The Filamentous Growth MAPK Pathway Responds to Glucose Starvation Through the Mig1/2 Transcriptional Repressors in *Saccharomyces cerevisiae*

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ABSTRACT In the budding yeast *S. cerevisiae*, nutrient limitation induces a MAPK pathway that regulates filamentous growth and biofilm/mat formation. How nutrient levels feed into the regulation of the filamentous growth pathway is not entirely clear. We characterized a newly identified MAPK regulatory protein of the filamentous growth pathway, *Opy2*. A two-hybrid screen with the cytosolic domain of *Opy2* uncovered new interacting partners including a transcriptional repressor that functions in the AMPK pathway, *Mig1*, and its close functional homolog, *Mig2*. *Mig1* and *Mig2* coregulated the filamentous growth pathway in response to glucose limitation, as did the AMP kinase *Snf1*. In addition to associating with *Opy2*, *Mig1* and *Mig2* interacted with other regulators of the filamentous growth pathway including the cytosolic domain of the signaling mucin *Msb2*, the MAP kinase kinase *Ste7*, and the MAP kinase *Kss1*. As for *Opy2*, *Mig1* overproduction dampened the pheromone response pathway, which implicates *Mig1* and *Opy2* as potential regulators of pathway specificity. Taken together, our findings provide the first regulatory link in yeast between components of the AMPK pathway and a MAPK pathway that controls cellular differentiation.

In eukaryotes, evolutionarily conserved signaling pathways regulate the cellular response to nutrients (Mantovani and Roy 2011). In the budding yeast *Saccharomyces cerevisiae*, nutrient sensing and regulation have been extensively studied, and receptors that sense nutrients and their cognate pathways have been defined (Dechant and Peter 2008; Xue *et al.* 2008; Ljungdahl 2009). Glucose is the preferred carbon source in yeast, and its detection and metabolism are highly regulated (Gancedo 2008; Zaman *et al.* 2008). Among the pathways that control the cellular response to glucose levels are the *Snf3/Rgt2* pathway (Ozcan and Johnston 1999), the *Ras2*-cAMP-protein kinase A (PKA) pathway (Thevelein and Voordeckers 2009), and a glucose repression pathway that is regulated by the ubiquitous nutrient-sensing AMP-dependent kinase (AMPK) *Snf1* (Carlson 1999). *Snf3* and *Rgt2* are glucose sensors that regulate the expression of

hexose transporters (*Hxt*) of appropriate affinities through the transcriptional repressor *Rgt1* (Ozcan and Johnston 1996; Sabina and Johnston 2009). *Gpr1* is another glucose sensor (Nakafuku *et al.* 1988; Kraakman *et al.* 1999; Harashima and Heitman 2002; Peeters *et al.* 2007; Thevelein and Voordeckers 2009) that, together with the major nutrient regulatory GTPase *Ras2* (Kataoka *et al.* 1984), converges on adenylate cyclase to regulate cellular cAMP levels and PKA activity (Toda *et al.* 1987; Robertson and Fink 1998; Pan and Heitman 1999; Robertson *et al.* 2000). Communication and signal integration among the different pathways results in a unified response to fluctuating nutrient levels (Kaniak *et al.* 2004; Kim and Johnston 2006).

Under nutrient-limiting conditions, *Snf1* regulates the utilization of poor carbon sources (Celenza and Carlson 1989; Woods *et al.* 1994; Lesage *et al.* 1996; McCartney and Schmidt 2001). One target of *Snf1* is the transcriptional repressor *Mig1*. *Snf1* phosphorylates *Mig1* (Ostling and Ronne 1998; Treitel *et al.* 1998; Smith *et al.* 1999), which relieves its transcriptional repression function and promotes its export from the nucleus. *Mig2* is a functional homolog of *Mig1* (Lutfiyya and Johnston 1996), but *Mig2* is not regulated by *Snf1* and has a localization (Lutfiyya *et al.* 1998;

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Fernandez-Cid *et al.* 2012) and turnover pattern (Lim *et al.* 2011) that is distinct from *Mig1*. In high-glucose conditions, *Mig1* and *Mig2* repress the expression of genes involved in the metabolism of poor carbon sources, in part through *Mig1*-dependent recruitment of the co-repressor *Tup1/Ssn6* (Treitel and Carlson 1995).

