The Regulation of Filamentous Growth in Yeast

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ABSTRACT Filamentous growth is a nutrient-regulated growth response that occurs in many fungal species. In pathogens, filamentous growth is critical for host–cell attachment, invasion into tissues, and virulence. The budding yeast *Saccharomyces cerevisiae* undergoes filamentous growth, which provides a genetically tractable system to study the molecular basis of the response. Filamentous growth is regulated by evolutionarily conserved signaling pathways. One of these pathways is a mitogen activated protein kinase (MAPK) pathway. A remarkable feature of the filamentous growth MAPK pathway is that it is composed of factors that also function in other pathways. An intriguing challenge therefore has been to understand how pathways that share components establish and maintain their identity. Other canonical signaling pathways—rat sarcoma/protein kinase A (RAS/PKA), sucrose nonfermentable (SNF), and target of rapamycin (TOR)—also regulate filamentous growth, which raises the question of how signals from multiple pathways become integrated into a coordinated response. Together, these pathways regulate cell differentiation to the filamentous type, which is characterized by changes in cell adhesion, cell polarity, and cell shape. How these changes are accomplished is also discussed. High-throughput genomics approaches have recently uncovered new connections to filamentous growth regulation. These connections suggest that filamentous growth is a more complex and globally regulated behavior than is currently appreciated, which may help to pave the way for future investigations into this eukaryotic cell differentiation behavior.

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FILAMENTOUS growth is a fungal differentiation behavior that occurs in response to extracellular stimuli. One stimulus that triggers filamentous growth is nutrient limitation, and filamentous growth is thought to represent a fungal scavenging response. Many different species undergo filamentous growth, including plant and animal pathogens and yeasts like the baker's (or budding) yeast *Saccharomyces cerevisiae*. Because budding yeast is readily amenable to a variety of genetic and genome-wide approaches (Botstein and Fink 2011), relatively recent studies using this organism have shed light on how filamentous growth is regulated, what cues cause it, and what genetic pathways mediate the morphological changes. In this review article, we focus on those advances. Other review articles discuss filamentous growth regulation in filamentous fungi (Steinberg 2007), and in fungal pathogens and the immune response (Netea and Marodi 2010; Hajishengallis and Lambris 2011; Kronstad *et al.* 2011; Moran *et al.* 2011) and summarize findings not described here.

Signal transduction pathways have taken center stage in the effort to understand filamentous growth regulation in yeast. Given that many signaling pathways regulate filamentous growth, and that some of these pathways are composed of proteins that function in multiple pathways, we will stress issues that relate to signal integration and signal insulation between pathways. We will also address the important question of how signaling pathways accomplish the change in cell type from the yeast mode to the filamentous mode. Other review articles have been published recently on filamentous growth regulation (Nobile and Mitchell 2006; Verstrepen and Klis 2006; Whiteway and Bachewich 2007; Zhao *et al.* 2007; Bruckner and Mosch 2011), nutrient-regulated signaling pathways (Hedbacker and Carlson 2008; Zaman *et al.* 2008; Sengupta *et al.* 2010), and mitogen activated protein kinase (MAPK) regulation (Bardwell 2006; Dohlman and Slessareva 2006; Chen and Thorner 2007; Saito 2010), which may offer different perspectives than those described here.

The Filamentous Growth Response

Filamentous growth is a fungal-specific growth mode in which cells adopt a unique morphological pattern that allows expansion into new environments. The filamentation response is highly variable among species, ranging from mycelial mat or hyphal formation in true filamentous fungi to subtle changes in cell shape in yeasts. The biology that attends this response is fascinating and mysterious and ranges from contact-responsive hyphal growth (Kumamoto 2005) to behavior modification of insect species, such as the erratic behavior exhibited by "zombie ants" infected with *Ophiocordyceps* (Pontoppidan *et al.* 2009), to the formation of lasso-type structures in *Nematode*-trapping parasites (Wang *et al.* 2009a). Some species, like the extensively studied fission yeast *Schizosaccharomyces pombe*, have only recently be shown to undergo filamentous growth as part of their life cycles (Amoah-Buahin *et al.* 2005).

The hyphal growth of filamentous fungi is morphologically striking. In *Neurospora crassa*, hyphal cells are multinucleate (Ramos-Garcia *et al.* 2009) and grow in bifurcating branches (Ziv *et al.* 2009) that can undergo cell-to-cell fusion (Steinberg 2007; Aldabbous *et al.* 2010). Fusion is a dynamic process that occurs by hyphal-cell recognition through a MAPK-dependent sensing mechanism (Fleissner *et al.* 2009). Hyphal cells grow rapidly, and cell polarity can be reorganized in response to many different cues. Polarized growth is regulated by a curious structure, the Spitzenkörper (Crampin *et al.* 2005).

Historically, much interest in understanding filamentous growth regulation has come from studies in fungal pathogens. Pathogens like *Candida albicans* and *Aspergillis fumagatus* pose a worldwide threat to human health (Netea *et al.* 2008; Gastebois *et al.* 2009). These pathogens are particularly harmful to individuals whose immune system has been compromised by AIDS or by suppression resulting from chemotherapies and other drug treatments (Ben-Ami *et al.* 2008). Fungal pathogens can also be devastating to plant communities, and harvest loss as a result of damage from fungal species is a serious problem (Rispail *et al.* 2009). In *C. albicans*, transition to the filamentous cell type is critical for virulence (Lo *et al.* 1997) and depends on a multitude of extracellular factors including temperature and nutrient availability (Berman 2006). Pathogenicity of *C. albicans* involves many interrelated processes that include cell-surface variation (Nather and Munro 2008), host–cell adhesion (Latge 2010), biofilm formation (d'Enfert 2009), and chromosome reorganization (Selmecki *et al.* 2010). In other fungal pathogens, like *Cryptococcus neoformans*, filamentous

growth is not tightly related to pathogenicity, as cells primarily exist in the yeast cell type (Lin 2009).

Progress in defining the genetic pathways that regulate filamentous growth has benefited from studies in the versatile fungal eukaryote *S. cerevisiae*. Lessons learned about filamentous growth regulation in budding yeast have turned out to be true for many fungal species. Identifying and characterizing the genetic pathways that regulate filamentous growth in yeast has contributed to understanding the genetic basis of virulence in fungal pathogens and has provided a model for how eukaryotic cells differentiate into morphologically distinct patterns in response to extrinsic cues.

Budding yeast does not undergo true hyphal growth, but rather a pseudohyphal growth pattern in which cells fully separate by cytokinesis—they are not multinucleate—and remain attached to each other by proteins in the cell wall. As for many fungal species, yeast cells can transition between yeast-form growth and filamentous-form growth as part of their life cycle (Figure 1). One of the triggers for filamentous growth in yeast and many other fungal species is nutrient limitation. Both haploid and diploid yeast cells undergo a version of the response, but the stimuli that trigger it, the underlying genetic machinery, and the resulting morphological changes differ slightly between the two cell types. The term invasive growth has been applied to the filamentation phenomenon shown by haploid cells because of their ability to invade agar substrates. The term pseudohyphal growth is sometimes used to describe the response in diploid cells. In this review article, we will use the phrase filamentous growth as a general term that applies to both haploid invasive growth and diploid pseudohyphal development. We will not distinguish between these two highly related responses unless it is crucial to do so in some experimental context.

Filamentous growth in yeast can be separated into three major changes: an increase in cell length, a reorganization of polarity, and enhanced cell–cell adhesion. Assays to study filamentous growth in yeast exist on the macroscopic and microscopic levels. The enhanced cell–cell adhesion of filamentous cells is visible by inspecting yeast colonies (Figure 2A). Cells on the underside of the colonies attach to and invade the agar substratum (Figure 2B), and this invasive growth has been used as a tool to determine whether filamentous growth occurs (Roberts and Fink 1994) and to screen for mutants that are defective at filamentous growth (or are better at it than wild-type cells, *e.g.*, Palecek *et al.* 2000). Changes in cell shape are visible by microscopic examination of cells, and specific assays are used to examine the response in haploid (Figure 2C) and diploid cells (Figure 2D). Using these and other assays, many of the genetic pathways that regulate filamentous growth have been uncovered. Below, we focus on the signaling pathways that regulate the response. We describe what the stimuli are, how they might be sensed, and how the activated pathways induce filamentous growth.

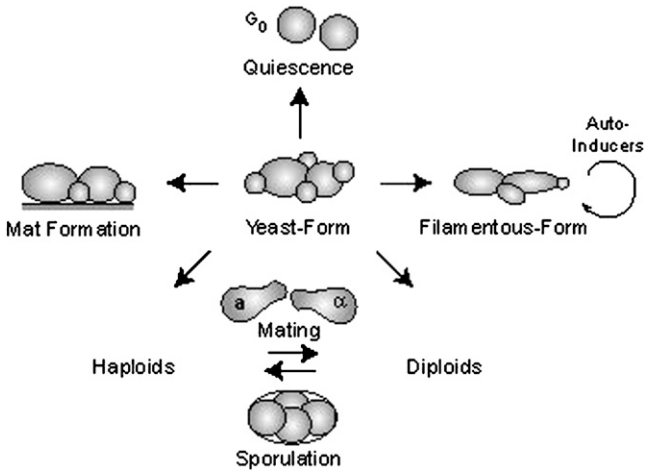

Figure 1 The life cycle of the budding yeast *Saccharomyces cerevisiae*. The diagram shows yeast-form cells, which can be induced to undergo different growth responses depending on ploidy and growth condition. Haploid and diploid cells interconvert between the two types by mating and sporulation, respectively. Both haploid and diploid cells can undergo filamentous growth, form biofilms, or enter stationary phase (quiescence) in response to nutrient (glucose or nitrogen) limitation. Diploid cells also sporulate in response to the limitation of carbon and nitrogen sources. Secreted alcohols act as autoinducers to stimulate filamentous growth.

Signaling Pathways That Regulate Filamentous Growth

Nutrient-sensing pathways

In 1992, the Fink lab rejuvenated a little known finding that the budding yeast *S. cerevisiae* undergoes filamentous growth as part of its life cycle (Gimeno *et al.* 1992). Their study drew attention to anecdotal observations about yeast's growth pattern (Gutlliermond 1920; Lodder 1970; Brown and Hough 1965; Eubanks and Beuchat 1982) and made use of genetic and molecular approaches to gain insights about the underlying mechanism of this unexplored behavior. The initial observation was that isolates of *S. cerevisiae* from a "wild" strain background (Σ1278b) form colonies composed of elongated cells that grow in connected chains on low-nutrient medium. This growth pattern resembles the morphology that is exhibited by some species of filamentous fungi. Filamentous growth is widely considered to represent a nutritional scavenging response, and the Fink lab connected this morphological behavior to nutrition in two important ways: first, strains defective for ammonium utilization were hyperfilamentous (Gimeno *et al.* 1992), suggesting a connection between nitrogen levels and filamentous growth. Subsequent studies have shown that the lack of fermentable carbon source can also be a trigger for filamentous growth (Cullen and Sprague 2000). Second, and more interesting, the global nutrient regulatory GTPase Ras2 was found to be required for filamentous growth regulation. The key experiment used an activated version of Ras2, in which the protein was "locked" in its activated (GTP-bound) state, dramatically stimulated the filamentous

