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## External signal mediated polarized growth in fungi

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## Summary

As the majority of fungi are non-motile, polarized growth in response to an external signal enables them to search for nutrients and mating partners, and hence is crucial for survival and proliferation. While the mechanisms underlying polarization in response to external signals has commonalities with polarization during mitotic division during budding and fission growth, the importance of diverse feedback loops regulating external signal mediated polarized growth is likely to be distinct and uniquely adapted to a dynamic environment. Here we highlight recent advances in our understanding of the mechanisms that are crucial for polarity in response to external signals in fungi, with particular focus on the roles of membrane traffic, small GTPases and lipids, as well as the interplay between cell shape and cell growth.

## Introduction

Polarized growth in fungi occurs by insertion of new material into the plasma membrane *via* the secretory pathway, and concomitant remodeling of the cell wall. Such growth can be triggered by internal cues, *e.g.* cell cycle progression or external cues, including changes in the environment or the presence of peptide mating pheromone. During external signal-mediated polarized growth in fungi, growth is either directed towards a source of attractant or occurs in a random direction. The presence of an external signal initiates polarized growth and typically overrides or masks growth dictated by an internal signal. Primary examples of fungal external

signal-mediated polarized growth are mating projection formation in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and hyphal cells in filamentous and dimorphic fungi, including *Neurospora crassa*, *Aspergillus nidulans* and *Candida albicans* (Figure 1). In fungi, polarized growth occurs in the presence of an internal hydrostatic pressure (turgor), which is on the order of 0.5 – 1.5 MPa for the aforementioned species, that is 0.1 mg/ $\mu\text{m}^2$  [1-8]. In each of these fungi, an initial asymmetry is triggered in response to an external signal. This limited asymmetry, which is typically observed as a cluster of an activated small GTPase, is reinforced by feedback loops and ultimately directs site-specific membrane traffic. Here, we will focus on new findings with respect to external signal mediated polarized growth in fungi, more specifically regarding membrane traffic, small GTPases and lipids, as well as the interplay between cell shape and polarized growth (Figures 2 and 3). We will not discuss chemotropism and cell fusion in *S. cerevisiae* and *S. pombe*, which have been extensively reviewed recently [9].

### ***Membrane traffic***

For polarized growth in fungi, new material has to be inserted into the plasma membrane at a spatially restricted location. Site-specific exocytosis is not sufficient to generate localized growth, as diffusion in the plane of the plasma membrane would result in an ever-extending zone of growth. Hence, it is critical to limit the zone of insertion of new membrane material, and several studies have revealed that endocytosis fulfills this function in budding and filamentous fungi [10-14]. Recent work in *S. cerevisiae* suggests that exocytosis is directly coupled to endocytosis *via*

an interaction between the Rab8 homolog Sec4 and the WASp protein homolog Las17/Bee1 at the plasma membrane [15]. While this molecular link between an exocytic Rab GTPase and a protein that promotes cortical actin patch formation and is required for endocytosis is intriguing, it remains to be seen whether this is a common theme in other fungi. In filamentous fungi, the endocytic collar is several microns back from the filament tip [16,17], in a region where homolog of Sec4 has yet to be observed. Nonetheless, endocytosis is clearly important for polarized growth in hyphal filaments, as well as in budding and fission yeast [13,16,18,19]. Indeed, membrane traffic is critical for morphogenesis in filamentous fungi, such as *N. crassa* and *A. nidulans* [16,20], as well as for the maintenance of polarized hyphal growth in *C. albicans* [21], and is regulated, in addition to Rab GTPases, by Arf GTPases. Recent studies investigating how membrane traffic regulates hyphal growth focused on Arf GTPases, as well as the AP-2 adaptor complex.

In *S. cerevisiae*, Arf3 (the homolog of the human Arf6) specifically interacts with cell polarity proteins and endocytosis factors [22,23] and the *A. nidulans* homolog ArfB, as well as the *Magnaporthe oryzae* homolog Arf6, regulate endocytosis and maintain polarized hyphal growth [24,25], yet surprisingly in *C. albicans* and *A. gossypii*, *arf3* mutants are unaffected for endocytosis and hyphal growth [26,27]. In contrast, in *C. albicans*, the Arf-like protein Arl1 is important for hyphal growth, with an *arl1* mutant exhibiting shorter hyphae and multiple germ tubes, indicating that Arl1 is required for maintaining growth to a single site [26]. Together, these studies highlight the importance of Arf GTPases in polarity maintenance in different fungi.