In response to nutrient limitation, yeast not only prepare to utilize less preferred carbon sources but also can switch their growth pattern. Depending on cell type and the specific nutritional challenge, yeast can undergo quiescence [*G₀* arrest (Gray *et al.* 2004)], sporulation (Neiman 2011), filamentous/invasive/pseudohyphal growth (Cullen and Sprague 2012), and/or biofilm/mat formation (Reynolds and Fink 2001). Many fungal species undergo similar responses. In pathogens like *Candida albicans*, filamentous growth (Lo *et al.* 1997) and biofilm/mat formation (Blankenship and Mitchell 2006) are required for virulence. In *S. cerevisiae*, filamentous growth occurs in response to limiting nitrogen (Gimeno *et al.* 1992) or glucose (Cullen and Sprague 2000) and is regulated by multiple signaling pathways including TOR (Rohde and Cardenas 2004), Ras-cAMP-PKA (Gimeno *et al.* 1992), and *Snf1* (Cullen and Sprague 2000) through the transcriptional repressors *Nrg1* and *Nrg2* (Kuchin *et al.* 2002, 2003), and a mitogen-activated protein kinase (MAPK) pathway commonly referred to as the filamentous growth pathway (Liu *et al.* 1993; Roberts and Fink 1994). Nutrient limitation stimulates the filamentous growth pathway (Pitoniak *et al.* 2009), although the plasma-membrane regulators *Msb2* and *Sho1* (O'Rourke and Herskowitz 1998; Cullen *et al.* 2000, 2004) are not thought to sense nutrients directly. Rather, the MAPK pathway is sensitized to nutrient levels by regulatory inputs from *Ras2*-cAMP-PKA (Mosch *et al.* 1996; Chavel *et al.* 2010) and regulated processing of *Msb2* (Cullen *et al.* 2004) by starvation-dependent induction of genes that encode its cognate proteases (Vadaie *et al.* 2008). Whether other nutrient-sensing pathways also regulate the filamentous growth pathway in response to nutrient levels is an open question (Figure 1A, question mark).

In this study, we identified a new connection between the major glucose-sensing (AMPK) pathway and the filamentous growth (MAPK) pathway. This connection was uncovered by characterizing the newly identified plasma-membrane regulator *Opy2* (Wu *et al.* 2006; Ekiel *et al.* 2009; Yang *et al.* 2009; Yamamoto *et al.* 2010; Cappell and Dohlman 2011), which regulates the *Ste11* branch of the high-osmolarity glycerol response (HOG) MAPK pathway (Hohmann *et al.* 2007; Saito 2010). *Opy2* is also thought to regulate the filamentous growth pathway (Yang *et al.* 2009; Yamamoto *et al.* 2010). We confirmed that *Opy2* is a major regulator of the filamentous growth pathway, and we identified *Mig1* and *Mig2* as *Opy2*-interacting proteins that coregulate the filamentous growth pathway. *Mig1* and *Mig2*, and the AMPK *Snf1*, were required for induction of the filamentous growth pathway in response to glucose limitation. We also show that *Mig1* and *Opy2* attenuated the pheromone response

pathway, which suggests a role for these proteins in regulating pathway specificity. The regulatory connection between *Mig1* and *Mig2* and the filamentous growth pathway provides a direct link between glucose sensing (via AMPK) and cell differentiation (via MAPK).

Materials and Methods

Yeast strains and plasmids

Yeast strains are listed in Table 1. Plasmids are listed in Table 2. Yeast and bacterial strains were manipulated by standard methods (Sambrook *et al.* 1989; Rose *et al.* 1990). All yeast strains were grown in standard YEP media supplemented with 2% glucose (GLU or D) or 2% galactose (GAL) for 16 hr at 30° unless otherwise indicated. Epitope tagging with hemagglutinin (HA) and c-MYC (MYC) epitopes were performed as described (Schneider *et al.* 1995). Gene disruptions and *GAL1* promoter fusions were made by PCR-based methods (Baudin *et al.* 1993; Longtine *et al.* 1998), including cassettes containing antibiotic-resistance markers (Goldstein and McCusker 1999). PCR analysis and phenotype were used to confirm integrations. Epitope tagging and *GAL1* promoter fusions were modified to generate N-terminal myristoylation tags by introducing codons for the amino acid sequence MGCTVSTQTI (Gillen *et al.* 1998; Jansen *et al.* 2005). The plate-washing assay (Roberts and Fink 1994) and single-cell invasive growth (Cullen and Sprague 2000) assay were performed as described. Biofilm and mat assays were carried out on YEP or YEPD plates containing 0.3 or 4% agar (Reynolds and Fink 2001). Mat perimeters were screened by microscopy as described (Karunanithi *et al.* 2012). β -Galactosidase assays were performed as described (Cullen *et al.* 2000). Values reported are the average of at least two independent experiments. Halo assays were performed as described (Jenness *et al.* 1987).