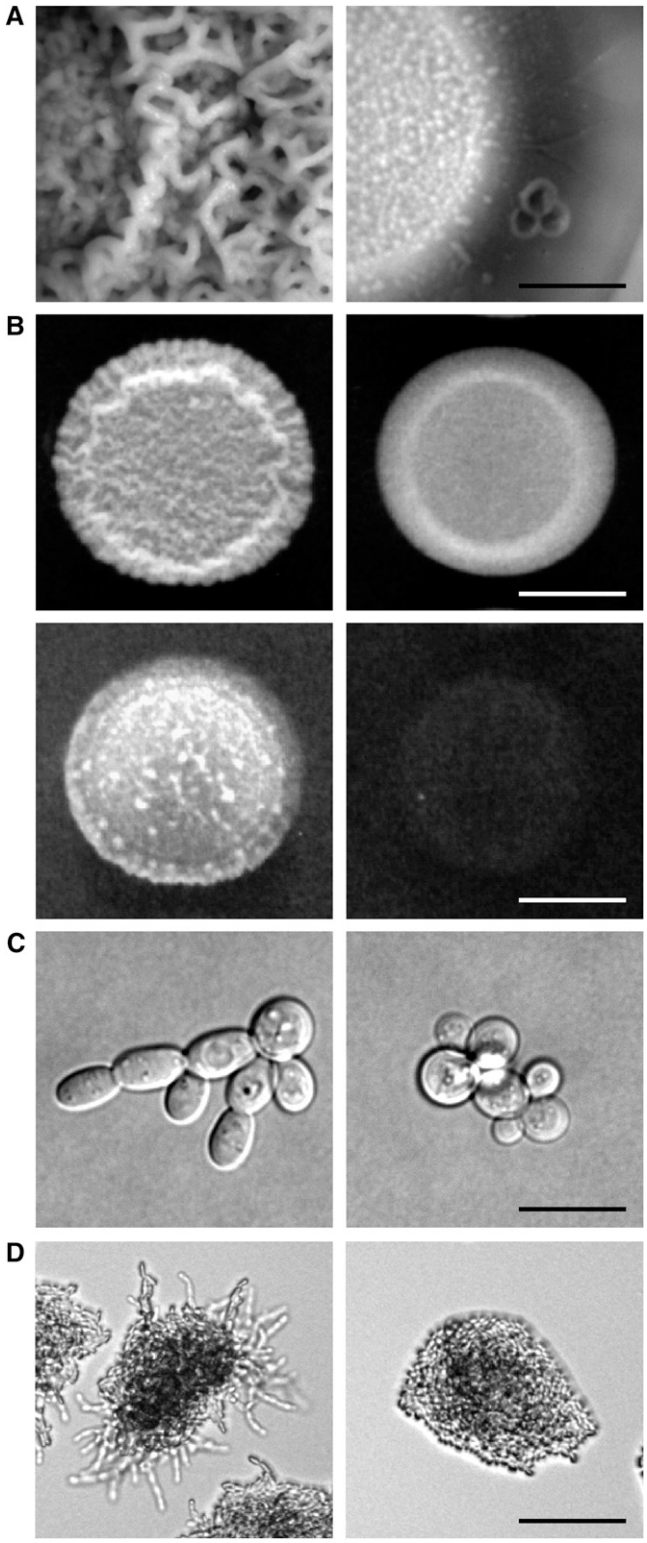

Figure 2 The filamentous growth response. Several biological assays permit the evaluation of the filamentous growth response in yeast, using the Σ1278b strain background. (A) Haploid wild-type (left) and *flo11* mutant (right) colonies grown on YEPD + 4% agar medium for 7 days show the Flo11-dependent colony ruffling. Bar, 0.5 cm. (B) The plate-washing assay (Roberts and Fink 1994). Haploid wild-type (left) and MAPK pathway mutant (right) cells were spotted onto YEPD medium (2% agar). After 3 days the plate was photographed (top), washed in a stream of water, and photographed again (bottom) to reveal invaded cells. Bar, 1 cm. (C) The single cell invasive growth assay (Cullen and Sprague 2000). Cells as in B were spread onto SC medium lacking glucose as a carbon source for 1 day. Bar, 10 μM. (D) Diploid pseudohyphal growth assay (Gimeno *et al.* 1992). Homozygous diploid versions of the strains described in B were examined on SLAHD (low nitrogen) medium. Bar, 50 μM.

properties of this organism (Gimeno *et al.* 1992). Altogether, four signaling pathways that regulate filamentous growth have been well characterized—rat sarcoma/protein kinase A (RAS/PKA), sucrose nonfermentable (SNF), target of rapamycin (TOR), and MAPK. We discuss each in turn below but concentrate on the MAPK pathway because it raises intriguing questions regarding signaling specificity.

Ras2/cAMP-PKA pathway and affiliated G-protein–coupled receptor Gpr1: The discovery that RAS is involved in filamentous growth provided a genetic context for elucidating components of the molecular pathway that plays a role in that growth habit (Figure 3). Yeast encode two *RAS* genes, *RAS1* and *RAS2*. The *RAS2* gene is expressed at higher levels than *RAS1* and is responsible for the majority of Ras function (Kataoka *et al.* 1984). Ras2 associates with and activates adenylate cyclase, a membrane-associated enzyme that produces the second messenger cyclic adenosine monophosphate (cAMP) (Toda *et al.* 1985). The Fink lab proposed that the levels of cAMP are critical for the decision of whether or not cells undergo filamentous growth (Mosch *et al.* 1996). Indeed, overexpression of the gene encoding the phosphodiesterase Pde2 dampened filamentous growth and suppressed the hyperfilamentation induced by activated RAS (Ward *et al.* 1995). As for many eukaryotes, cAMP regulates the activity of a family of protein kinases, referred to as protein kinase A (PKA). Binding of cAMP to a regulatory subunit (in yeast Bcy1) releases PKA, activating its kinase activity. Budding yeast has three different PKAs, Tpk1, Tpk2, and Tpk3, which are ~75% homologous in their catalytic domains but differ in their N-terminal regions (Toda *et al.* 1987). Subsequent studies have shown that all three Tpks associate with the regulatory subunit Bcy1 (Pan and Heitman 1999) and that Ras2/cAMP activation of PKA is required for filamentous growth (Pan and Heitman 2002).

What roles do the three Tpk proteins play in filamentous growth regulation? A breakthrough came when it was discovered that deletion of each *TPK* gene caused different phenotypes with respect to filamentous growth. Deletion of *TPK2* abolished filamentous growth, whereas deletion of *TPK1* had no effect. Deletion of *TPK3* caused hyperfilamentous growth, suggesting that Tpk3 may function in an inhibitory capacity (Robertson and Fink 1998; Pan and Heitman 1999). The three Tpks induce different target genes that regulate diverse metabolic outputs ranging from trehalose metabolism to iron uptake (Robertson *et al.* 2000). Among the substrates of Tpk2 is the transcription factor Flo8. Phosphorylation of Flo8 by Tpk2 results in activation

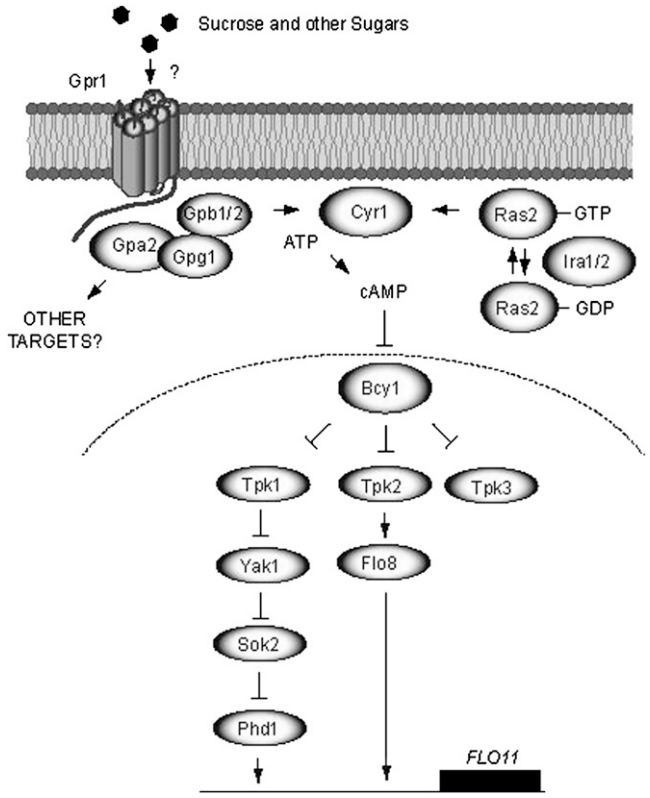

Figure 3 The RAS/PKA pathway. The G-protein coupled receptor (GPCR) Gpr1 and its associated heterotrimeric G protein regulate the Ras2 GTPase activating proteins (GAPs), Ira1 and Ira2. Ras2 regulates adenylate cyclase, which produces cAMP. cAMP binds to Bcy1, inactivating the protein, and releasing Tpk1, Tpk2, and Tpk3 to activate Flo8 and other targets that contribute to nutrient-regulated filamentous growth. Filled hexagons represent sucrose and other sugars.

of Flo8 and expression of filamentation target genes (Pan and Heitman 1999).

The PKA pathway regulates filamentous growth not only by regulating the transcription factor Flo8, via Tpk2, but also by regulating the dual-specificity tyrosine-regulated kinase (DYRK) Yak1, via Tpk1 (Figure 3). Yak1 has a positive role in regulating filamentous growth (Zhang *et al.* 2001). Specifically, Yak1 is phosphorylated by Tpk1, which inactivates the protein (Deminoff *et al.* 2006). In its nonphosphorylated (active) form, the protein regulates *FLO11* expression through the transcription factors Sok2 and Phd1 (Figure 3) (Malcher *et al.* 2011).

What are the upstream regulators of the RAS/PKA pathway? The search for upstream regulators of the RAS pathway has led to the identification of a G-protein–coupled receptor (GPCR) Gpr1 (see below) and glucose limitation as key triggers of filamentous growth. The yeast genome encodes two Gα subunits, *GPA1*, which functions in the mating pathway, and *GPA2*, which was identified by homology to mammalian Gα subunits (Nakafuku *et al.* 1988). Several observations connect the Gα Gpa2 to Ras2. First, the addition of glucose to glucose-starved cells causes a rapid but transient rise in cAMP levels. High-copy *GPA2* enhanced this rise in cAMP levels (Nakafuku *et al.* 1988; Papasavvas *et al.* 1992). Second, the glucose-induced increase in cAMP levels was inhibited by the mating pathway, which was mediated in some way through Gpa2 and Ras2 (Arkinstall *et al.* 1991; Papasavvas *et al.* 1992). On the basis of these observations, the Heitman lab reasoned that Gpa2 might regulate RAS-dependent filamentous growth, and they showed that *gpa2/gpa2* homozygous mutant diploid cells are indeed defective for filamentous growth. Using both gain- and loss-of-function alleles of *GPA2*, in combination with various *RAS* alleles, a model has emerged in which Gpa2 and Ras2 converge on regulating adenylate cyclase (Figure 3) (Kubler *et al.* 1997; Lorenz and Heitman 1997). Adding to this finding is the observation that the glucose-dependent rise in cAMP levels is mediated specifically through Gpa2 (Colombo *et al.* 1998), suggesting provocatively that Gpa2 might function in some manner through a type of sugar receptor.

The involvement of a Gα subunit suggests obvious questions: what are the interacting partners (Gβ and Gγ), and what receptor associates with the G protein? These questions are relevant because the precise triggers of filamentous growth have been (until relatively recently) ill defined. Several labs independently identified a seven-transmembrane receptor of the β-adrenergic type that associates with Gpa2 called Gpr1 (Yun *et al.* 1997; Xue *et al.* 1998; Kraakman *et al.* 1999). Together with Gpa2, Gpr1 regulates filamentous growth (Lorenz *et al.* 2000b). Gpr1 is thought to be a sugar-sensing receptor (specifically sucrose) not a sugar transporter (Thevelein and Voordeckers 2009). The strongest evidence in support of this claim comes from Thevelein and coworkers, who used cysteine scanning mutagenesis of Gpr1 to identify potential sites of sucrose binding (Lemaire *et al.* 2004). Hence, Gpr1 functionally resembles the glucose sensor Rgt2/Snf3 (Ozcan *et al.* 1996, 1998). A rigorous test to prove that Gpr1 binds to sucrose would be a ligand-binding assay using radiolabeled sucrose. This type of experiment has not been reported for either Gpr1 or Rgt2. In this and subsequent investigations it was also shown that sucrose may stimulate the filamentation response (Lemaire *et al.* 2004; Van de Velde and Thevelein 2008). There is some controversy surrounding this issue. In contrast to the report that the glucose-dependent rise in cAMP levels is thought to be mediated directly through Gpa2 (Colombo *et al.* 1998), it has subsequently been shown that glucose induces GTP binding to Ras2 independently of Gpr1 and Gpa2 (Colombo *et al.* 2004).

Two Gβ subunits were subsequently identified (Gpb1 and Gpb2) that inhibit Gpr1 (Harashima and Heitman 2002; Batlle *et al.* 2003; Peeters *et al.* 2006). Gpb1/2 do not have the seven WD-40 repeats typically found in Gβ subunits but instead contain seven kelch repeats, a related protein–protein interaction motif, that results in the formation of a seven-bladed β-propeller structure typical of Gβ subunits (Harashima and Heitman 2002). Gpa2 (Gα) interacts with Gpb1/2 (Gβ) and with Gpg1 (Gγ) (Harashima and Heitman

2002). The idea that Gpb1/2 are the Gβ subunits for Gpa2 is not universally accepted, as recently reviewed by Peeters *et al.* (2007). For one thing, Gpb1/2 do not associate with Gpa2 at the switch interface regions, which is where the classical Gβ subunits bind to Gα subunits (Niranjan *et al.* 2007). Furthermore, another candidate Gβ subunit has been identified, Asc1, which contains the seven WD-40 repeats typically found in Gβ subunits (Zeller *et al.* 2007).

Mutants lacking Gpr1 or Gpa2 are defective for cAMP production and filamentous growth; thus, they are positive factors in controlling filamentous growth. Mutants lacking Gpb1/2 on the other hand are hyperfilamentous, which indicates that the Gβ subunits play an inhibitory role in pathway activation (Harashima and Heitman 2002; Batlle *et al.* 2003). This inhibition can be explained by the formation of an inactive Gα–Gβ complex, based on genetic observations that *gpa2 gpb1 gpb2* triple mutants are less hyperinvasive (Harashima and Heitman 2002). Interestingly however, the triple mutant is somewhat hyperinvasive, indicating that the inhibitory role of Gβ is also mediated by interaction with an as yet unidentified factor (Harashima and Heitman 2002). The Heitman lab reasoned that Gβ mediates its inhibitory effect through the RAS pathway, on the basis of the fact that the hyperfilamentation of Gβ mutants was fully suppressed by deletion of Tpk2 (Harashima and Heitman 2002).