The heterotetrameric complex AP-2 has been shown to be critical for relocalization of the cell wall stress sensor Mid2 to the tip of a mating projection in *S. cerevisiae* [28] and, in *S. pombe*, the AP-2 alpha subunit Apl3 is important for cell wall synthesis and morphogenesis [29]. Although dispensable for fungal endocytosis in general, AP-2 was very recently shown to be essential for the maintenance of hyphal polarity in *A. nidulans* [30], *Fusarium graminearum* [31] and *C. albicans* [18], via the regulation of lipid polarization and cell wall deposition. In *A. nidulans* and *F. graminearum* AP-2 interacts with lipid flippases (DnfA and DnfB) [30,31], and it maintains ergosterol polarization specifically at the *C. albicans* hyphal tip and not at the bud tip [18], highlighting the difference between these two modes of growth. Furthermore, in *C. albicans*, the AP-2 mu subunit (Apm4) was shown to be critical for the recycling of the cell wall biosynthesis enzyme, chitin synthase 3 (Chs3), resulting in elevated chitin synthesis, associated with morphological defects in the *apm4* deletion mutant [18]. Together, these studies point to a critical role of AP-2 in lipid polarization, necessary to sustain fungal polarized growth.

### ***Lipids***

Phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol phosphates (PIPs) are the major phospholipids of the yeast plasma membrane, together comprising over 80% of phospholipids [32], and the sterol ergosterol comprises ~half of the lipids on the inner leaflet of the plasma membrane [33]. In yeast, over 80% of the phospholipids are found on the inner leaflet of the plasma membrane [34], and it is likely that the negative charge of PS and PIPs is important

for protein localization and clustering. PS is enriched at sites of growth, such as *S. cerevisiae* and *S. pombe* tip of mating projections [35,36] and *C. albicans* hyphae [26], and is critical for polarized growth in response to an external signal, during shmoo and hyphal formation [35,37]. Upon perturbation of PS levels, defects in Cdc42 localization in *S. cerevisiae* shmoos and *S. pombe*, as well as in *C. albicans* hyphae, and Rho1 localization in *S. pombe* and *C. albicans* [35,36,38] were observed, indicating that this negatively charged lipid is important for Rho GTPase distribution. In these fungi, PS forms a gradient from the growth site [26,35,36]. Interestingly, PS appears to regulate the size of Cdc42 nanoclusters in *S. cerevisiae* budding cells, dependent on the Bem1 scaffolding protein [39]. Recent work in this organism indicates that multivalent anionic lipid interactions are crucial for establishing polarity and occur *via* the Bem1-Cdc42 GEF (Guanine nucleotide Exchange Factor) complex at the site of bud growth [40]. This lipid – polarity protein association affects membrane rigidity in a sterol-dependent fashion and limits the diffusion of Cdc42. Intriguingly, it was recently shown that PS similarly stabilizes nanoclusters of the Rho GTPase ROP in plants [41]. Whether such lipid dependent clustering is critical for cell polarity in response to external signals in shmoos and hyphae is not yet known.

Phosphatidylinositol phosphates are also important for polarized growth in fungi. In particular, plasma membrane PI(4)P and PI(4,5)P<sub>2</sub> are critical for external signal mediated polarized growth in *S. cerevisiae*, *C. albicans* and *N. crassa* [17,42-45]. PI(4,5)P<sub>2</sub> is enriched at the tip of the mating projection in *S. cerevisiae* [46] and forms a long-range gradient in the *C. albicans* hyphal filament emanating from the