Bacterial strains were grown in Luria-Bertani broth at 37° (Sambrook *et al.* 1989). Plasmid pBS34 was obtained from the University of Washington Yeast Resource Center (Shaner *et al.* 2004). Filamentous growth pathway reporter plasmids *pYLR042c-lacZ* (Roberts *et al.* 2000) and *pFRE-lacZ* (Madhani and Fink 1997) have been described. Plasmids expressing a library of *P_{GAL}*-driven ORFs were obtained from Open Biosystems (Gelperin *et al.* 2005). *pSTE11-4* (Stevenson *et al.* 1992), *pRS316-SHO1-GFP* (Marles *et al.* 2004), *pHA-MSB2* (Vadaie *et al.* 2008), and *pMSB2-GFP* (Cullen *et al.* 2004) have been described. Genomic full-length *OPY2-GFP* was cloned into *SalI* and *EcoRI* sites in *pRS316* (Sikorski and Hieter 1989) and *pRS426* (Christianson *et al.* 1992) by engineering restriction sites in the primers. The cytoplasmic domain (116–360 amino acids) of *Opy2* was cloned into *EcoRI* and *SalI* sites in *pGBDU-C1* (James *et al.* 1996) for two-hybrid analysis. Truncations of the cytosolic domain of *Opy2* were generated by PCR amplification of codons corresponding to amino acids 246–360 (*pGBDU-C1*-

Figure 1 Opy2 regulates the filamentous growth pathway. (A) The MAPK pathway that regulates filamentous growth and biofilm/mat formation. It is not clear how glucose limitation leads to pathway activation. (B) The plate-washing assay. Wild-type, *msb2Δ*, *opy2Δ*, *ste50Δ*, and *ste12Δ* strains were spotted onto YEPD medium and incubated for 2 days at 30°. The plate was photographed (left panel, YEPD), washed under a stream of water, and photographed again to reveal invaded cells (right panel, Washed). (C) Microscope-based assays and colony morphology assays to evaluate the role of Opy2 in the filamentous growth pathway. (Far left column) The single-cell invasive growth assay. Wild-type, *opy2Δ*, and *ste12Δ* cells were spread at low concentrations onto synthetic complete medium lacking glucose (SC-GLU). Plates were incubated for 24 hr and examined by DIC microscopy at ×100 magnification. More than 100 microcolonies were examined, and representative images are shown. Arrows point to axial (nondistal) budding patterns commonly seen in the *opy2Δ* and *ste12Δ* mutants. Bar, 5 μm. (Second column from left) Biofilm/mat assay. Strains were grown for 16 hr in YEPD and spotted onto YEPD media with 0.3% agar (YEPD + 0.3% agar). Plates were incubated at 30° for 4 days and photographed. The *opy2Δ* and *ste12Δ* mats were smaller and less ruffled. Bar, 1 cm. (Third column from left) Strains were spotted onto YEPD + 4% agar atop a nitrocellulose filter and incubated at 30° for 7 days and photographed. Bar, 1 cm. (Far right column) Cells were spotted onto YEP + 0.3% agar for 21 days. Microscopic images (×20 magnification) of mat perimeters are shown. Dense mats containing pseudohyphae at borders can be seen for wild type but not for the *opy2Δ* and *ste12Δ* mutant. Bar, 200 μm. (D) Phosphorylation of the MAP kinase Kss1 in wild-type cells and the indicated mutants. Immunoblots are shown to detect phosphorylated Kss1 (P~Kss1, 42 kDa) and Fus3 (P~Fus3, 40.7 kDa) with p44/42 antibodies. Blots using antibodies to Pgk1 (44.7 kDa) are shown as a loading control for protein levels. Blots using antibodies to the Kss1 protein (Kss1, 42 kDa) are shown. The asterisk refers to a nonspecific band. (E) β-Galactosidase assays to measure expression of the pFRET-LacZ and pYLR042c-LacZ reporters in wild-type and *opy2Δ* strains. Enzymatic activity is expressed in Miller units. Two independent experiments were performed, with error bars showing differences between trials. (F) Immunoblots showing the level of the Flo11-HA protein (>220 kDa) in culture supernatant (S) and pellet (P) fractions. The level of Flo11 in supernatants probably reflects the levels seen in pellets.