What is the connection between the GPCR and the RAS pathway? In an elegant study, genetic epistasis analysis showed that Gβ functions at the same level in the pathway as Ras2 (Harashima *et al.* 2006). In further support of the Ras2/GPCR connection, the two Ras2 GTPase activating proteins (GAPs), Ira1 and Ira2, were identified as Gpb1/2 interacting proteins by mass spectrometry (Harashima *et al.* 2006). Gpb1/2 associates with Ira1/2, resulting in inhibition of the Ras2 GTPase (Harashima *et al.* 2006). What is the effect of the association between Gpb1/2 with Ira1/2? The answer to this question is under some contention. In one report, Gpb1/2 are thought to associate with and stabilize Ira1/2 (Harashima *et al.* 2006), whereas, more recently, it has been proposed that Gpb1/2 target Ira1/2 for degradation (Phan *et al.* 2010). The resolution of these two opposing models will have important implications for understanding how the pathway regulates Tpk activity (Figure 3). A related discrepancy is the connection between nutrition and Ras2/Tpk2 signaling. If Gpr1 is a sucrose sensor, then does binding to sugars activate or repress Tpk2 activity? A tool that might be useful in addressing these questions is transcriptional reporters for Tpk target genes.

Snf1 pathway: Depletion of fermentable carbon sources, like glucose, can also trigger the filamentous growth response. The finding that glucose depletion is a trigger for filamentous growth came from observations from our lab, in experiments to define the stimuli that regulate the response. By removing and adding back various nutrients and examining the effects on cell and colony morphology, we showed that depletion of fermentable carbon sources, like glucose, triggers filamentous growth (Cullen and Sprague 2000). To determine how glucose levels feed into filamentous growth regulation, several established nutrient-sensing pathways were examined, which uncovered a role for the protein kinase Snf1 in regulating filamentous growth (Cullen and Sprague 2000). Snf1 operates in a separate pathway from Gpr1, by regulating the repressors Nrg1 and Nrg2 at the *FLO11* promoter (Kuchin *et al.* 2002; Vyas *et al.* 2003), a gene required for filamentous growth (see below for further discussion of *FLO11*). Nrg1 and Nrg2 function by recruitment of the Cyc8–Tup1 complex to promoters. Thus, two different glucose-sensing pathways, Gpr1/Gpa2/Ras2/PKA and Snf1, regulate filamentous growth in yeast.

TOR pathway: Initial observations of filamentous growth showed that limiting fixed nitrogen (specifically ammonia) is a trigger of filamentous growth (Gimeno *et al.* 1992). Specifically, mutants defective for ammonium transport were hyperfilamentous, which suggests that ammonium starvation might be a trigger for filamentous growth (Gimeno *et al.* 1992). In addition, Lorenz and Heitman (1998b) showed that the high-affinity ammonium transporter Mep2 is required for filamentous growth. The filamentation defect of the *mep2* mutant arises apparently not from a defect in ammonium transport, as one might expect, but rather from a specific role for that transporter in communicating a signal through a small region in its cytosolic domain. The signal may be conveyed through a mechanism that is not well understood via the MAPK pathway (Rutherford *et al.* 2008). Nitrogen signals have subsequently been shown to be interpreted by the TOR pathway (Crespo *et al.* 2002), an evolutionarily conserved nutrient-regulatory pathway (Heitman *et al.* 1991). The serine/threonine protein kinase TOR regulates cellular homeostasis by coordinating metabolic processes with cellular nutrient levels (Sengupta *et al.* 2010). The TOR pathway regulates the transcription factor Gcn4, which is a regulator of *FLO11* expression (Braus *et al.* 2003; Boeckstaens *et al.* 2008). The TOR pathway regulates filamentous growth in a manner that is apparently independent of the RAS/PKA and MAPK pathways. Evidence for this conclusion comes from the fact that rapamycin inhibits filamentous growth under nitrogen-limited conditions, an inhibition that is mediated by the TOR pathway phosphatases Tap42 and Sit4 (Cutler *et al.* 2001).

To summarize, limiting for nitrogen or glucose can induce filamentous growth. The fact that the glucose response was first observed in haploid cells (invasive growth, Figure 2, B and C) and the nitrogen limitation response first characterized in diploid cells (pseudohyphal growth, Figure 2D) may have led to the impression that the different cell types respond to different stimuli. In fact, glucose depletion induces filamentous growth in both haploid and diploid cells (Cullen and Sprague 2002; Kuchin *et al.* 2002), and nitrogen limitation also induces filamentous growth in both cell types (P. J. Cullen and G. F. Sprague, unpublished observations). How different are

haploid and diploid cells with respect to filamentous growth? The answer to this question is complicated: relatively few studies directly compare filamentous growth in the two cell types, and different assays are used for haploid (Figure 2, C and D) and diploid cells (Figure 2E). A further complication comes from incongruous results. It was initially reported that haploid cells undergo invasive growth better than diploid cells (Roberts and Fink 1994), although we found the opposite to be true (Cullen and Sprague 2002). Nevertheless, the expression of filamentation target genes is regulated by different stimuli in haploid compared to diploid cells (Lo and Dranginis 1998), and regulatory pathways Ras2/PKA and MAPK (discussed below) have different roles in regulating the response in haploid and diploid cells (Chen and Thorner 2010).

At this point, an important paradox should be discussed. One the one hand, glucose limitation induces filamentous growth in both haploid and diploid cells. Indeed, cells grown in nutrient-rich (high glucose) conditions do not produce pseudohyphae. But on the other hand, as stated above, glucose/sucrose is required for filamentous growth in a Gpr1-dependent manner. What is the basis for this discrepancy? Although this point has not been explicitly addressed in the literature, there are several possibilities. One is that glucose/sucrose is required for pseusohyphal growth in diploid cells enduring a low-nitrogen stress, the conditions used by the Thevelein group, to establish the requirement. A less interesting alternative is that different strains are sensitized to different nutritional requirements.

Diploid cells starved for both nitrogen and glucose undergo sporulation, which raises an important point: how do cells decide whether to undergo filamentous growth, enter stationary phase, or sporulate in response to limiting nutrients (Figure 1)? Sporulation has been extensively studied in yeast (Neiman 2011), and many of the signals that trigger meiosis and spore formation are well characterized (Engebrecht 2003). One protein that controls the sporulation/filamentation decision is the repressor of meiosis Rme1 (van Dyk *et al.* 2003). Rme1p is a zinc-finger type transcriptional factor that promotes the mitotic/meiotic decision (Mitchell and Herskowitz 1986). Rme1 binds directly to the *FLO11* promoter to induce cell–cell adhesion and invasive growth (van Dyk *et al.* 2003). Given that Rme1 is not regulated by Ras2 or the MAPK pathway (van Dyk *et al.* 2003), presumably other pathways regulate Rme1-induced filamentous growth. Regulators of the sporulation pathway, Ime1 and Ime2, are also required for filamentous growth (Strudwick *et al.* 2010), although this is true only in the SK1 genetic background. Even in that background, the requirement for Ime2 is extremely weak. Neither protein is required for agar invasion by haploids, but rather only for colony morphology changes shown by diploids (Strudwick *et al.* 2010).

Other sensory pathways: Several other metabolites have been identified that influence filamentous growth. One is alcohol byproducts like 1-butanol (Dickinson 1996; Lorenz *et al.* 2000a). Response to alcohols has now been identified as a quorum-sensing behavior. Budding yeast undergo filamentous growth in response to cell density using secreted alcohols as a gauge of its population levels (Chen and Fink 2006). Quorum sensing also occurs in *C. albicans* via sensing different secreted alcohol derivatives (Chen *et al.* 2004). An intact respiratory pathway, as mediated by a signaling pathway referred to as the retrograde pathway (Butow and Avadhani 2004), also regulates filamentous growth (Jin *et al.* 2008b). Several other metabolites that induce filamentous growth have also been identified, including tetrahydrofolate (vitamin B9). B9 levels feed into *FLO11* expression through signaling mechanisms that have not been well characterized (Guldener *et al.* 2004). External pH may also be sensed in some manner through a signaling pathway that regulates the transcription factor Rim101 (Lamb and Mitchell 2003).

The filamentation MAPK pathway: expeditions into signaling specificity

Early studies from the Fink lab uncovered two signaling pathways that regulate filamentous growth. As discussed above, one major pathway is the Ras2 pathway. The other major pathway is a MAPK pathway composed of kinases that also function in the mating or pheromone response pathway (Figure 4A). The logic underlying testing for a role for the MAPK pathway in filamentous growth was that elements of the pheromone response pathway are expressed in diploid cells, even though diploid cells do not mate. What might the pathway's function in diploids be? Liu *et al.* (1993) reported that four proteins required for mating in haploid cells, the p21-activated (PAK) kinase Ste20, the MAPKKK Ste11, the MAPKK Ste7, and the transcription factor Ste12 (Figure 4A), are also required for filamentous growth in diploids. In contrast, the genes encoding the pheromone receptors Ste2/Ste3, the associated hererotrimeric G protein (Gpa1, Ste4, and Ste18), and the MAPK Fus3 are not required for filamentous growth in diploids (or haploids). Thus, it seemed that haploid cells utilize the "core module" of Ste20→Ste11→Ste7→Ste12 for mating, whereas diploid cells utilize that same core module for filamentous growth regulation (Figure 4A).

Although the separation of function by cell type seems a tidy way to establish specificity, the tidiness is superficial and specificity questions loom large. First, the transcription factor Ste12 functions in both pathways. How are different gene sets activated in mating and filamentous growth? Second, it soon became apparent that haploid cells execute a similar filamentous growth program that requires the same core module (Roberts and Fink 1994). An even more fundamental question therefore is how does the same module direct two distinct physiologic programs in the same cell type?

An example of this quandary comes from studies of the global regulatory Rho-family GTPase Cdc42 (Park and Bi

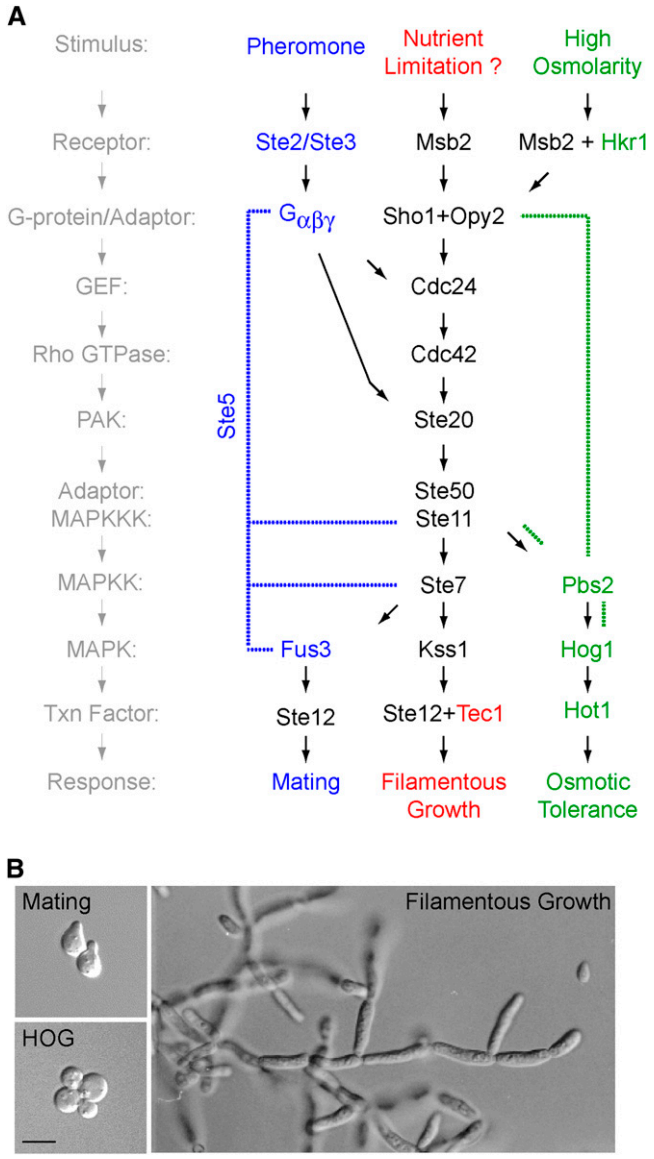

Figure 4 Three MAPK pathways in yeast share common components and also contain pathway-specific factors. (A) Three MAPK pathways are shown. Colored proteins represent pathway-specific factors; protein shown in black function in multiple pathways. Scaffold-mediated interactions are shown by colored, dashed lines. Not all protein interactions are shown. Hot1 is one of a number of transcription factors for the HOG pathway. The red question mark indicates that how nutritional signals feed into filamentous growth pathway regulation is not well understood. (B) Examples of MAPK morphogenesis in yeast. The pheromone response (Mating) pathway induces distinctive polarized structures called shmoos to promote cell fusion and diploid formation. The Filamentous Growth pathway induces filamentous growth, branched chains of elongated and connected cells. Activation of the HOG pathway does not induce polarized growth. Bar, 5 μm.

