the essential enzyme CDK Cdc28 and the cyclins Cln1 and Cln2. Even though Pho85 is non-essential, cells lacking Pho85 have pleiotropic defects such as G1 checkpoint delay [192], morphological defects [182][193] and glycogen accumulation [176]. My results suggest a novel role for Pho85/pcl1 in the mating pathway. Consistent with this in a review it was suggested that Pho85 could have a role under special growth conditions such as starvation or presence of pheromone (reviewed in [194]).

Pho85 is a functional homologue of the mammalian Cdk5. Over-expression of Cdk5 in *S. cerevisiae* rescues Pho85 null phenotypes [178], [195]. Similar to Pho85, Cdk5 is also not required for cell division. During embryogenesis, Cdk5 is essential for normal brain development [196]. In the adult brain it is necessary for multiple functions such as pain signaling [197] and associative learning [198]. More importantly, during axonal growth which is a chemotropic process, Cdk5 localizes to the growth cone [199] where it is thought to phosphorylate numerous substrates that include micro-tubule associated protein 1B [200] and Pak1 which are involved in cytoskeletal regulation [180], [201]. Like its mammalian homologue, it seems likely that Pho85 also has a previously unappreciated role in chemotropism and direction sensing in Yeast. Although Cdk5 is important for axon guidance, its exact role is still not clearly understood. Perhaps we can also learn more about Cdk5’s role in axon guidance by studying the role of Pho85 in the mating pathway and identifying novel substrates.
The expression and localization pattern of Pcl1 are suggestive of a role in the mating pathway. First of all, *PCL1* expression and activity peaks at late G1 phase which coincides with the only time during cell division that cells can respond to pheromone. The localization of Pcl1 to the presumptive shmoo site, tip of mating projection in early shmoo and later at the neck in mature shmoos is also quite intriguing. Gβ also localizes to the incipient shmoo site and the mating projection [113] suggestive of an interaction of with Pcl1. The results demonstrate a Gβ-Pcl1 genetic interaction. However, by nature of genetic interactions, they might not necessarily be direct. My quest to identify a physical interaction by BiFC was perplexing. Although a mating specific signal is only identified in the BiFC strain and not the experimental strain, there were only 1% of the cells where a reconstitution of the signal was observed. This raises the question why is a signal observed only in 1% of the cells? It is possible that this could be a technical issue. Perhaps changing the orientation of the tags in one or both proteins of interest would help boost the signal. It is also likely that, Gβ and Pcl1 does not interact directly but indirectly through another protein or set of proteins. Thoughts on how to address this question will be discussed in the future directions section.

It is worthy of also noting that Septins have been recorded to localize as bars to the neck of the mating projection [202]. Septins are complex of GTP binding proteins that are involved in formation of higher ordered structures and act as barriers during cell division and cytokinesis. It has been suggested that Septins have distinct roles in mating like acting as a scaffold (reviewed in [203]). Septins are thought to promote localization of certain proteins involved in mating like Afr1 [202] and Glc7 [204]. It is possible that Septins also play a barrier role in the mating projection, perhaps by promoting localization of proteins involved in the mating pathway by preventing them from diluting away from the front of the cell (reviewed in [203]). Pho85/Pcl1
has been demonstrated to phosphorylate Shs1, a non-essential component of the septin ring [185] which like Pcl1 localizes to the tip of the mating projection [205]. This raises the immediate question if localization of Pcl1 is dependent on Shs1, vice versa or both. Also how would receptor polarity and mating projection appear in shs1 mutant cells.

Our results suggest that Pho85/Pcl1 is required for the establishment of receptor polarity and chemotropism. Although the cyclins Pcl1 and Pcl2 share many redundant functions, that does not seem to be the case here. It is likely that Pcl1 targets specific substrate(s) in response to pheromone. I propose a model in which Pho85/Pcl1 promotes receptor polarization by phosphorylating Gβ (Fig. 33). As previously mentioned in the introduction, Gβ phosphorylation is crucial for chemotropism and receptor polarization [96], [113]. Gβγ gets phosphorylated perhaps by multiple kinases Fus3, Pho85/Pcl1 and Yck1/2. On the up gradient side, slightly more receptor activation results in more G-protein Ga and Gβγ activation at the front compared to the back. Yeast casein kinases normally would phosphorylate the receptor which triggers the ubiquitination and internalization of the receptor. It is possible that Gβ gets sequentially phosphorylated perhaps by Fus3 or Pho85/Pcl1 first which then makes it a better substrate for YCKs thereby protecting the receptor from internalization. Better protection of receptor then facilitates more activation of G-proteins which then results in a positive feedback loop that aids in the establishment of receptor polarity.