apex [45], while PI(4)P forms a steep gradient in *C. albicans* hyphae [17]. It is likely that such polarized distributions of these PIPs play a critical role in reinforcing cell polarity by binding small GTPases and their effectors. For example, the polybasic region of Rho GTPases binds acidic phospholipids, and PIPs, including PIP<sub>2</sub>, are important for targeting these GTPases to the plasma membrane [47]. In addition, a number of proteins with varied roles in polarized growth bind PI(4,5)P<sub>2</sub>, including the PAK kinase Ste20, the CRIB containing Cdc42 effector Gic2, septins [48-50], exocyst subunits Exo70 [51] and Sec3 [52,53]. Interestingly, studies in mammalian cells show that protein targeting to the plasma membrane requires both PI(4)P and PI(4,5)P<sub>2</sub>, and suggest that PI(4)P has functions at the plasma membrane independent of its role as a PI(4,5)P<sub>2</sub> precursor [54]. Indeed, in *S. cerevisiae*, PI(4)P is required to localize the PAK kinase Cla4 to sites of bud growth [55].

In *S. cerevisiae* budding cells, Osh proteins, which are homologs of the oxysterol-binding proteins, are important for polarized exocytosis [56,57], and recent work in *S. pombe* shows that ER-plasma membrane contact sites spatially confine regions of exocytosis [58]. Either Osh4 or Osh6 is sufficient for polarized exocytosis in *S. cerevisiae*, suggesting that PI(4)P (which binds both of these Osh proteins) has a critical role in this process [59]. It remains to be seen whether plasma membrane PI(4)P has a specific function in external signal-mediated polarized growth, and whether ER-plasma membrane contact sites, which play a crucial role in regulating this phospholipid [60], are important for such growth responses.

### ***Small GTPases***

The highly conserved Rho GTPase Cdc42 is critical for cell polarity in a range of systems and a small asymmetry in the distribution of this activated GTPase, which is subsequently amplified by positive feedback that include local activation, typically defines sites of growth [61]. Two key characteristics of the feedback are that active Cdc42 recruits additional activators to growth sites and that the various polarity components exhibit different mobilities in the membrane [62]. The association of active Cdc42 with Bem1 increases the activation of additional Cdc42, *via* the sole Cdc42 activator, the GEF Cdc24 [63]. In addition, Bem1 increases Cdc24 phosphorylation *via* the PAK kinase Cla4, which ultimately results in Cdc24 becoming insensitive to stimulation by Bem1. Bem1 interactions with anionic lipids are also critical for Cdc42 nanoclustering and reduced mobility of active Cdc42 at sites of growth [39,40]. A recent optogenetic study in *S. cerevisiae* budding cells revealed that local recruitment of either Cdc24 or Bem1 is sufficient to trigger cell polarization, yet the specific response depends on whether Cdc24 or Bem1 was recruited, the cell cycle stage and pre-existent polarization sites [64]. How site-specific recruitment of these proteins affects cell polarity in response to an external signal, such as mating pheromone, is currently unknown. Very recent studies in *C. albicans* show that recruitment of constitutive active Cdc42 all over the plasma membrane resets polarity in highly asymmetric hyphal cells [65]. Increasing the level of active Cdc42 on the plasma membrane disrupts the localization of Sec3 and results in a striking *de novo* clustering of secretory vesicles, which is highly dynamic

until it settles down at a new growth site. These results highlight that even in the absence of directional growth, membrane traffic retains some organization.

GTPase activating proteins (GAPs) play an important role in restricting the distribution of active Cdc42. During vegetative growth, Cdc42 GAPs prevent *S. cerevisiae* budding within the previous division site [66], and restrict active Cdc42 to *S. pombe*, cell tips [67,68], yet the role of these GAPs in external signal mediated growth is less clear. For example, *C. albicans* cells can form hyphae even in the absence of the GAPs Rga2 and Bem3 [69], and *S. pombe* cells lacking all three Cdc42 GAPs (*RGA3*, *RGA4* and *RGA6*) were able to polarize in response to pheromone [70]. Nonetheless, in the absence of Rga4, which localizes to cell sides, *S. pombe* cells are somewhat wider [67,71]. In *S. cerevisiae* and *C. albicans*, there is a sole GEF for Cdc42 at the growth site, yet, in *S. pombe*, there are two Cdc42 GEFs, one localized at the growth site (Scd1) and one broadly distributed in the cytoplasm (Gef1) that is normally antagonized by Rga4, which may provide an additional level of regulation of Cdc42 activation [72]. While deletion of *RGA3* does not result in morphological defects during *S. pombe* vegetative growth, during mating, strains had longer shmoos, which appear to be less well oriented towards a mating partner [70]. Together, these results indicate that GAP activity would only play a minor role in external-signal mediated fungal polarized growth.