2007). Cdc42 is an essential protein that is required to establish cell polarity (Bender and Pringle 1989; Adams *et al.* 1990; Shimada *et al.* 2004; Gao *et al.* 2007; Tong *et al.* 2007 and references therein). It has subsequently been shown that Cdc42 functions in the mating pathway and is required for transduction of the signal initiated by the GPCR (Simon *et al.* 1995). Although it was known that temperature-sensitive mutations in *CDC42* were defective for mating (Reid and Hartwell 1977), the assumption was that this resulted from a defect in the overall cell polarity. However, the studies of Simon *et al.* (1995) suggested a more direct involvement of Cdc42 in the mating pathway. The salient finding was that temperature-sensitive versions of Cdc42 and its guanine nucleotide exchange factor (GEF) Cdc24 were defective in MAPK signaling, as assessed by a pheromone-inducible transcriptional reporter. Cdc42 associates with the PAK Ste20, based on two-hybrid analysis and *in vitro* pull downs using recombinant proteins (Simon *et al.* 1995; Zhao *et al.* 1995; Peter *et al.* 1996). More recently, it was shown that Cdc42 and Ste20 function in both the mating pathway and the filamentation pathway (Figure 4A) (Peter *et al.* 1996; Leberer *et al.* 1997), again raising the question of how specificity among MAPK pathways is achieved.

The depth of this puzzle has been magnified by the fact that some of the common or shared components function in yet another MAPK pathway. The Saito lab showed that elements of that same core module—Cdc42, Ste20, and Ste11 (Figure 4A)—are required to activate one of the branches of the high osmolarity glycerol response (HOG) pathway. The HOG pathway responds to changes in external osmolarity caused by exposure to media containing salt, sugar, and other small molecules (Posas and Saito 1997; O'Rourke and Herskowitz 1998; Raitt *et al.* 2000; Tatebayashi *et al.* 2006; Hohmann *et al.* 2007). As another example, the Ste11-interacting protein Ste50 also functions in all three MAPK pathways (Figure 4A) (Posas *et al.* 1998; Ramezani-Rad 2003; Tatebayashi *et al.* 2006; Truckses *et al.* 2006; Wu *et al.* 2006).

Hence, a common or core module regulates the expression of nonoverlapping target genes (Roberts *et al.* 2000) and evokes distinct morphogenetic responses depending on the stimulus. Visual inspection of cells illustrates this point. Nutrient limitation induces filamentous growth (Figure 4B). Mating pheromone induces shmoo formation (Figure 4B). Activation of the HOG pathway does not induce a morphological change (Figure 4B). Indeed, external osmolarity causes rapid depolymerization of the actin cytoskeleton (Yuzyuk *et al.* 2002; Yuzyuk and Amberg 2003), which might be expected to prevent cell polarization during mating and filamentous growth. The question of whether cells can simultaneously activate multiple pathways in response to multiple stimuli has been examined. Cells challenged simultaneously with pheromone and salt activate either one or the other pathway but not both (McClean *et al.* 2007), although this finding may represent an oversimplification of the true decision-making response (Patterson *et al.* 2010).

These discoveries raise important questions: (1) What is the MAPK for the filamentation pathway? (2) How is specificity maintained between kinases that function in multiple pathways? (3) What is the receptor for the filamentation MAPK pathway? Answering these questions

is relevant to filamentous growth regulation and to understanding how signaling pathways maintain specificity. Given that signaling pathways in diverse organisms share common components, insights in this area may shed light on the general mystery of signaling pathway insulation. As will be seen in the discussion below, the quest to understand signaling specificity has repeatedly identified mechanisms or new pathway components that were thought to confer specificity. However, in most cases, the apparent solution was short lived. Further studies often showed that the specificity problem remained, and it is fair to say that fundamental questions regarding specificity still exist. The discussion below summarizes the history of the quest and highlights the extant questions.

Kss1 is the MAP kinase for the filamentation pathway: Two MAP kinases (Fus3 and Kss1) were discovered around the same time in genetic screens for regulators of the mating pathway. The protein kinase Fus3 was established early on as a regulator of the mating pathway, because it was required for pheromone-induced growth arrest, and because its overexpression resulted in heightened sensitivity to pheromone (Elion *et al.* 1990). However, *fus3* mutants showed only a partial mating defect, suggesting other proteins could carry out Fus3 function. The protein kinase Kss1 was identified as a high-copy suppressor of the cell-cycle arrest phenotype induced by pheromone (Courchesne *et al.* 1989), which suggested that it might function in opposition to the mating pathway. Indeed, *kss1* mutants showed elevated growth arrest in response to pheromone (Courchesne *et al.* 1989) and normal or slightly elevated *FUS1* expression (Elion *et al.* 1991b). Nevertheless, *fus3 kss1* double mutants were completely deficient for mating, implying that the two kinases function redundantly in the mating pathway.

Despite the above results, it was also suspected that Kss1 might be the filamentation MAPK. The *kss1* mutant had a strong invasive growth defect (Roberts and Fink 1994) and showed reduced activity of a filamentation response element (FRE) (Mosch *et al.* 1996). The breakthrough in assigning functions to Fus3 and Kss1 came from observations that were at first paradoxical. Deleting *FUS3* restored invasive growth to the *kss1* mutant (Roberts and Fink 1994). Moreover, Thorner and colleagues found that *ste7 fus3 kss1* triple mutants invaded the agar as well as wild-type cells (Cook *et al.* 1997). This new finding flew in the face of the established result that the MAPKK Ste7 was required for invasive growth. Thorner and colleagues reasoned that Fus3 and Kss1 (mainly Kss1 from genetic evidence) had an inhibitory function in filamentous growth, and that Ste7 was required to relieve that inhibition. A dual role for Kss1 in MAPK regulation could be explained by changes in its phosphorylated (active) state. Unphosphorylated Kss1 functions as an inhibitor, whereas phosphorylated Kss1, catalyzed by Ste7, functions as an activator (Cook *et al.* 1997).

In a parallel study, Madhani, Fink and colleagues corroborated these findings by showing that the inhibitory effect of Kss1 was mediated through the transcription factor Ste12 (Madhani *et al.* 1997). Using kinase-inactive versions of the Kss1 and Fus3 proteins, which maintained protein–protein interactions with their respective factors and thereby prevented cross-talk, they showed that Kss1 functions in the mating pathway only when Fus3 is absent (Madhani *et al.* 1997). One conclusion from these two studies is that Fus3 is the MAPK for the mating pathway, whereas Kss1 is the MAPK for the filamentous growth pathway.

It should be recognized that Kss1 also plays a role in the mating pathway. Cells lacking the mating pathway MAPK Fus3 can mate (Elion *et al.* 1991a), and pheromone induces the phosphorylation/activation of Fus3 and Kss1 to a similar degree (Gartner *et al.* 1992). Specificity between the pathways may involve transient *vs.* sustained pathway signaling (Sabbagh *et al.* 2001; Bruckner *et al.* 2004) rather than the more simplistic view that each MAPK pathway has its own MAPK. In this way, Kss1 can also be viewed as a shared component between the mating and filamentous growth pathways (Figure 4A).

How does Kss1 mediate its inhibitory function? To begin to answer this question, two-hybrid analysis (Fields and Song 1989) was performed using Kss1 as bait. In addition to identifying the transcription factor Ste12, two novel proteins were identified, Dig1 and Dig2 (Cook *et al.* 1996). Biochemical tests confirmed that Kss1 and Ste12 associate with Dig1 and Dig2, and invasive growth assays showed that the *dig1* and *dig2* mutants were strongly hyperinvasive, demonstrating that the proteins were potent negative regulators of filamentous growth. Kss1 phosphorylates Dig1 and Dig2, which suggests a mechanism for relieving the inhibitory effects of these transcriptional repressors (Cook *et al.* 1996). Dig1 and Dig2 also associate with Fus3 and function as negative regulators of the mating pathway (Tedford *et al.* 1997; Roberts *et al.* 2000). Subsequent experiments showed that Dig1/2 function in pathway discrimination by conferring differences in the binding to mating (Ste12) and filamentation (Ste12 and Tec1, see below) promoters (Bardwell *et al.* 1998).

The transcription factor Ste12 functions in both the mating and filamentation pathways (Figure 4A). How does a transcription factor induce one set of target genes in one setting (pheromone) and a different set of targets in another (nutrient limitation)? One possibility is that Ste12 associates with a protein that specifies it to filamentation regulated genes. The transcription factor Tec1 was identified as a member of the TEF-1, Tec1p, and AbaAp (TEA) or AbaAp, TEF-1, Tec1p, and Scalloped (ATTS) family that coregulates the expression of transposable elements along with Ste12 (Laloux *et al.* 1994). Tec1 was also required for filamentous growth (Gavrias *et al.* 1996). Madhani and Fink (1997) interpreted these findings to indicate that Tec1 may be the coregulator of Ste12 function. Support for their hypothesis came from the finding that purified versions of Ste12 and

Tec1 bind cooperatively to FREs. The distribution of Ste12 and Tec1 at target promoters *in vivo* largely bears out the hypothesis that Tec1 and Ste12 exhibit combinatorial control over filamentation pathway targets (Kohler *et al.* 2002; Zeitlinger *et al.* 2003; Chou *et al.* 2006).

Given that Tec1 specifies Ste12 to filamentation-specific targets, one might expect that *TEC1* is not expressed during the mating response. Unexpectedly, the gene encoding the Tec1 protein is induced by pheromone (Oehlen and Cross 1998). But paradoxically, immunoblot analysis showed that the Tec1 protein is not present in cells exposed to mating pheromone. Therefore, a mechanism for regulating the levels of the Tec1 protein must exist in mating cells. Such a mechanism was identified and has come to represent a fundamental way of maintaining specificity between pathways. In response to pheromone, the activated MAPK Fus3 phosphorylates Tec1. Phosphorylated Tec1 is recognized by a ubiquitin ligase that targets Tec1 for degradation by the proteasome (Bao *et al.* 2004; Bruckner *et al.* 2004; Chou *et al.* 2004). Failure of Tec1 to be degraded results in cross-talk between the filamentation and mating pathways. Tec1 is subject to complex regulation, being phosphorylated at multiple residues (Bao *et al.* 2010) as well as being sumoylated (Wang *et al.* 2009b). Ste12 itself and other components of the mating/filamentation pathways are also ubiquitinated and degraded to attenuate signaling generated by these pathways (Esch *et al.* 2006). Persistence of Ste12, for example, can lead to a shift in filamentation over the mating response (Esch *et al.* 2006). Among the proteins that may regulate the turnover of Ste12 is the CDK Srb10/Cdk8 (Nelson *et al.* 2003). Therefore, the regulated degradation of pathway-specific proteins can result in signal discrimination.

Scaffolding proteins insulate signaling by proteins in the core module: Genetic screens identified a number of *STE* genes as encoding potential mating pathway components. Despite rigorous genetic epistasis analysis to order components into the pathway, the function of several Ste proteins had remained elusive. One of these was Ste5, a large protein whose amino acid composition suggested little of its function. To determine how Ste5 regulates the mating pathway, a directed two-hybrid approach was employed. Using this approach, three labs independently made an important discovery— Ste5 associates with multiple components in the MAP kinase cascade (Choi *et al.* 1994; Kranz *et al.* 1994; Marcus *et al.* 1994; Printen and Sprague 1994). Elion and colleagues expanded on this exciting finding by showing that Ste5 physically associates with Ste11, Ste7, and Fus3. They also showed that Ste5 is required for Ste11 function in the mating pathway (Choi *et al.* 1994). Together these studies establish Ste5 as a scaffold for the mating pathway.

In addition to its interaction with MAPK pathway kinases, Ste5 associates with the heterotrimeric Gβ (Ste4) subunit for the mating pathway (Whiteway *et al.* 1995). Gβ also associates with the PAK Ste20 (Leeuw *et al.* 1998), which thereby connects upstream signals to both the PAK and the MAPK scaffold. Ste5 functions in the cell as a dimer (Yablonski *et al.* 1996; Inouye *et al.* 1997; Feng *et al.* 1998) and is recruited to the plasma membrane upon binding of pheromone to receptor (Pryciak and Huntress 1998). Hence, one has a picture of a multiprotein complex localized to the site of ligand bound receptors controlling the entire mating pathway.

The identification of a scaffold has implications about how signaling through the pathway might be regulated. How does Ste5 in fact contribute to pathway specificity? First, Ste5 promotes the interaction among kinases to increase the efficiency of signal transmission (and signal attenuation) (Choi *et al.* 1994). Thus, Ste5 might tether general components (Ste11 and Ste7) to a pathway-specific factor (Fus3) to prevent erroneous signaling of the upstream kinases.