Although most of my work is phenomenological, it raises a lot of questions that are readily testable and would help gain insight into the mechanism of how Pho85/Pcl1 facilitates in the receptor polarization. For example, does Pho85/Pcl1 directly phosphorylate Gβ or is it indirect? What other substrates do Pho85/Pcl1 phosphorylate in a pheromone specific manner and are those events required for chemotropism or receptor polarization?
Figure 33: Proposed model for Pcl1s role in receptor polarization.

The model illustrates the postulated feedback loops involved in directional sensing. Phosphorylation of Gβ have been shown to be crucial for differential internalization of receptor and chemotropism. Pho85/Pcl1 or Fus3 could phosphorylate Gβ first (loop 1), making it a better substrate for Yck1/2 thereby locally inhibiting receptor internalization (loop 2). Gβ would then be able to better protect the receptor from internalization from the membrane which would in turn result in the activation or recruitment of more G-proteins on the up-gradient side. These positive feedback loops would result in the polarization of the receptor at the chemotropic site. The red box marks the loop in which Pho85/Pcl1 is postulated to function.
Image credit to Dr. Nicholas Waszczak
e. Future directions

i. Does deletion of PCL1 result in hypo-phosphorylation of Gβ by MS analysis

In order to directly ask if Pcl1 can contribute to phosphorylation of Gβ, MS analysis could be performed with vegetative or pheromone treated, WT and pcl1Δ cells and screen for Gβ hypo-phosphorylated in pcl1Δ cells. If so, one could analyze which residues are specifically hypo-phosphorylated. A comparison of the vegetative dataset with the pheromone-induced data set would reveal pheromone-induced phosphorylation sites that are dependent on Pcl1. Lastly, other substrates that are hypo-phosphorylated in pheromone-induced pcl1Δ cells could also be identified by this assay.

ii. Identify interacting partners for Pcl1

An affinity-capture assay with Pcl1 could be performed to identify its interacting partners. Since Pcl1 is only predicted to be around 600 copies/cell, a pull down with native levels of Pcl1 would most likely have technical difficulties. Therefore, a pull down can be performed with cells over-expressing GST-Pcl1 and assay all the proteins that come along with Pcl1 by MS analysis. If Gβ is one of the hits from this assay, it would further strengthen the claim for Pcl1-Gβ interaction and that Gβ is a target for Pho85/Pcl1.

iii. In vivo kinase assay to test whether and analog sensitive Pho85 phosphorylates Gβ

In order to directly demonstrate that Gβ is a target of Pho85-Pcl1, an in vivo kinase assay could be performed. Analog-sensitive (AS) kinases have been used previously to identify its substrates [206]. The AS kinases have been mutated at the binding pocket which makes it larger to fit bulky analogs such as radiolabeled ATP. Only Pho85-AS kinase can use the bulky radioactive analog of ATP to phosphorylate its substrates, whereas all other cellular kinases would
only be able to use regular ATP. Lysates from cells expressing Pho85-AS and HA-Gβ should be incubated with the radiolabeled ATP and immuno-purified HA-Gβ should be assayed for the incorporation of radiolabel in the substrate.

iv. **Role of septins in receptor polarization**

Since Pho85/Pcl1 have known septin component targets such as Bni4 and Shs1 and that the localization of Pcl1 in mating cells resemble septin ring localization pattern, it would be worthy of testing if septins play a role in receptor polarization. Key questions of interest would be whether Pcl1 localization is dependent on Bni4 or Shs1 or if localization of septin ring components are affected in *pcl1Δ* cells. Another key experiment to do would be to analyze whether receptor polarization is affected in *bni4Δ* or *shs1Δ* cells.
VI. Perspectives

Chemical gradient-directed cell movement and growth i.e. chemotaxis and chemotropism respectively, are crucial to all eukaryotes during the development and beyond. Chemotaxis underlie cell migration during development, neutrophil and macrophage migration. Angiogenesis and axonal growth are guided by chemotropism. Both chemotaxing and chemotroping cells, sense the chemical gradient by specific receptors expressed on the cell surface. These receptors belong to the family of receptors called GPCRs. In nature, the chemical gradients perceived by these cells are very shallow and dynamic. Therefore, one of the main question that has driven the scientists in the field has been, how do cells interpret such subtle cues and what are the molecular mechanisms involved? The process by which the cells interpret the gradients is termed directional sensing. The mating of budding yeast has served as an excellent model system to study chemotropism and directional sensing.