OPY2^{Δ1-245}), 116–268 (pGBDU-C1-OPY2^{Δ1-115, 269-360}), and 116–323 (pGBDU-C1-OPY2^{Δ1-115, 324-360}) from the parent plasmid pGBDU-C1-OPY2^{CT} (James *et al.* 1996). The cytosolic domain of Opy2 was subcloned from two-hybrid constructs into pMAL-C2 vector (New England Biolabs, Ipswich, MA) for expression in *Escherichia coli*. Full-length MIG1 from pBM3315 (De Vit *et al.* 1997) was used as a template to PCR-amplify and clone into BamHI and SalI sites in pGAD-C1 (James *et al.* 1996) and pGEX4T1 (Amersham Biosciences, Piscataway, NJ). Genomic full-length FUS3 was amplified by PCR and cloned into EcoRI and SalI sites in pGBDU-C1. Other two-hybrid constructs

are described by A. Pitoniak, C. Chavel, N. Vadaie, J. Smith, S. Karunanithi, D. Camara, and P. Cullen (unpublished results).

Microscopy

Differential interference contrast (DIC) and fluorescence microscopy using GFP and rhodamine filter sets were performed using an Axioplan 2 fluorescent microscope (Zeiss) with PLAN-APOCHROMAT 100X/1.4 (oil) objective (numerical aperture 0.17). Digital images were obtained with the Axiocam MRm camera (Zeiss). Axiovision 4.4 software (Zeiss) was used for image acquisition.

Table 1 Yeast strains used in this study

Strain	Genotype	Reference
PC313 ^a	<i>MATa ura3-52</i>	Liu et al. (1993)
PC538	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52</i>	Cullen et al. (2004)
PC560	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 snf1::URA3</i>	Cullen and Sprague (2000)
PC948	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 msb2::KanMX6</i>	Cullen et al. (2004)
PC999	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA</i>	Cullen et al. (2004)
PC586	<i>MATα ura3-52 leu2</i>	Cullen et al. (2004)
PC1029	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 flo11::KanMX6</i>	Karunanithi et al. (2010)
PC1079	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA ste12::URA3</i>	Cullen et al. (2004)
PC2043	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA::KanMX6</i>	Karunanithi et al. (2010)
PC1516	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA$\Delta^{100-818}$</i>	Vadaie et al. (2008)
PC2382	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste12::KanMX6</i>	Cullen et al. (2004)
PC474 ^b	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sho1::KIURA3</i>	Cullen et al. (2004)
PC1523	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ssk1::NAT</i>	Pitoniak et al. (2009)
PC284	<i>(PJ69-4a) MATa trp1-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2::GAL1-HIS3GAL2-ADE2 met2::GAL7-lacZ</i>	James et al. (1996)
PC2053	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pbs2::nat</i>	Cullen et al. (2004)
PC2691	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA::KanMX6 ste12::KIURA3</i>	Chavel et al. (2010)
PC3752	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 opy2::KIURA3</i>	This study
PC3784	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA opy2::KIURA3</i>	This study
PC3786	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA opy2::NAT</i>	This study
PC3788	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2$\Delta^{100-818}$ opy2::KIURA3</i>	This study
PC3819	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ssk1::NAT opy2::KIURA3</i>	This study
PC3823	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 OPY2-GFP::KanMX6</i>	This study
PC3861	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste11::NAT</i>	This study
PC3894	<i>MATa ura3-52 opy2::NAT</i>	This study
PC3896	<i>MATa ura3-52 leu2 opy2::NAT</i>	This study
PC3970	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 P_{GAL}-OPY2::KanMX6</i>	This study
PC3976	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 P_{GAL}-MYR-STE50::KanMX6 opy2::KIURA3</i>	This study
PC4107	<i>MATa ura3-52 P_{GAL}-OPY2::KanMX6</i>	This study
PC4244	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 P_{GAL}-MYR-OPY2^{CT}::KanMX6</i>	This study
PC4470	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA opy2::NAT rga1::URA3</i>	This study
PC4703	<i>MATa ura3-52 OPY2-GFP::KanMX6</i>	This study
PC4843	<i>MATa ura3-52 mig1::KIURA3</i>	This study
PC4862	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 P_{GAL}-OPY2-GFP::KanMX6:: NAT</i>	This study
PC5208	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 opy2::NAT</i>	This study
PC5484	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MYR-HA-STE50 opy2::NAT</i>	This study
PC3863	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste50::NAT</i>	This study
PC3974	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 P_{GAL}-MYR-STE50::KanMX6</i>	This study
PC5469	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MYR-HA-STE50</i>	This study
PC4016	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 P_{GAL}-STE50::KanMX6</i>	This study
PC4108	<i>MATa ura3-52 P_{GAL}-STE50::KanMX6</i>	This study
PC4202	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 HA-STE50</i>	This study
PC4982	<i>MATa ura3-52 ste50::NAT</i>	This study
PC5009	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig1::KIURA3</i>	This study
PC4860	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 P_{GAL}-MIG1::KanMX6</i>	This study
PC4866	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MIG1-GFP::KanMX6</i>	This study
PC5020	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig1::KIURA3 mig2::NAT</i>	This study
PC5023	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig2::NAT</i>	This study
PC5058	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig2::HYG</i>	This study
PC5060	<i>MATa ura3-52 mig2::HYG</i>	This study
PC5175	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig1::KIURA3 ssk1:: NAT</i>	This study
PC5178	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig2::HYG ssk1::KIURA3</i>	This study
PC5181	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig1::NAT mig2::HYG ssk1::KIURA3</i>	This study
PC5184	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig1::NAT mig2::HYG</i>	This study
PC5187	<i>MATa ura3-52 mig1::NAT mig2::HYG</i>	This study
PC5214	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA mig1::NAT mig2::HYG</i>	This study
PC5204	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA mig1::NAT</i>	This study
PC5205	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA mig2::NAT</i>	This study
PC5200	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig3::NAT mig2::HYG</i>	This study
PC5210	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig3::NAT</i>	This study
PC5212	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig1::NAT mig3::HYG</i>	This study
PC5222	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig1::NAT mig2::HYG mig3::TRP</i>	This study