Second, Ste5 selectively recruits proteins to the plasma membrane (Pryciak and Huntress 1998; van Drogen *et al.* 2001; Maeder *et al.* 2007). Plasma-membrane recruitment is a general way of increasing the local concentration of protein complexes. Ste5 is recruited to the plasma membrane in response to pheromone (Pryciak and Huntress 1998; van Drogen *et al.* 2001; Maeder *et al.* 2007; Yu *et al.* 2008) through its PM domain (Winters *et al.* 2005), its PH domain (Garrenton *et al.* 2006), and by Gβ recruitment (Pryciak and Huntress 1998; Mahanty *et al.* 1999; Winters *et al.* 2005). Directly targeting Ste5 to membranes substantially activates MAPK signaling (Pryciak and Huntress 1998). The PH and PM domains of Ste5 associate with phosphorylated inositol lipids in the plasma membrane (Winters *et al.* 2005; Garrenton *et al.* 2006). Specifically, PI(4,5)P2 is highly enriched in shmoo tips, which results in the polarized localization of Ste5 and associated proteins to that site (Jin *et al.* 2008a; Garrenton *et al.* 2010). In cells not exposed to pheromone, Ste5 is localized to the nucleus (Pryciak and Huntress 1998; Mahanty *et al.* 1999), where it is degraded by ubiquitin-mediated proteolysis by the proteasome (Garrenton *et al.* 2009).

Third, Ste5 causes a conformational change in Fus3 that makes it competent to be phosphorylated by Ste7 (Flatauer *et al.* 2005; Good *et al.* 2009). The way in which Fus3 and Kss1 are activated by Ste7 is fundamentally different. On the one hand, Ste7 recognizes a specific docking site in the CD/sevenmaker region of Kss1 and Fus3 that is common to MAPKs of many different species and that promotes interactions with key regulatory proteins (Kusari *et al.* 2004). On the other hand, whereas Ste7 readily phosphorylates Kss1, it cannot phosphorylate Fus3 without Ste5. Evidence comes in part from the fact that hyperactive versions of Ste7 induce invasive growth and Kss1 phosphorylation but not mating and Fus3 phosphorylation (Maleri *et al.* 2004). Ste5 contributes to specific activation of the mating pathway in response to pheromone (Flatauer *et al.* 2005). Induced-fit recognition between Ste7, Fus3, and Kss1 may allow docking peptides to achieve discrimination through differences in kinase

flexibility (Remenyi *et al.* 2005). Pathway-specific activation of different MAPK pathways also involves differences in pathway kinetics (Sabbagh *et al.* 2001). Kss1 induces a transient response, whereas Fus3 induces a sustained response. Studies stemming from the crystal structure determination of Fus3 have shown that Ste5 functions to unlock the Fus3 kinase for phosphorylation by the MAPKK Ste7 (Good *et al.* 2009). In addition, activated Fus3 (by phosphorylation) exists in a gradient, concentrating at the shmoo tip (Maeder *et al.* 2007).

Pbs2, the MAPKK for the HOG pathway (Brewster *et al.* 1993), is also thought to provide an example of a scaffold (Figure 4A). In a landmark study, Saito and colleagues identified the MAPKKK Ste11 as being required to transmit a signal in the HOG pathway (Posas and Saito 1997). Ste11 activates the HOG pathway by associating with and activating Pbs2. Pbs2 also associates with the cell-surface protein Sho1 (Maeda *et al.* 1995). Pbs2 therefore functions as both the scaffold and MAPKK for the HOG pathway.

To summarize, pathway-specific complexes for MAPK pathways can be constructed from general factors by pathway-dedicated scaffolds. Ste5 promotes Ste11 function in the mating pathway, whereas Pbs2 promotes Ste11 function in the HOG pathway. This overall picture is satisfying but may represent only part of the actual connections that underlie pathway specificity. For one thing, a scaffold for the filamentation pathway, although theorized, has yet to be identified (Saito 2010). Therefore, other specificity factors may also contribute to signal insulation. For another thing, the protein interactions depicted here probably represent an oversimplified view. A more accurate picture, but harder to visualize, is that proteins in these pathways exist in multiprotein complexes. For example, Sho1 associates directly with Ste11 (Zarrinpar *et al.* 2004; Tatebayashi *et al.* 2006) and with Ste50 (Tatebayashi *et al.* 2006), suggesting that like Pbs2, it may also serve a scaffolding role. Whether such complexes are contiguous or whether there are multiple different protein subcomplexes in the cell remains to be determined.

Proteins that function at the head of the MAPK pathway: The discovery that haploid cells require a core module for mating and filamentous growth implies that different receptors activate the two pathways. The receptor for the mating pathway is a seven-transmembrane heterotrimeric GPCR. But the receptor for the filamentation MAPK pathway—any protein that functions above Ste11—had not been identified.

A cell-surface regulator for the HOG pathway had been identified, called Sho1 (Maeda *et al.* 1995). Sho1 contains four transmembrane helices and a cytosolic SH3 domain. In the HOG pathway, Sho1 associates with the MAPKK Pbs2 by a SH3 domain–polyproline domain interaction. In a pioneering study, O'Rourke and Herskowitz (1998) reasoned that Sho1 might also function in the filamentation pathway. Their reasoning was motivated by the discovery that cross-talk occurs from the HOG pathway to the pheromone pathway in cells lacking downstream components of the HOG pathway, Pbs2 or Hog1. For example, the addition of salt to *hog1* or *pbs2* mutants caused activation of the mating pathway. Activation required Sho1, Ste20, and the MAPK cascade. Cross-talk was not dependent on the mating receptor, the heterotrimeric G protein (Ste4), or Ste5. Thus, Sho1 could be functionally connected to the Ste12-dependent MAPK pathway in some contexts. Further support for the identification of a Sho1→Ste12 pathway came from analysis of an "amalgamated" pathway that functioned in protein glycosylation mutants (Cullen *et al.* 2000). Therefore, at least one protein that might function at the head of the filamentous growth pathway is Sho1 (Figure 5).

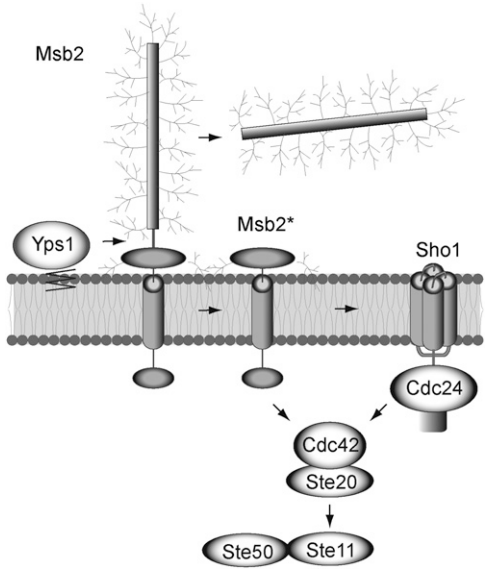

Figure 5 Model of the filamentous growth MAPK pathway. Upon nutrient limitation, expression of the gene encoding the aspartyl protease Yps1 is induced. Yps1 processes the signaling mucin Msb2 in its extracellular domain, which is required for MAPK activation. Processed Msb2 (Msb2*) associates with and functions through Sho1 to activate cytosolic signaling modules. Msb2 associates with the Rho GTPase Cdc42, and Sho1 functions in a complex with the GEF Cdc24. A straightforward possibility is that the association of activated Msb2 with Sho1 brings the GEF into close proximity with its GTPase. Activated Cdc42 binds effector proteins including the PAK Ste20, which when activated, phosphorylates the MAPKKK Ste11, thereby activating the MAPK cascade.

Although the studies summarized above have the pleasing virtue of identifying a membrane protein that operates in the filamentation pathway, they compound the specificity problem because Sho1 works in the HOG pathway as well (Figure 4A). In a study to identify new regulators of filamentous growth (Cullen *et al.* 2004), two high-throughput approaches were employed that began to shed light on filamentous growth pathway signaling and specificity. In one approach, DNA microarray analysis identified a small number of highly induced transcriptional targets of the filamentation pathway. In a second approach, phenotypic analysis of a collection of ordered deletion mutants identified factors required for invasive growth. Comparing the two datasets

revealed a single common protein, Msb2. The *MSB2* gene was previously identified as a high-copy suppressor of temperature-sensitive *cdc24* and *cdc42* alleles (Bender and Pringle 1992), and the protein had been implicated in HOG pathway regulation (O'Rourke and Herskowitz 2002). Moreover, the amino acid sequence of the Msb2 protein suggested it was a large cell-surface glycoprotein, with an N-terminal signal sequence and large S/T-rich extracellular domain. Together these findings suggested that Msb2 might be a candidate cell-surface regulator of Cdc42 in the filamentation pathway.

Does Msb2 regulate the filamentous growth pathway? Deletion of *MSB2* resulted in a defect in pathway activity equivalent to deletion of the *SHO1* gene. Msb2 also formed a protein complex with Sho1 and with Cdc42, preferentially the active (GTP-bound) form of Cdc42 (Cullen *et al.* 2004). Therefore, two proteins (Msb2 and Sho1) function at the head of the filamentation MAPK pathway and interface with cytosolic regulators (like Cdc42) to transmit a signal to downstream components (Figure 5).

Most intriguingly, Msb2 was not required to regulate the mating pathway and had a relatively minor role in the HOG pathway, making it the first cell-surface protein to be identified with a specific role in regulating the filamentation pathway (Cullen *et al.* 2004). A convincing piece of evidence that Msb2 plays a specific role in the filamentation pathway was that the *MSB2* gene is a transcriptional target of the pathway. Many examples exist of pathway-specific components encoded by genes that are induced by activation of their cognate pathways, creating a positive-feedback loop. Positive feedback loops can lead to bistable activation states, a type of cellular "memory" (Ingolia and Murray 2007) that has been reported throughout eukaryotes (Xiong and Ferrell 2003).

Msb2 is a member of the signaling mucin family of proteins, which are general regulators of MAPK pathways that have been identified in a variety of species. The most highly characterized signaling mucin is MUC1, which regulates the RAS–rapidly accelerated fibrosarcoma (RAF)-mitogen activated protein kinase kinase (MEK)-extracellular signal regulated kinase (ERK) pathway (Singh and Hollingsworth 2006). Signaling mucins are single pass transmembrane proteins that are glycosylated in their extracellular domains (Kufe 2009; Bafna *et al.* 2010). A defining feature is the presence of heavily glycosylated tandem repeats that are rich in proline, threonine, and serine residues (PTS domain). To determine whether the PTS domain of Msb2 was required for its function in the filamentation pathway, that domain was disrupted and replaced with an epitope tag. Unexpectedly, Msb2 lacking the PTS domain was hyperactive for MAPK activity, which suggested an inhibitory role for the PTS domain in signaling mucin regulation (Cullen *et al.* 2004).

Signaling mucins differ in their overall structure and regulation from the highly characterized and more well-understood GPCR-type receptors. Unlike GPCRs, signaling mucins have not been studied in model systems where genetic approaches are readily available. In mammalian cells, signaling mucins are shed by post-translational processing (Litvinov and Hilkens 1993; Parry *et al.* 2001; Brayman *et al.* 2004). In many cases, the proteases that process signaling mucins have not been identified (Parry *et al.* 2001), and the relationship between processing and mucin regulation remains unclear (Singh and Hollingsworth 2006). We found that most of the glycosylated extracellular domain of Msb2 is shed from cells (Vadaie *et al.* 2008). Given that Msb2 is encoded by a single polypeptide, we hypothesized that Msb2 might be subject to proteolytic processing. Examining Msb2 shedding in a panel of protease mutants uncovered the aspartyl protease Yps1 as being required for processing and release of the protein (Vadaie *et al.* 2008).

Given that the PTS domain (698–818 residues) of Msb2 is inhibitory, we further explored the extent of that inhibition. Deletion analysis showed that most of the extracellular domain (100–950 residues) had an inhibitory function. A version of Msb2 lacking this large domain was strongly hyperactive for MAPK signaling (Vadaie *et al.* 2008). The finding that Msb2 is processed and its extracellular inhibitory domain released from cells suggests an activation mechanism (Figure 5). Cleavage-dependent activation may be a general regulatory feature of signaling mucins.

An appealing aspect to defining Msb2 as an upstream regulator of the filamentation pathway is that it provides an explanation for how specificity is achieved at the head of the pathway: Msb2 is a protein that functions in filamentation but not mating or shmoo response. This model is not completely satisfying, however because Msb2 might function in at least some capacity in the HOG pathway (O'Rourke and Herskowitz 2002). This tidy notion of Msb2 as a filamentation specific component was further challenged by the recent discovery by the Saito lab that a second signaling mucin, Hkr1, functions together with Msb2 in the HOG pathway (Tatebayashi *et al.* 2007). That is, Msb2 and Hkr1 are redundant for function in the HOG pathway. The *HKR1* gene was identified in a genetic screen for mutants that were osmosensitive in an *msb2*Δ (and *ssk2*Δ/*ssk22*Δ) background. Saito and colleagues showed that both Msb2 and Hkr1 associate with Sho1 to transmit a signal to downstream components (Tatebayashi *et al.* 2007). Therefore, Msb2 cannot be thought of as solely functioning in the filamentation pathway (Figure 4).