Membrane domains and their role in cell polarization and chemotropism: My work has implicated a protein that belongs to the claudin superfamily to be involved in PM lipid organization. The existence of membrane domains and their role in cell polarization is a relatively new and upcoming field. The role of lipid rafts in cell polarity and signaling is only just now being explored. A detailed study of how these domains are generated and maintained would provide crucial insights into cell polarization dynamics that were previously underestimated. Although further investigations are required to demonstrate Dcv1’s direct role in organizing lipid domains, the yeast mating system would nevertheless serve as a good model to study how these membrane domains are regulated and if they are necessary for cell polarization, signal transduction and chemotropism.
**Identification of novel mechanisms that contribute to directional sensing:** Like its eukaryotic counterparts, budding yeast senses the external gradient via a GPCR. The GPCR’s are the most upstream component of the pathway. Unlike most mammalian cells, in yeast the GPCR’s physically polarize toward the up gradient. The premise of my project was that the GPCR polarization to the up gradient side was essential for chemotropism. However, recent evidence from our lab suggest otherwise. Although the polarization of GPCR is important for directional sensing, cells lacking the ability to polarize the receptor can still sense the gradient and chemotrop albeit defectively [96]. On the other hand, the phosphorylation state of the receptor also seems crucial for directional sensing. Cells that are completely unable to polarize and phosphorylate the receptor have a severe defect in chemotropism. Perhaps cells lacking the ability to polarize the receptor are still able to sense the gradient by relying on other feedback loops or other unidentified mechanisms. The unphosphorylated receptor has been shown to bind to the RGS protein Sst2 [207]. Even cells that cannot physically polarize the receptor, can concentrate the unphosphorylated receptor and Sst2 on the up gradient side and in mating projections [96]. This suggests that the unphosphorylated (perhaps active) receptor can interact with downstream effectors to guide the cell toward the right direction. Historically, RGS proteins were considered as only negative regulators that shut down the pathway. In a recent screen, it was identified that Sst2 was important for orientation and re-orientation [208]. These observations are suggestive of the existence of other novel mechanisms that contribute to directional sensing and should be investigated in detail. Since most mammalian GPCR’s do not polarize, the newly identified mechanisms would likely be applicable to directional sensing in mammalian cells as well.
Mathematical modeling: In recent years, mathematical modeling has become an integral part of understanding complex biological processes. Since these processes often involve numerous critical components and various feedback loops, biological testing of how all parameters behave in a given cell is not possible. In biological experiments, we are limited to altering only one or two components at a time and the output assayed is also limited. However, mathematical modelling gives the advantage of altering one component and assaying how every parameter in the system behave or is affected. This will help us identify key parameters involved. As we learn more from the computation cell, these can be tested experimentally. Experimental results would in turn feed into the computational model thereby advancing our learning of how the system works. In collaboration with Dr. Jie Liang, we have constructed a computational yeast cell that can polarize in response to pheromone gradients. Although the computational cell has helped us identify components/molecular processes that are critical for yeast chemotropism, this computational cell’s behavior is not as efficient as the real cell. Therefore, constantly combining biological and computational approaches will advance our goal to understand eukaryotic directional sensing.
VII. Appendix-I

a. **Yeast two hybrid screen to identify interacting partners for Dcv1**

i. **Introduction and background:**

A lot can be learned about a protein's function by learning about its molecular interactors. At the outset of my project Dcv1’s biological function was unknown. There were no known molecular interactors as well. Hence I sought to do a yeast two hybrid (YTH) analysis to identify interactors. Since Dcv1 was predicted to be a membrane protein, a regular yeast two hybrid analysis would not work, as YTH require proteins to localize to the nucleus and localizing a hydrophobic membrane protein to nucleus would result in misfolding and aggregation. Hence I performed Y2H by expressing just the three predicted loops and the C-terminal domain (CTD) of Dcv1.

ii. **Material and methods**

The required plasmids and strains were obtained from Matchmaker Gold Yeast two hybrid system (Clonetech). To generate bait plasmids, the three loops and the CTD were ordered as gene blocks (IDT). The inserts and the bait vector pGBK7 were digested with EcoR1 and BamH1, the products were ligated together to generate the bait vectors. The bait vectors containing L1, L2, L3 and CTD were transformed into Y2HGold MA7a strain to generate the bait strains MSY279, MSY282, MSY284, MSY285. The yeast cDNA prey library was purchased from dualsystems. The prey library was transformed into the Y187 prey vector by following the procedure outlined in the Matchmaker Gold user manual. The manual protocols were followed for performing the screen and to sequence colonies.
iii. Results and discussion