### ***Effects of cell shape***

In *S. pombe*, the size of the active Cdc42 domain scales with local cell-surface radius of curvature over a large range, and is independent of cell volume and surface [73].

In another fission yeast, *Schizosaccharomyces japonicus*, active Cdc42 also scales with changes in cell size, presumably cell surface radius of curvature [74]. These studies suggest that local cell surface curvature is, in some fashion, sensed and responded to by the cell. Cell curvature can be directly sensed, as during *S. cerevisiae* mating Cdc42 localization is negatively affected by the positive curvature of the zone of cell fusion [75]. Computational studies also suggest that cell geometry has striking effects on reaction-diffusion models of fungal polarization, with the active Cdc42 domain not stably localized to the tip of cells with shmoo-like morphology, while it is stable at filament tips, similar to those of hyphal filaments [76]. Consistently, recent results from optogenetic perturbation of cell polarity in *C. albicans* show that the polarity site is easier to disrupt in cells with short filaments, compared to cells with longer filaments [65]. In addition, mechanical perturbation, *e.g.* when a cell encounters a barrier, destabilizes regions of active Cdc42, suggesting that there is a dynamic feedback between growth and stable cell polarity [77]. Cell geometry can also affect spatial chemoattractant signaling, for example pheromone is released from sites of growth during *S. pombe* mating [78]. Together, these studies point to a critical role of cell geometry on polarized growth, as local cell curvature could be recognized on larger scales by the actin network and/or on smaller scales by BAR containing proteins [79].

## Conclusion

Research in the past several years has shed light on different mechanisms that play key roles during polarized growth in response to external stimuli in fungi. Cellular

processes that establish an asymmetric state, such as directed exocytosis, site specific incorporation of lipids and localized activation of GTPases have been extensively studied, and more recent work has focused on cellular processes that limit the location of an active species, thereby creating a boundary or a sharp gradient. These processes include a collar of endocytosis for membrane uptake, hydrolysis or transport of lipids and GTPases, as well as GEF phosphorylation, for spatially and temporally limiting GTPase activation. We have emphasized the common themes in external signal-mediated polarized growth in fungi, yet polarization mechanisms vary within fungi and in response to different environments, depending on the speed of the response, cell shape and robustness of response required. In the future it will be critical to quantitate the contributions of different positive and negative feedback loops on polarized growth in fungi with different growth rates, cell sizes and cell shapes to identify how polarity is regulated in an ever-changing environment.

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### **Figure legends**

**Figure 1. Fungal polarized growth in response to external stimuli.** Polarized growth in response to mating pheromone in *S. pombe* (left panel) and *S. cerevisiae* (middle panel), as well as in dimorphic fungi such as *C. albicans* in response to a range of external cues including serum (right panel). The upper row depicts cells that are polarized in response to internal stimuli with grey arrows indicating where new growth is directed. The yellow star indicates a source and gradient of mating pheromone and the graded black arrow (lower row) depicts location of growth in response to external stimuli.

**Figure 2. Contribution of membrane lipids, membrane traffic and Rho GTPases to polarized growth in response to external stimuli.** Schematic shows the different processes critical for initiation and maintenance of polarity in response to an external signal (top panel). The left panel highlights the asymmetric distribution of membrane lipids, depicted by a red to yellow gradient at the plasma membrane, for example of PS, PI(4)P or PI(4,5)P<sub>2</sub>. The green patch at the tip depicts polarity factors, including targeted membrane insertion and GTPases with lipid nanoclusters indicated in blue. The middle panel shows directed membrane traffic, with a cluster of vesicles (Spitzenkörper), which is typically found in filamentous fungi, and green patch indicating zone of membrane insertion. Note the brown dashed ring depicts the endocytic collar, which is several microns back from the growth site. The right panel shows the distribution of a Rho GTPase, such as Cdc42, with the active form indicated in red. The grey arrows indicate diffusion in the plane of the membrane

and GAP proteins limiting the cluster active Cdc42, with Cdc42 inactive form shown in blue.