Does Hkr1 function in the filamentation pathway? To address this question, the role of Hkr1 in regulating the filamentous growth pathway was tested (Pitoniak *et al.* 2009). Unlike for Msb2, cells lacking Hkr1 were not defective for filamentous growth pathway signaling. Moreover, the genes encoding the two mucins exhibited different expression patterns, and their overproduction induced non-overlapping sets of target genes. Therefore a model can be drawn where Msb2 functions preferentially in the filamentation pathway, whereas Hkr1 functions preferentially in the HOG pathway (Pitoniak *et al.* 2009). This model is

reinforced by the recent finding that underglycosylation of Msb2 activates the filamentous growth pathway but not the HOG pathway (Yang *et al.* 2009). Examining the role of the two mucins in promoting the phosphorylation of downstream kinases would lend further support to this possibility. Hence, differential MAPK activation by signaling mucins represents a new point of discrimination between MAPK pathways.

An important unresolved question is what do signaling mucins "sense" to induce a downstream signal? This question is unanswered for any such mucin. Specifically, in yeast it remains unclear how nutritional information is sensed or conveyed through Msb2/Sho1 or how a change in external osmolarity is sensed by Msb2/Sho1/Hkr1. Msb2 may be a mechanoreceptor that monitors mechanical stress between the plasma membrane and cell wall during osmotic stress (O'Rourke and Herskowitz 2002). Intriguingly, the mammalian signaling mucin MUC1 has been proposed to detect mechanical shear (Macao *et al.* 2006).

The legitimacy of studies focused on mucin-like glycoproteins in yeast is further validated by the fact that most (three of the four) MAPK pathways in yeast for which cell-surface proteins have been identified are regulated by large mucin-like glycoproteins. Msb2 functions in the filamentous growth pathway (Cullen *et al.* 2004), Msb2/Hkr1 in the HOG pathway (Tatebayashi *et al.* 2007), and Wsc1,Wsc2,Wsc3, Mid2, and Mtl1 (Rodicio and Heinisch 2010) in the cell wall integrity or protein kinase C pathway (Levin 2005). These glycoproteins are structurally and mechanistically dissimilar from GPCRs. The yeast pheromone receptors bind to well-defined peptide ligands, oligomerize (Gehret *et al.* 2006), exist in inactive and activated (ligand-bound) states (Boone *et al.* 1993; Stefan and Blumer 1994), are differentially internalized depending on whether or not they are bound to ligand and cleared from the cell surface by ubiquitination (Roth and Davis 1996; Tan *et al.* 1996; Jenness *et al.* 1997; Roth and Davis 2000; Chen and Davis 2002). It will be interesting to learn how mucin-like glycoproteins are regulated to modulate MAPK activation.

Yet another cell-surface component of the HOG pathway has recently been identified. The *opy2* mutant was uncovered in a synthetic genetic array (SGA) screen (Tong *et al.* 2001) by the Whiteway lab in a search for salt-sensitive mutants in an *ssk1* background (Wu *et al.* 2006). Opy2 was initially postulated to function exclusively in the HOG pathway (Wu *et al.* 2006), although it has subsequently been suggested to operate in the filamentation MAPK pathway as well (Yamamoto *et al.* 2010). Two-hybrid analysis, *in vitro* pull down, and co-immunoprecipitation showed that Opy2 associates with the adapter protein Ste50 (Wu *et al.* 2006; Ekiel *et al.* 2009). Ste50 associates with Cdc42 (Truckses *et al.* 2006) and Ste11 (Posas *et al.* 1998; Jansen *et al.* 2001; Tatebayashi *et al.* 2006; Truckses *et al.* 2006; Garcia *et al.* 2009) and is thought to function in the membrane recruitment of Ste11 to activated complexes at the cell surface. Evidence supporting this conclusion comes from the fact that membrane tethering of Ste11 can bypass the requirement for Ste50 (Wu *et al.* 2006).

Why do different MAPK pathways require the same core module? The answer to this question is not known but hints may come from an antagonistic relationship between two of the MAPK pathways. Early findings showed that cells exposed to osmotic stress fail to undergo filamentous growth (Davenport *et al.* 1999). Inhibition of the filamentation pathway requires the HOG pathway MAPK Hog1 (O'Rourke and Herskowitz 1998). Together with other studies, it has been proposed that Hog1 phosphorylates a component of the filamentation pathway to inactivate it (Westfall and Thorner 2006; Shock *et al.* 2009). Therefore, the sharing of components between pathways may contribute to an either/or response.

Recently, the Dohlman and Saito labs have moved our understanding of this attenuation mechanism forward (Hao *et al.* 2008; Yamamoto *et al.* 2010). Preliminary studies uncovered a surprising result: Kss1 is phosphorylated in response to osmotic stress (Hao *et al.* 2008). The phosphorylation of Kss1 is transient and (as expected during crosstalk) is dramatically stimulated in cells lacking Hog1. In fact, the phosphorylation of Hog1 itself is stimulated in cells containing a kinase-inactive version of Hog1 (Hao *et al.* 2008). How then are multiple MAPK pathways inhibited by Hog1? Dohlman and colleagues reasoned that Hog1 phosphorylates a shared component between the two pathways to attenuate signaling. By testing several shared components, they found that Ste50 is phosphorylated by Hog1(Hao *et al.* 2008). The phosphorylation of Ste50 was at first thought to limit the duration of Kss1 (and Hog1) activation (Hao *et al.* 2008) but this claim has been more recently questioned (Shock *et al.* 2009; Patterson *et al.* 2010). Saito and colleagues confirmed that Ste50 is a target for Hog1 and went on to show that two protein phosphatases, Msg5 and Ptp3, synergistically contribute to MAPK downregulation by Ste50 (Yamamoto *et al.* 2010). Nevertheless, the precise target of Hog1 in dampening the filamentous growth pathway remains unclear, and it has been suggested that an as yet unidentified target of Hog1 contributes to pathway specificity (Saito 2010). Future studies in this area will undoubtedly move forward our understanding of signal discrimination between related MAPK pathways.

Mechanisms of signal integration during filamentous growth

A fundamental question in pathway regulation is how information from different pathways is integrated into a coordinated response. As discussed above, the differentiation from yeast-form to filamentous-form cells requires multiple pathways: TOR, SNF, RAS, and MAPK. How do signals sent through these pathways become integrated into a coherent response? Recently, several examples of signal integration have been elucidated (Figure 6).

One way in which filamentation signals become integrated is by convergence at common target genes.

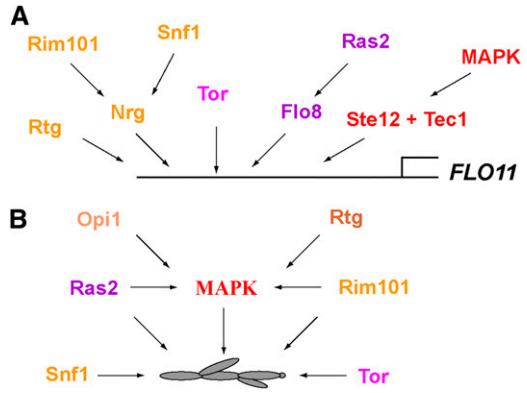

Figure 6 Mechanisms of signal integration among regulatory proteins and pathways that control filamentous growth. (A) Multiple signaling pathways converge on the *FLO11* promoter to modulate gene expression (Rupp *et al.* 1999). Both Snf1 and Rim101 are thought to function through the transcriptional repressors Nrg1 and Nrg2 (Kuchin *et al.* 2002; Lamb and Mitchell 2003). (B) Multiple signaling pathways regulate the activity of the filamentation MAPK pathway, adapted from (Chavel *et al.* 2010). Rtg refers to the retrograde mitochondrial signaling pathway (Liu and Butow 2006). Other pathways also converge on *FLO11* that are not shown here (Bruckner and Mosch 2011).

The most extensively studied example is the *FLO11* promoter (Figure 6A). The gene encoding the cell-adhesion flocculin *FLO11* has one of the largest promoters in the yeast genome (>2.8 kb). In one study, transcription factor binding sites were mapped along the *FLO11* promoter. Rupp and colleagues showed that the MAPK-dependent transcription factors Ste12 and Tec1, and the RAS/cAMP–PKA-dependent transcription factor Flo8 each binds to the *FLO11* promoter (Rupp *et al.* 1999). Chen and Thorner (2010) followed up on this study by showing that the two pathways contribute additively to the filamentation response. When maximally activated, either pathway can fully induce filamentous growth, which suggests that, normally, both pathways are required because neither pathway is maximally active. In addition to Ras2/PKA and MAPK, other pathways also feed into *FLO11* gene regulation (Figure 6A). The TOR pathway likewise regulates the *FLO11* promoter through the transcription factor Gcn4 (Braus *et al.* 2003). In addition, the transcriptional repressors Nrg1/2 mediate signals initiated by the glucose-regulatory kinase Snf1 (Vyas *et al.* 2003) and by the pH sensing Rim101 (Lamb and Mitchell 2003) pathways. An intriguing recent finding is that *FLO11* expression is regulated by long noncoding RNAs that are produced by antisense transcription (Hongay *et al.* 2006). These *cis*-interfering noncoding RNAs toggle *FLO11* expression back and forth to variegate gene expression (Bumgarner *et al.* 2009; Octavio *et al.* 2009). The noncoding RNAs themselves are regulated by chromatin remodeling proteins like the histone deacetylasee Rpd3(L) (Bumgarner *et al.* 2009). Other proteins also regulate *FLO11* expression through mechanisms that may or may not result from direct binding to the promoter, such as the Opi1 transcription factor (Reynolds 2006).

A second mechanism of signal integration involves coregulation of signaling pathways involved in filamentous growth. Two major pathways that regulate filamentous growth, RAS and MAPK, are functionally connected to each other. Specifically, Ras2 regulates the activity of the MAPK pathway at or above Cdc42 (Mosch *et al.* 1996). The critical experiments demonstrating this result came from gain- and loss-of-function alleles of *RAS2* and *CDC42*. A dominant active version of Ras2, Ras2V19, activated the filamentation-specific *FG(Ty)-lacZ* reporter and filamentous growth. This stimulation was not observed in cells containing loss-of-function alleles of *CDC42*, implying that Ras2 functions at or above Cdc42 in the filamentous growth pathway (Mosch *et al.* 1996).

There are several ways in which Ras2 might regulate the MAPK pathway above Cdc42. Ras2 may associate with and modulate upstream components of the pathway (like Msb2, Sho1, and Cdc42). Alternatively, Ras2 may indirectly modulate MAPK activity, for example by regulating the expression of a MAPK regulatory gene. We found evidence to support the latter possibility. Specifically, Ras2 was found to regulate expression of the *MSB2* gene in a Ste12-independent manner (Chavel *et al.* 2010). The key finding was that activated versions of Msb2 failed to bypass the *ras2* mutant, whereas overexpression of the *MSB2* gene did bypass *ras2*. This result supports the idea that Ras2 regulates the MAPK pathway indirectly, by modulating *MSB2* expression. In further support of this possibility, Tpk2 was also required for *MSB2* expression (Chavel *et al.* 2010).

Although one cannot formally exclude the possibility that Ras2 is a component of the filamentous growth pathway, experiments to date do not support that possibility. Msb2's cytosolic domain associates with Cdc42 by two-hybrid analysis, but not with Ras2 (Cullen *et al.* 2004). Ste50 associates with Cdc42, but not Ras2, by its RA domain (Truckses *et al.* 2006). Ras2 regulates MEK–ERK signaling in mammalian cells through the protein kinase RAF, which is not present in yeast (Zebisch *et al.* 2007). How Ras2–Tpk2 regulates *MSB2* expression remains to be determined. As stated above, the Ras2–Tpk2 pathway and the MAPK pathway also converge on the *FLO11* promoter (Rupp *et al.* 1999) and exhibit nonoverlapping effects on filamentous growth (Chen and Thorner 2010).

Several pieces of evidence suggest that coordination among signaling pathways is greater even than the foregoing discussion suggests. One study by Snyder and colleagues showed that several key transcription factors regulate each others' expression, implicating these factors as target hubs for filamentous growth. These hubs serve as master regulators that integrate different aspects of the response into a coordinated behavior (Borneman *et al.* 2006). Likewise, it has been suggested that an integrated molecular network may be involved in the overall regulation of filamentous growth (Prinz *et al.* 2004). This transcriptional network is likely to be extensive, given the number of different transcription factors that have been identified that

contribute to filamentous growth, such as the forkhead proteins (Zhu *et al.* 2000), Mss11 (Van Dyk *et al.* 2005), Sok2 (Pan and Heitman 2000), and Hms1 (Lorenz and Heitman 1998a), whose binding site has recently been identified (Chua *et al.* 2006).