From the yeast two hybrid screen, I had 59 unique colonies for loop2 and 42 unique colonies for CTD. Upon sequencing these were narrowed down to 13 genes that were identified as potential interactors from this screen. Of these, there were some hits that interacted with both CTD and L2 and others hits that had interacted only with L2. But there were none that were unique to CTD. However, none of these hits gave more clue into Dcv1 function. I performed a GO term process and component search to find common themes between the hits. There was no significant similarity for the component search. 4 out of 13 genes were found to involved in purine synthesis. Again, this did not advance our understanding about how Dcv1 could contribute to receptor polarization. Expressing just small domains of Dcv1 might not be sufficient to identify all its interactors. Perhaps more than one domain is required for the critical interactions and such interactions would not be identified from this screen as only one domain is expressed in these cells. It is possible that, if these domains were co-expressed more hits would be identified. This problem could be solved by performing a MYTH screen with the full length protein as described in IV.e.ii (Future directions for chapter IV). At the beginning of this experiment, I hadn’t yet successfully tagged DCV1 with a fluorescent protein. Since the MYTH screen requires the protein of interest to be tagged with N-terminal half of ubiquitin (NubG), I was unable to do the MYTH screen. With Dcv1 successfully tagged with mCherry, the MYTH screen can be performed by tagging the NubG domain at the same site in loop 2.
Table 4. Hits from Yeast Two Hybrid screen

<table>
<thead>
<tr>
<th>Identified homology region</th>
<th>Description</th>
<th>Redundancy in clones (%)(^a)</th>
<th>Clone hit(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sim1</td>
<td>Protein of the SUN family (Sim1p, Uth1p, Nca3p, Sun4p); may participate in DNA replication;</td>
<td>16.66</td>
<td>CTD and L2</td>
</tr>
<tr>
<td>Pnc1</td>
<td>Nicotinamidase that converts nicotinamide to nicotinic acid</td>
<td>5.4</td>
<td>CTD and L2</td>
</tr>
<tr>
<td>Bye1</td>
<td>Negative regulator of transcription elongation; contains a TFIIS-like domain that associates with chromatin and a PHD domain that interacts with H3K4me3</td>
<td>48.9</td>
<td>CTD and L2</td>
</tr>
<tr>
<td>RPL20A/RPL20B</td>
<td>Ribosomal 60S subunit protein L20A; homologous to mammalian ribosomal protein L18A, no bacterial homolog</td>
<td>3.3</td>
<td>CTD and L2</td>
</tr>
<tr>
<td>Adh2/3</td>
<td>Alcohol dehydrogenase II/III</td>
<td></td>
<td>CTD and L2</td>
</tr>
<tr>
<td>Sba1</td>
<td>Co-chaperone that binds and regulates Hsp90 family chaperones; plays a role in determining prion variants; important for pp60v-src activity in yeast; homologous to the mammalian p23 proteins, and like p23 can regulate telomerase activity</td>
<td>3.3</td>
<td>CTD and L2</td>
</tr>
<tr>
<td>SBA1 AND ADH1/2</td>
<td>Mostly Sba1. Pieces of Adh1 and 2</td>
<td>3.3</td>
<td>CTD and L2</td>
</tr>
<tr>
<td>RPS4b</td>
<td>Protein component of the small (40S) ribosomal subunit</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Adh1</td>
<td>Alcohol dehydrogenase; fermentative isozyme active as homo- or heterotetramers; required for the reduction of acetaldehyde to ethanol, the last step in the glycolytic pathway</td>
<td>3.3</td>
<td>CTD and L2</td>
</tr>
<tr>
<td>Gpm1</td>
<td>Tetrameric phosphoglycerate mutase; mediates the conversion of 3-phosphoglycerate to 2-phosphoglycerate during glycolysis and the reverse reaction during gluconeogenesis</td>
<td>1</td>
<td>L2</td>
</tr>
<tr>
<td>Pfk1</td>
<td>Alpha subunit of hetero-octameric phosphofructokinase; involved in glycolysis</td>
<td>1</td>
<td>L2</td>
</tr>
<tr>
<td>Por1</td>
<td>Mitochondrial porin (voltage-dependent anion channel); outer membrane protein required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability</td>
<td>1</td>
<td>L2</td>
</tr>
<tr>
<td>Ser3/33</td>
<td>3-phosphoglycerate dehydrogenase; catalyzes the first step in serine and glycine biosynthesis</td>
<td>1</td>
<td>L2</td>
</tr>
</tbody>
</table>

\(^a\) - Redundancy was calculated as a percentage of the number of clones that were positive for that particular gene to the total number of hits for either C-terminal domain (CTD) or Loop2 (L2).

\(^b\) - The clone hit represents which domain of Dcv1 interacted with the respective gene.
VIII. Cited literature


IX. VITA

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