**Figure 3. Mechanisms critical for Cdc42 membrane recruitment, Cdc42 activation and formation of a polarity cluster.** A) Cdc42 GTPase cycle. GAPs include Rga and Bem3 proteins and their homologs, GEFs include Cdc24, Gef1 and Scd1 and their homologs and guanine nucleotide dissociation inhibitor (GDI) homologs. B) Clustering of polarity proteins and lipids on the inner face of the plasma membrane. PAKs include Ste20 and Cla4 proteins and their homologs, scaffold proteins include Bem1 and Scd2 and their homologs and anionic phospholipids include phosphatidylserine and phosphatidylinositol phosphates. Dashed arrow indicates higher order interactions between polarity clusters (nanoclusters) to generate larger signaling platforms. Figure based upon that of [40] which investigated internal signal mediated polarity in *S. cerevisiae*. Note that the transduction of an external cue to the inner leaflet could be *via* proteins and/or lipids.

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The restriction the location of exocytosis is important for polarized growth, however in fission yeast actomyosin-dependent delivery of exocytic vesicles

is not necessary for polarized secretion. This work shows that endoplasmic reticulum-plasma membrane contact sites limit the location of exocytosis and are critical for morphogenesis in *S. pombe*.

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The authors generated a *S. cerevisiae* strain that effectively lacks endoplasmic reticulum-plasma membrane contact sites and showed that this strain grows in rich media, suggesting that contact sites are not critical for polarized growth. They further demonstrated that plasma membrane PI(4)P is critical for the viability of this strain, as deletion of the OSBP *OSH4* or the PI(4)P phosphatase *SAC1* is synthetically lethal in this strain lacking endoplasmic reticulum-plasma membrane contact sites.

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This study uses a combination of biochemistry, imaging and genetics to show how a scaffold protein (Bem1) regulates cell polarity in the budding yeast *S. cerevisiae*. The authors show that Bem1 increases Cdc24 guanine nucleotide exchange factor (GEF) activity, as well as facilitates the Cdc24 phosphorylation by the PAK Cla4, which abolishes the positive effect of Bem1 on Cdc24 GEF activity.

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The authors use optogenetics to dissect polarization in the budding yeast *S. cerevisiae* and show that the recruitment of the GEF Cdc24 or the scaffold protein Bem1 is sufficient to polarize cells. They demonstrate that site-specific recruitment of Cdc24 or Bem1 activates Cdc42, as well as recruits more polarity proteins to this specific site.

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The authors used optogenetics to localize active Cdc42 all over the plasma membrane in filamentous *C. albicans* cells and show that this resets cell polarity. Optogenetic recruitment of active Cdc42 to plasma membrane disrupts exocytosis by perturbing the localization of the exocyst subunit Sec3 and results in a striking *de novo* cluster of secretory vesicles, which is highly dynamic.

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The authors identify the Cdc42 GTPase-activating protein (GAP) Rga3 in fission yeast as important for regulating the lifetime of unstable patches of Cdc42-GTP during mating. Surprisingly, a triple mutant that lacks all Cdc42 GAPs, is still able to polarize in response to mating pheromone, suggesting that the GAP dependent inactivation of Cdc42 is not critical during external signal-mediated polarization process.

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Intriguingly, the fission yeast *S. pombe* has two Cdc42 GEFs, Scd1 and Gef1. Here, the authors show that while Scd1 (a homolog of Cdc24) is localized to sites of growth, Gef1 is found throughout the cytoplasm. Gef1 activity at the cell sides is regulated by the GTPase-activating protein (GAP) Rga4, providing additional means of regulating cell polarity.

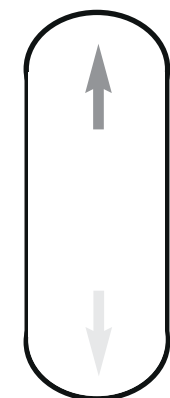
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This work investigates the mechanisms underlying size scaling, by interfering with this process in the fission yeast *S. japonicas*. The authors show that the Cdc42 module is used in fission yeast to modulate its geometry to change cell size.