A second study by Kumar and colleagues used a large-scale approach to determine whether the localization of any of the predicted protein kinases showed an altered distribution during filamentous growth (Bharucha *et al.* 2008). The reasoning was that altered kinase localization might reflect a role for the kinase in the filamentation response. In the study, five kinases (Fus3, Kss1, Tpk2, Sks1, and Ksp1) and the regulatory subunit Bcy1 shuttled from the cytosol to the nucleus under conditions permissive for filamentous growth (Bharucha *et al.* 2008). Kss1, Tpk2, and Bcy1 might be expected to have this pattern, given that they play a role in filamentous growth. Fus3 is not known to enter the nucleus in response to nutrient limitation, and this result was unexpected. Sks1 is involved in the cellular response to glucose limitation (Vagnoli and Bisson 1998) and Ksp1 is poorly characterized. The surprising finding came when it was discovered that the colocalization of these kinases was interdependent (Bharucha *et al.* 2008). Therefore, interlocking kinase localization (through a mechanism that remains to be determined) may coordinate the activity of different pathways that regulate filamentous growth.

A third example comes from a novel screen designed to identify regulators of the signaling mucin Msb2, which functions at the head of the MAPK pathway (Cullen *et al.* 2004). A high-throughput approach called secretion profiling was used to measure release of the extracellular domain of Msb2 (Chavel *et al.* 2010). Secretion profiling of complementary genomic collections showed that some of the proteins that regulate filamentous growth—including Opi1 (Reynolds 2006), retrograde (Rtg) (Liu and Butow 2006), and Rim101 (Lamb and Mitchell 2003)—were also required to activate the MAPK pathway (Figure 6B). Some of these regulators functioned by regulating *MSB2* expression. Therefore, the *MSB2* gene, like other hub genes, may be a place where multiple signals converge. Accessibility to the *MSB2* promoter was regulated by the histone deacetylase (HDAC) Rpd3p(L), which positively regulates filamentous growth (Chavel *et al.* 2010).

Together, these examples provide a glimpse of the regulatory hierarchy that controls filamentous growth. Integration of signaling circuitry at a systems level may be important for regulating complex behaviors like nutritional cell differentiation in eukaryotic cells and will likely shape future studies where systems biology approaches become more heavily utilized.

How Do Signaling Pathways Accomplish Nutritional Cell Differentiation?

Cells undergoing filamentous growth have different properties than yeast-form cells. Filamentous cells are commonly thought of as having differentiated into a distinct cell type. This idea is supported by the fact that filamentation regulatory pathways induce the expression of hundreds of genes to reconstruct the cell's shape and biochemical properties. Three major changes are associated with filamentous growth, which can account for the morphological changes and cell-surface properties of filamentous cells. These changes include the expression of the cell adhesion molecule Flo11, a switch in polarity, and an extension of different phases of the cell cycle. Although the changes occur synchronously, each aspect is thought to be regulated by different mechanisms. For example, each response can be genetically separated from the others (Mosch and Fink 1997; Palecek *et al.* 2000; Cullen and Sprague 2002). As a result of these changes, cells grow away from colony interiors, become elongated, and adhere to each other and to surfaces. Robust filamentous growth is a property of certain strain backgrounds (typically Σ1278b). Most laboratory strains of *S. cerevisiae* have acquired mutations, presumably as a result of genetic manipulation in the laboratory, that compromise the filamentous response (Liu *et al.* 1996).

Cell adhesion regulation by the flocculin Flo11

One change that is associated with filamentous growth is cell–cell adhesion. Unlike yeast-form cells that fully separate from each other after each cell cycle, filamentous cells remain connected in chains or filaments. The situation in budding yeast differs from that of filamentous fungi, which fail to undergo cytokinesis and grow as multinucleate hyphae. Filamentously growing yeast cells undergo cytokinesis but remain attached to each other through protein and polysaccharide attachments. That is, they form pseudohyphae by virtue of adhesive contacts in the cell wall.

The major cell adhesion molecule that controls filamentous growth is the adhesion/flocculin Flo11 (or Muc1) (Lambrechts *et al.* 1996; Lo and Dranginis 1998; Guo *et al.* 2000). Flo11 is one of the most intensively studied fungal adhesion molecules, and its overall structure and properties are typical of adhesion molecules in other fungal species (Verstrepen and Klis 2006). Flo11 contains a putative N-terminal signal sequence and transmembrane domain, an external Ser/Thr/Pro-rich repeat region that is heavily glycosylated, and a C-terminal glycosylphosphatidylinositol (GPI) anchor. *FLO11* is a member of the *FLO* gene family (*FLO1*, *FLO5*, and *FLO9*–*FLO11*) and is the major expressed flocculin. Other *FLO* genes, which are located at subtelomeric loci, are transcriptionally silent (Guo *et al.* 2000; Verstrepen *et al.* 2004). If by some means they become expressed, the encoded Flo proteins have different and potentially unique adherence properties (Guo *et al.* 2000). For example, *FLO10* is expressed in mutants lacking transcriptional repressors Sfl1 or Rst1/Dig1, and in these settings can substitute for *FLO11* (Breitkreutz *et al.* 2003). In contrast, Flo1 can promote biofilm formation (Smukalla *et al.* 2008).

As discussed above, the *FLO11* gene is regulated by an unusually large promoter where multiple signaling pathways converge. Changes in the *FLO11* gene/promoter can have dramatic effects on cell adhesion. For example, altering the levels and adherence properties of Flo11 can induce novel responses, which range from the flocculation of cells in a dense pellet that falls out of solution, to the formation of buoyant aggregates of cells on broth surfaces (Fidalgo *et al.* 2006). Changes in *FLO11* expression occur rapidly and are subject to epigenetic regulation, which can result in a heterogenous population of cells with different adherence properties (Halme *et al.* 2004; Verstrepen *et al.* 2005). In pathogens, variation of proteins at the cell surface is an important feature of virulence, and is thought for example to allow fungal cells to evade detection by the immune system (Heinsbroek *et al.* 2005; Nather and Munro 2008).

Flo11 is required for invasive growth of cells into agar-based substrates. Indeed, natural isolates of yeast exhibit high levels of agar invasion (Casalone *et al.* 2005). What is the physiological basis for this response? Yeast cells undergo invasive growth in at least one "natural" setting, the fruiting bodies of grapes. The filamentous growth pathway and *FLO11* are required for full colonization of this environment (Pitoniak *et al.* 2009). Presumably, therefore, invasive growth mediated by Flo11-dependent contacts allows the penetration of cells into a variety of different environments.

Flo11 is also required to mediate colonial surface expansion in a connected mat of cells or biofilm (Reynolds and Fink 2001). Biofilms are a common growth pattern in many microbial species (Parsek and Greenberg 2005). Mat formation in yeast is regulated by some of the same signaling pathways that regulate filamentous growth, as well as by nonoverlapping pathways (Martineau *et al.* 2007). The role that Flo11 plays in biofilm/mat expansion is not entirely clear, which highlights the fact that certain aspects of Flo11 regulation have yet to be elucidated. For example, it is not clear what Flo11 binds to to mediate its adherence properties. As a second example, cells in a biofilm are thought to glide past each other during colonial expansion in a Flo11-dependent manner (Reynolds and Fink 2001). How does a potent cell-adhesion molecule promote cellular sliding? More generally, are adherent cells capable of separating in response to changes in environmental conditions?

One hint toward answering some of these questions has come from the unexpected finding that Flo11 can be shed from cells (Karunanithi *et al.* 2010). Flo11 shedding was tested because two other yeast mucin-like glycoproteins, Msb2 and Hkr1, are also shed. Flo11 shedding provides a mechanism to interrupt adherence. Indeed, the overall balance in adherence properties—not maximal adherence—optimizes filamentous growth and mat formation (Karunanithi *et al.* 2010). Shed Flo11 surrounds yeast mats in a fluid layer that may be functionally equivalent to the mucus secretions of higher eukaryotes. Secreted mucin-like proteins may play unexpected roles in the adherence properties and virulence of microbial pathogens (Karunanithi *et al.* 2010).

Understanding Flo11 properties and regulation provides a direct connection to pathogenesis. The *ALS* gene family in *C. albicans* (Hoyer 2001) and *EPA* family in *C. glabrata* (De Las Penas *et al.* 2003) are Ser/Thr-rich glycoproteins that are connected to the cell wall by a C-terminal GPI anchor (Verstrepen and Klis 2006). In *C. albicans*, adhesion molecules regulate the attachment of cells to medical devices. They promote the compaction of cells into specialized biofilms that are resistant to high levels of antibiotics and contribute to the formation of filaments of interconnected hyphal cells (Chandra *et al.* 2005; Blankenship and Mitchell 2006; Nobile and Mitchell 2006). The regulated expression of adhesion molecules also appears to control the specific adherence properties of cells in a surface-dependent manner (Verstrepen and Klis 2006). Therefore, cell adhesion regulation is an important feature of filamentous growth, biofilm formation, and pathogenesis.

A potentially related response is the formation of ruffled colonies in a Flo11-dependent manner on high-agar surfaces (Figure 2A) (Granek and Magwene 2010). Despite the visually striking nature of these colonies, the functional significance of this patterning is not clear. The degree of colony ruffling corresponds to the level of the Flo11 protein, and it has been speculated that Flo11 (St'ovicek *et al.* 2010) and other flocculins like Flo1 (Beauvais *et al.* 2009), contribute to the formation of an extracellular matrix in this setting. Given that Flo11 exists in a form that is shed from cells (Karunanithi *et al.* 2010), this possibility, although relatively unexplored, is intriguing.

Cell polarity reorganization by bud-site–selection proteins

The reorganization of cell architecture that occurs during filamentous growth involves many different processes and proteins that control cell polarity, the cell cycle, and the switch from apical to isotropic growth. Cell polarity in yeast is primarily dictated by proteins that mark the ends of the cell, which connect to signaling GTPases that regulate the actin cytoskeleton. Budding yeast exhibit different growth patterns depending on cell type and nutrient levels (Chant and Pringle 1991; Sanders and Field 1995; Ni and Snyder 2001; Nelson 2003). During yeast-form growth, haploid cells bud in an axial pattern, whereas diploid cells bud in a bipolar pattern (Figure 7). The different patterns result from differential utilization of cortical landmarks, or bud-site–selection proteins, that mark the poles of the cell and that are chosen by a cascade of Ras and Rho-like GTPase modules (Park and Bi 2007). Early observations of cells undergoing filamentous growth showed a change in growth pattern (Gimeno *et al.* 1992). Specifically, diploid cells, which typically bud at either pole (bipolar), switched to budding only at the distal pole (distal-unipolar). As a result, cells grew away from their mothers into virgin territories in chains of connected cells.

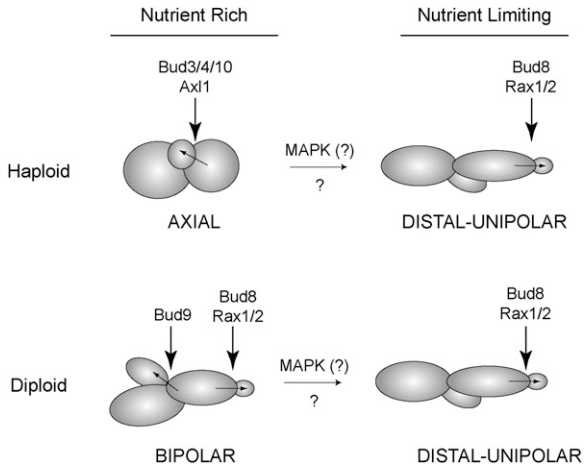

Figure 7 Patterns of budding and bud-site–selection proteins required during yeast-form and filamentous growth. In nutrient-rich conditions, haploid cells bud axially, using cortical landmarks Bud3, Bud4, Bud10, and Axl1 that are localized to the mother-bud neck. Diploid cells grow at both poles in a bipolar pattern using Bud8, Rax1, and Rax2 at the distal pole and Bud9 at the proximal pole. Under nutrient-limiting conditions, both cell types switch to a distal-unipolar pattern and bud more or less exclusively at the distal pole.

The switch in budding pattern is particularly striking in haploid cells, which switch from budding in an axial pattern to a distal-unipolar pattern (Figure 7) (Roberts and Fink 1994). How is this new growth pattern established? The first clues to the answer to this question came from the identification of *BUD* genes in a genetic screen to identify the molecular pathway underlying this growth pattern (Chant and Herskowitz 1991). By examining microcolony peripheries, *bud* mutants were isolated, and the resulting genes began to map out a now well-established molecular pathway. All of the initially identified *BUD1–BUD5* genes were found to be required for proper budding pattern and filamentous growth of haploid cells (Roberts and Fink 1994). Therefore, to a first approximation, the same pathway that is required to establish cell polarity during yeast-form growth is also required for the change in budding pattern of filamentous cells.

