something countable, such as trees flashing past. Even if you are not actually counting, you are still having a sensation of countable quantity. Perhaps this is how numerosity is measured in animals. In a range of vertebrate species, the representation of time and numerosity seem to share common principles [14,18], as Kant posited; Boisvert and Sherry's [12] elegant demonstration of a timing sense in bees opens the way for further investigation of these fundamental questions in invertebrates.

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G-Protein Signaling: A New Branch in an Old Pathway

A recent study provides evidence for a new branch of the yeast mating pathway in which a G-protein alpha subunit directly activates phosphatidylinositol 3-kinase at endosomes.

Lee Bardwell

The signal transduction pathway by which yeast cells respond to peptide mating pheromone secreted by nearby cells is arguably one of the most well-understood signaling pathways in eukaryotes [1]. Nevertheless, workers in the field confidently expect the pathway to provide important insights into fundamental signaling mechanisms for decades to come. Few anticipated, however, that a completely new branch of the pathway had remained hidden from thousands of person-years of genetic and biochemical assault, waiting to be revealed by the right approach. Now Slessareva *et al.* [2], by cannily combining functional genomics with a revealing mutant allele, appear to have found such a branch.

G-protein-coupled receptor pathways respond to hormones, neurochemicals, light, odors, and tastes, and constitute a plurality of known drug targets [3]. As with other G-protein-coupled receptor pathways, when the yeast pheromone receptor binds ligand, it stimulates the alpha subunit of an associated heterotrimeric G protein to bind GTP. GTP-bound Gα then detaches from the receptor and releases the Gβγ subunits. One or both members of the newly liberated pair (Gα and Gβγ) then go on to bind to downstream effectors and thus propagate the signal. Gα is also a GTPase; after hydrolyzing GTP to GDP, it rebinds Gβγ, thus terminating signaling. In the yeast pheromone response pathway (Figure 1), Gβγ transmits the mating signal to a scaffolded mitogen-activated protein (MAP) kinase cascade [4]. The two MAP kinases in this pathway, Kss1 and Fus3, then phosphorylate transcription factors, cell-cycle regulators, and other targets that coordinate mating.

Gα proteins were first discovered in mammalian cells as 'transducers' that propagated signals from hormone receptors to second-messenger producers like adenylate cyclase [5]. For many years it was thought that Gβγ did nothing but bury the business end of Gα so that Gα could not signal until it scored some GTP and disengaged. Studies of the yeast mating pathway helped turn that dogma on its head, however, when genetic and (eventually) molecular studies showed that Gβγ transmitted the mating signal to downstream effectors like the Ste5 scaffold protein and the Ste20 protein kinase. As the evidence favoring a signaling role for yeast Gβγ mounted, most workers presumed that Gα did nothing more to transmit the signal than release Gβγ (and perhaps activate a desensitization pathway [6]). Now the dogma is chasing its tail, as the new work indicates a positive signaling role for yeast Gα as well.

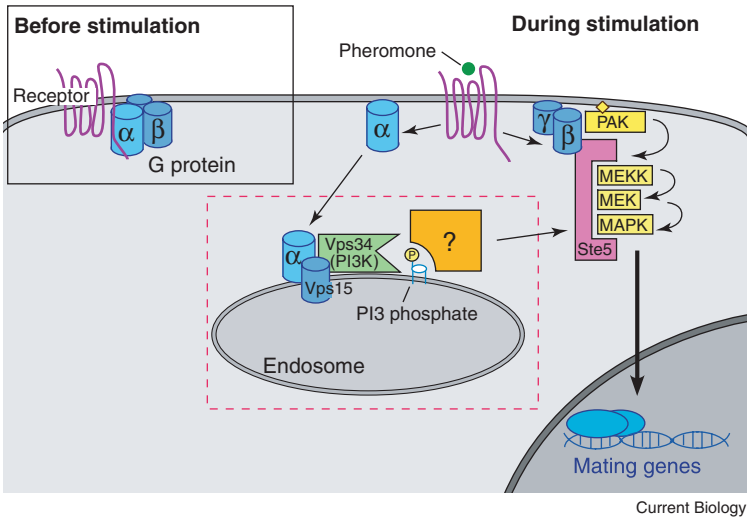

Figure 1. A new branch of the yeast mating pheromone response pathway.

In the 'classic' pathway, pheromone binding to a cognate seven-transmembrane receptor leads to the activation of the Gα subunit of a receptor-coupled heterotrimeric G-protein. Active Gα releases Gβγ, which then binds to both the Ste20 protein kinase (PAK) and the Ste5 scaffold protein. Ste5 tugs Ste11 (MEKK), the first domino in the MAP kinase cascade, to the membrane, where it is phosphorylated and thereby activated by $Ste20^{PAK}$. The new work by Slessareva *et al.* [2] provides evidence for the existence of a new branch of the pathway (surrounded by dotted frame) that modulates the efficiency of signaling through the classic branch. In the new branch, active Gα is proposed to move to the endosome, where it binds PI 3-kinase (Vps34) and to an associated protein kinase (Vps15) and stimulates the production of PI 3-phosphate at the endosome. The figure shows the PI 3-phosphate recruiting an unknown phospholipid-binding protein (represented by ?), perhaps Bem1, which reconnects to the classic pathway at an unknown point upstream of MAP kinase activation.