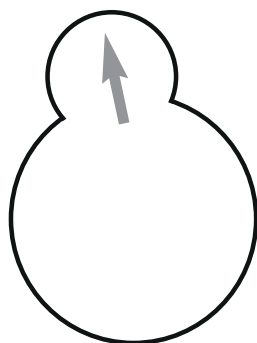
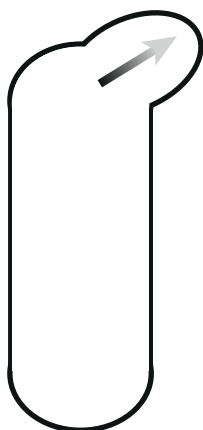
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This study shows that the spatial stability of active Cdc42 domains in *S. pombe* depends on the rate of growth. The authors used a number approaches to reduce growth rate, which results in unstable, wandering domains of active Cdc42, whereas increasing growth rate stabilizes this domain. This effect of growth rate may be a conserved means of responding to mechanical challenges in the fungal environment.

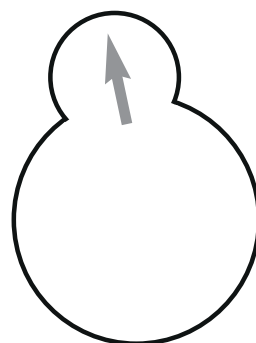
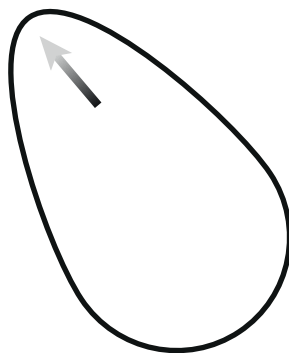
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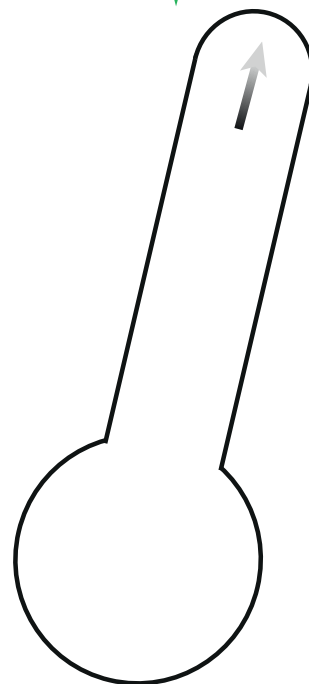
**mating  
pheromone**