The critical barrier to progress in this area was that the protein that marked the distal pole had not been identified. Detailed analysis by Chant and Pringle (1995) of the budding pattern of yeast cells grown under a regimen of starving and refeeding showed that diploid cells bud at the distal pole through a persistent mark or set of marks. The validation of this prediction occurred when the bipolar cues were uncovered in a genetic screen. In a landmark study by Pringle and colleagues, the bipolar cues that regulate budding in diploid cells were identified, which consisted of a pair of related proteins that mark the distal (Bud8) and proximal poles (Bud9) (Harkins *et al.* 2001). As might be expected, Bud8 was required for the distal-unipolar budding pattern of filamentous diploid (Taheri *et al.* 2000) and haploid cells (Cullen and Sprague 2002). Therefore, the switch in cell polarity during filamentous growth requires predominate selection of Bud8 over other positional cues. In fact, filamentous growth provides part of the overall rationale for why yeast cells adopt different budding patterns in the first place: haploid and diploid cells bud distally to escape the colony and enter new territories, whereas haploid cells bud back toward each other to rapidly identify and select a mating partner (Gimeno and Fink 1992).

How is Bud8 preferentially established as the major polar landmark for filamentous cells? Although this question is not yet resolved, several pieces of information are available. Bud8 is required in haploid cells exclusively under nutrient-limiting conditions (Cullen and Sprague 2002); therefore a change in Bud8 or its regulators would be expected to occur preferentially in nutrient-limiting environments. Such a change is not likely to result from loss of the proximal or axial marks, given that they are present in filamentous cells (Cullen and Sprague 2002).

One might expect a signal transduction pathway to interface with the bud-site–selection machinery and orchestrate the change in budding pattern. Addressing this possibility is complicated for several reasons. One is that in diploid cells, in which many studies on filamentous growth have been performed, the switch from bipolar to distal-unipolar budding is subtle, given that the first bud typically emerges from the distal pole in both high- and low-nutrient conditions (Chant and Pringle 1995). Examining the change in budding in haploid cells, which switch from an axial to a distal-unipolar pattern under glucose-limiting conditions, may be easier and more fruitful. We developed the single cell invasive growth assay (Figure 2C), which in principle surmounted these obstacles by allowing quantitation of the changes in budding pattern and cell elongation of individual cells. Using this assay, we showed that components of the filamentous growth MAPK pathway play a role in the change in budding pattern. Specifically, a *ste12* mutant showed a partial defect, and a *ste20* mutant a more severe defect (Cullen and Sprague 2000). A role for the MAPK pathway in regulating distal-unipolar budding conflicts with a previous report (Roberts and Fink 1994). In that study, however, cells were examined on the agar surface and did not show as robust a change in budding pattern as invading cells (Roberts and Fink 1994). A greater role for Ste20 than Ste12 in promoting distal-pole budding is supported by the fact that Ste20 has a specific role in bipolar budding in diploid cells (Sheu *et al.* 2000). More recently, we reported a bud site defect for the *msb2*, *sho1*, and *ste12* mutants, although the *msb2* and *sho1* mutants had a less severe cell elongation defect than the *ste12* mutant (Cullen *et al.* 2004). Recently, it has been reported that neither Ras2 nor the MAPK is required for distal-pole budding (Chen and Thorner 2010). Although we contend that the MAPK pathway does play a role in the switch to distal-unipolar budding, it seems clear that other pathways are also required. Identifying these pathways is an important future goal for research in this area.

Cell elongation due to changes in the cell cycle and polarized growth

During filamentous growth, yeast cells become elongated. How does a cell change its shape? At least two different mechanisms underlie the change in behavior. One is that cells undergo a change in polarity (Pruyne and Bretscher 2000a,b). Specifically, cells undergoing filamentous growth exhibit an increase in apical growth, the highly polarized growth that occurs at the very tip of cells, which differs from isotropic growth that occurs uniformly around the cell cortex. There is clear evidence to support this mechanism. Cells undergoing filamentous growth show a highly polarized actin cytoskeleton, and the polarisome machinery (Gladfelter *et al.* 2005), which is composed of the formin Bni1 and other proteins (Evangelista *et al.* 1997) and is regulated by the polarity control GTPase Cdc42, is required for cell polarization during filamentous growth. How do signaling pathways regulate polarisome function? One possibility is that the PAK kinase Ste20 may phosphorylate Bni1 under conditions of MAPK activation (Goehring *et al.* 2003). Nevertheless, there are many other possible ways in which Cdc42/Bni1 and other proteins might promote enhanced apical growth. Perhaps they interface with proteins whose molecular functions are to regulate tip growth or to regulate the switch between apical and isotropic growth. Potential targets include actin cables, Cln1/2–Cdc28, and GAPs and GEFs for Rho1/Cdc42.

A second mechanism that underlies the change in cell length involves an extension of the cell cycle. Differential extension of one phase of the cell cycle can tip the balance toward more apical growth over isotropic growth (Kron *et al.* 1994). The MAPK pathway regulates the expression of the cyclin *CLN1*, which encodes a G_1 cyclin (Madhani *et al.* 1999). The different G_1 cyclins have different effects on the filamentation response (Loeb *et al.* 1999; Colomina *et al.* 2009). Elements of the morphogenetic checkpoint (Lew and Reed 1995) including the protein kinase Swe1 (Sia *et al.* 1998; La Valle and Wittenberg 2001) are also important for filamentous growth regulation.

Transcriptional targets of filamentation signaling pathways

Filamentous growth can be explained in large part by changes in polarity, the cell cycle, and *FLO11* expression. Over the past decade, hundreds of genes have been identified by various screening approaches that implicate many different cellular processes in the regulation of filamentous growth. Genetic screens (Mosch and Fink 1997; Lorenz and Heitman 1998a; Lorenz *et al.* 2000a), large scale genomic screens (Jin *et al.* 2008b), proteomic approaches (Xu *et al.* 2010), and genome-wide expression profiling (Madhani *et al.* 1999; Roberts *et al.* 2000; Breitkreutz *et al.* 2003) have unveiled a new picture of filamentous growth regulation that impinges on many different cellular processes. Comparative genome sequencing between a standard laboratory strain and the Σ1278b background coupled with genome-wide deletion analysis of all nonessential genes in the Σ1278b background show multiple functional differences between the two genetic backgrounds, and support the notion of a globally regulated cellular response (Dowell *et al.* 2010). Together these genetic and high-throughput approaches reinforce the idea that the dimorphic transition to filamentous growth is a cellular differentiation response that involves the reorganization of many aspects of cellular machinery to produce a specific cell type.

How complicated is filamentous growth? One way to explore the complexity of the response is to examine the outputs of the signaling pathways that regulate the behavior. A diverse collection of genes is induced by the filamentation MAPK pathway. One target encodes the polygalacturonidase Pgu1, an enzyme that metabolizes a component found in plant cell walls (Madhani *et al.* 1999). Filamentous growth occurs in the grape-producing plant *Vitus vinifera* (Pitoniak *et al.* 2009), one environment in which Pgu1 may be required. Another prominent group of transcriptional targets are Ty1 transposons. The fact that transposition is induced by the filamentation pathway in response to environmental perturbation may provide a mechanism for adaptive evolution in response to stress (Morillon *et al.* 2000).

In addition to *PGU1* and Ty, there are many (hundreds of) targets of the signaling pathways that control filamentous growth. What are all of these genes doing? Many of the main targets and highly induced genes do not at present have a clear cellular function. For example, several genes that are considered canonical reporters for the filamentation pathway, like *YLR042c* and *SVS1*, have no clear phenotype when deleted and no established cellular function (P. J. Cullen and G. F. Sprague, unpublished data). An existing challenge is to understand at a functional and phenotypic level the roles that the target genes play in filamentous growth. One reason for the lack of phenotype could be genetic redundancy. A second reason may be that yeast undergoes critical behaviors for filamentous growth that are not obvious under standard laboratory conditions. For example, Pgu1 may be critical for yeast cells to colonize plant tissue but *pgu1* mutants would not be expected to show a clear phenotype in laboratory settings.

Filamentous growth is also tied into core cellular processes. These include transcription by RNA polymerase II (Singh *et al.* 2007), protein translation (Strittmatter *et al.* 2006; Gilbert *et al.* 2007), tRNA modification (Murray *et al.* 1998; Abdullah and Cullen 2009), protein glycosylation (Cullen *et al.* 2000), the unfolded protein response (UPR) (Schroder *et al.* 2000, 2004), autophagy (Ma *et al.* 2007), and the proteasome (Prinz *et al.* 2004). Together these findings resonate with the current picture of the yeast genetic interaction network, where many cellular processes are connected in some manner to each other (Costanzo *et al.* 2010). It will be interesting to overlay onto this network the changes in basic cellular machinery that occur during filamentous growth.

One connection may exist between protein glycosylation, the UPR, and the MAPK pathway. The rationale goes as follows: defects in protein glycosylation or protein folding reduce the glycosylation/stability of the extracellular domain of Msb2 (Yang *et al.* 2009). Underglycosylated Msb2 mimics the activated form of the protein, because the extracellular domain is inhibitory and activates the MAPK pathway. In protein glycoslyation mutants, Msb2 is underglycosylated (Cullen *et al.* 2004; Yang *et al.* 2009), and the MAPK pathway is active (Cullen *et al.* 2000). Indeed, most perturbations to Msb2's mucin homology domain (which is heavily glycosylated in mammalian mucins) (Silverman *et al.* 2003) results in a hyperactive protein (Cullen *et al.* 2004). Most intriguingly, protein glycosylation provides a readout of nutrition, because mannosyl substrates are derived from glucose-6-phosphate. Therefore, underglycosylation of Msb2 may represent a signal to the MAPK pathway for entry into low-nutrient environments. More generally, the rates of core cellular processes may reflect overall nutritional status that becomes sensed and incorporated into the filamentation response.

Interestingly, the UPR has an inhibitory role in sporulation (Schroder *et al.* 2000) and mediates its inhibitory effect by recruitment of the HDAC Rpd3 to early meiotic genes through the transcription factor Ume6 (Schroder *et al.* 2004). In contrast, Rpd3 plays a positive role in filamentous growth and is required for the expression of the *MSB2* and *STE12* genes (Chavel *et al.* 2010). The reciprocal roles of Rpd3 in promoting filamentous growth and dampening meiosis suggest that Rpd3 may be involved in the decision of whether cells should undergo filamentous growth or sporulate (Figure 1). The mechanism by which a HDAC, traditionally considered to function as a repressor of gene transcription, promotes the expression of filamentation regulatory genes is not clear. Nevertheless, microarray analysis reveals that many genes are downregulated in HDAC mutants, and Rpd3 also positively regulates the HOG pathway (de Nadal *et al.* 2004).

Perspectives

Studies of filamentous growth regulation in budding yeast have had at least two major biological impacts. The first is that yeast provides a roadmap to identify and characterize elements of the response that also occurs in other fungal species, particularly fungal pathogens. Many of the genetic pathways that regulate filamentous growth in *C. albicans* and other pathogens have been uncovered through studies in *S. cerevisise*. As one of many possible examples, Msb2 homologs have recently been identified in *C. albicans* and in three plant fungal pathogens. In all cases, Msb2 presides over a MAPK pathway that is important for filamentous growth and virulence (Roman *et al.* 2009; Lanver *et al.* 2010; Liu *et al.* 2011; Perez-Nadales and Di Pietro 2011). The second is that filamentous growth regulation is a model for understanding eukaryotic cell differentiation. Cell differentiation in mammals involves processes that are at present complex and poorly defined. Specifically, the concept of a globally connected network of signaling pathways working in concert, although accepted, is not well understood at the molecular level. Budding yeast provides a working template to understand how signals that initiate from different pathways become routed through common modules to induce a specific behavior.

What lies ahead for studies on filamentous growth regulation in yeast? Filamentous growth represents a point of convergence between many cellular pathways— the cell cycle, cell polarity, and nutrition—and therefore is an attractive system to understand the connection between different biological processes. Of course, one of the main places where future progress is needed is in further defining the signaling pathways that regulate the response. The Gpr1/Gpa2/Ras2 pathway is filled with controversies that make drawing a coherent picture of that pathway difficult. Paramount in this regard is resolving the paradox of whether and how carbon sources are sensed and interpreted into the decision of whether or not to undergo filamentous growth. Another area in which much progress is needed is in understanding how the MAPK pathway that regulates filamentous growth maintains its identity. It could be reasonably argued that all of the components of that pathway (Msb2, Sho1, Cdc42, Ste20, Ste50, Ste11, Ste7, Kss1, and Ste12), with the exception of Tec1, are general components that function in multiple pathways. Solving this identity crisis represents a daunting challenge in the field of cellular signaling. Probably the most important and mysterious aspect of filamentous growth regulation involves the integration of signals from multiple pathways into a coherent response. Do the MAPK, TOR, and Ras2 pathways talk to each other, and if so, to what extent? This area in particular is ripe for future investigations.

In addition to filamentous growth, bakers' yeast undergoes other nutrient-limitation–dependent responses (Figure 1). These include entry into a quiescent state, sporulation (in diploids), microbial mat expansion, and quorum sensing. As mentioned above, an important question is how does a cell choose among these different lifestyles? Similarly, is there a relationship between these various responses? For example, *C. albicans* forms microbial mats or biofilms that are composed of multiple cell types including filamentous cells and that interface with other communities of microorganisms (Parsek and Greenberg 2005). Ultimately, the ecology of filamentous growth regulation—especially of cell populations in native settings—will be the most fun and challenging to explore.

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