Slessareva *et al.* [2] started their work by expressing a GTPase-deficient allele of Gα in yeast cells. They observed that it hyper-activated the pathway in a dominant fashion. Because it can bind to GTP but cannot hydrolyze it, GTPase-deficient Gα becomes 'stuck' in the 'on' configuration, that is, constitutively active. But active Gα binds neither to Gβγ nor to the receptor, so the fact that the constitutively active allele had a dominant phenotype indicated that it must bind to something else — a mystery effector. To find this effector, the researchers expressed the constitutively active allele (CA-Gα) and an appropriate reporter gene in almost 5,000 individual yeast strains, each deleted for a different (non-essential) gene. The logic was that strains lacking the mystery effector would not respond to CA-Gα. Among a short list of candidates so generated, one — a protein named Vps34 — was the only phosphatidylinositol 3-kinase (PI 3-kinase) in yeast, and another — Vps15 — was a binding partner of Vps34.

Type I PI 3-kinases are important mediators of growth factor signaling (in which they are activated by receptor tyrosine kinases) and chemotaxis (in which they are activated by G-protein-coupled receptors) [7,8]. PI 3-kinases act at membranes to add a phosphate group to the third position of the inositol ring of phosphatidylinositol. Proteins containing PI 3-phosphate-binding domains (notably Akt/PKB) then congregate at the membrane-localized increase in PI 3-phosphate and start regulating one another. But Vps34, the only PI 3-kinase in yeast, is a member of the type III family, a family that had not previously been implicated in signaling [9].

To support the idea that Gα might regulate PI 3-kinase, Slessareva *et al.* [2] showed that these proteins colocalized in the cell and bound to each other in pull-down assays. Indeed, $Vps34^{PI3K}$, as expected for a *bona fide* effector, bound to Gα in its GTP-bound conformation, and CA-Gα stimulated the production of PI 3-phosphate in cells. In contrast, Vps15 bound to Gα in its GDP-bound conformation, suggesting that Vps15 may play a role similar to a Gβ subunit.

Importantly, the workers also provided some evidence that PI 3-kinase influences signaling during the physiological response to pheromone, and not just in response to the CA-Gα allele: cells lacking PI 3-kinase mated at about one-quarter of the efficiency of their wild-type counterparts, and exhibited a diminished transcriptional response to pheromone. (It must be noted, however, that these defects are modest compared to the phenotype of mutants of the previously known components of the mating pathway.) The defects appeared to be a consequence of a reduced level of activation of the MAP kinase Fus3; hence, the new branch apparently modulates the efficiency of signaling through the 'classic' pathway. Interestingly, the activation of the MAP kinase Kss1 was not affected in cells lacking PI 3-kinase. Thus the new branch selectively regulates one mating MAP kinase and not the other.

Almost as surprising as the existence of this new branch of the mating pathway is where it takes place — at endosomes [10]. Yeast PI 3-kinase has long been known to have an endosomal address, consistent with its original identification as a protein required for proper vesicular sorting [11]. Slessareva *et al.* [2] show that a portion of the pool of CA-Gα colocalizes with PI 3-kinase, and suggest that Vps15 and a protein called Atg14 may act as a Gβγ stand-in to make Gα feel more at home at endosomes. It will be important to determine whether wild-type Gα translocates to endosomes and stimulates the production of PI 3-phosphate during physiological mating. If so, yeast may provide a model for G-protein signaling at the endosome [12].

How could an increase in endosomal PI 3-phosphate augment MAP kinase phosphorylation? Presumably the

mechanism involves the recruitment of a protein(s) containing a PI 3-phosphate-binding domain. Indeed, the authors show that one such protein, Bem1, is recruited to endosomes in cells expressing CA-Gα. But Bem1 cannot be the sole mediator, because it is not required for CA-Gα signaling. One (highly speculative) possibility is that PI 3-kinase promotes the scaffolding function of the Ste5 protein. Ste5 associates with membranes [13] and can execute its scaffold function at internal membranes [14]. Indeed, Ste5 has recently been shown to have a cryptic PH domain that associates with phosphoinositides (although PI 4,5-phosphate, not PI 3-phosphate) [15]. A Ste5–PI 3-kinase connection would explain the observed selective effect of PI 3-kinase on $Fus3^{MAPK}$, as Ste5 scaffolding selectively channels upstream signals to this MAP kinase [16,17]. Furthermore, the integration of Gα- and G$\beta\gamma$-promoted signaling at Ste5 would explain why G$\beta\gamma$ is required for CA-Gα signaling. The identity of the PI 3-kinase effector is just one of many intriguing questions raised by the new work.

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