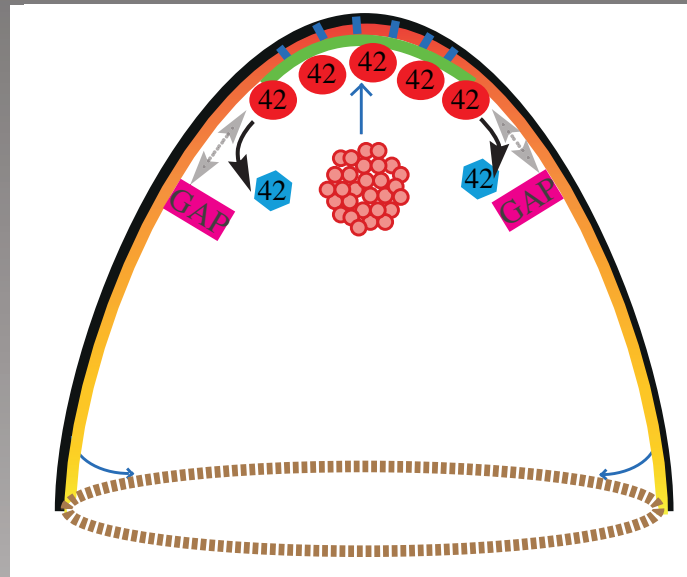


**mating  
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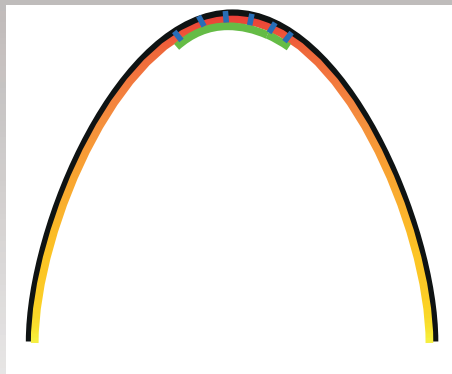


**Serum, 37°C  
*etc.***

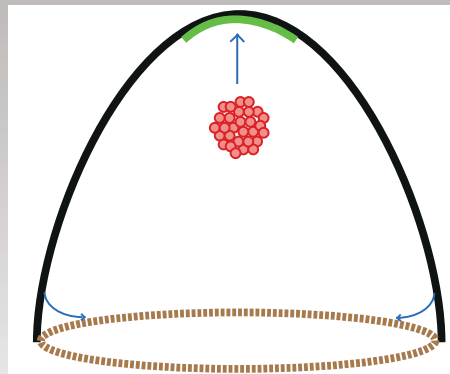




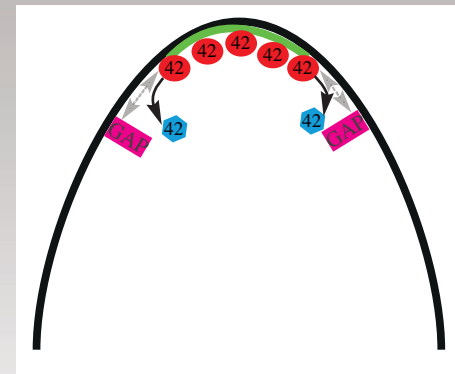
**Membrane lipids**

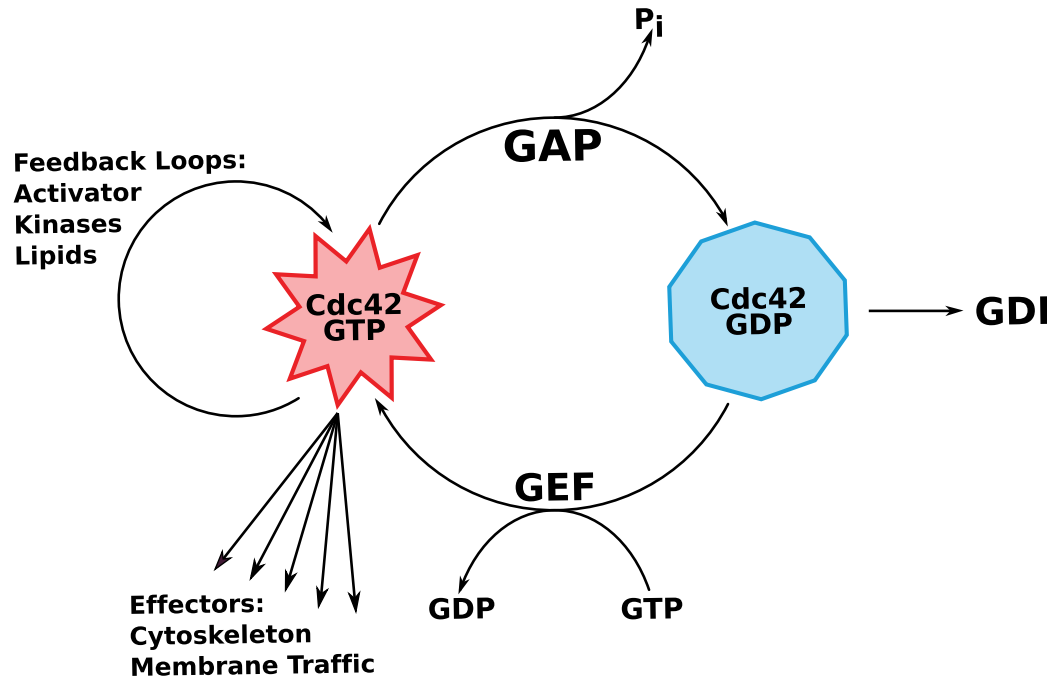


**Membrane traffic**



**Rho GTPase**



**A****B**