(continued)

Table 1, continued

Strain	Genotype	Reference
PC5216	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 trp mig1::NAT mig2::HYG</i>	This study
PC5601	<i>MATa ura3-52 P_{GAL}-MYR-STE50::KanMX6</i>	This study
PC5603	<i>MATa ura3-52 opy2::NAT P_{GAL}-MYR-STE50::KanMX6</i>	This study
PC5645	<i>MATa ura3-52 P_{GAL}-MYR-STE50-GFP::KanMX6::NAT</i>	This study
PC5599	<i>MATa ura3-52 P_{GAL}-MIG1::KanMX6</i>	This study

^a All strains are in the Σ 1278b background unless otherwise indicated.

^b *KIURA3* refers to the *Kluyveromyces lactis* *URA3* cassette.

OPY2 plasmid mutagenesis

Plasmid mutagenesis was performed as previously described (Vadaie *et al.* 2008). Approximately 25 μ g of pRS316-*OPY2-GFP* DNA was mutagenized using hydroxyl-amine mutagenesis (Rose and Fink 1987). Mutation efficiency was $\sim$ 12% as assessed by the frequency of *opy2* null mutations. Null alleles were selected on synthetic media lacking histidine, using the *FUS1-HIS3* reporter integrated into the *opy2* Δ *ste4* Δ Σ 1278b strain (PC3786). A total of 150 URA⁺ colonies were assessed, of which 14 were initially identified as HIS⁻. Of the 14, 2 isolates were found to be plasmid-dependent and were confirmed by sequencing at the Roswell Park sequencing facility (Roswell Park Cancer Institute, Buffalo, NY) to contain mutation in the extracellular domain (*OPY2*^{C30Y}) and in the cytosolic tail (*OPY2*^{G207D}), respectively.

Two-hybrid analysis

Two-hybrid analysis was used to assess protein interactions (Fields and Song 1989). Two-hybrid analysis using the cytosolic domain of *Opy2* against an ordered library of yeast ORFs fused downstream of the *Gal4*-activation domain constructs (pOAD) (Uetz *et al.* 2000) was performed. *Opy2*'s cytosolic tail was cloned downstream of a *Gal4*-binding domain vector (pGBDUC1) and transformed into the library by standard transformation protocols. A total of 200,000 transformants were screened. Transformants were selected on synthetic media lacking uracil and leucine to select for the plasmids as well as lacking histidine, in the presence of 7 mM 3-amino-1,2,4-triazole (ATA), which allowed the detection of 40 ATA-resistant colonies. Isolates were selected on 5-fluoroorotic acid (Boeke *et al.* 1987) containing media to lose the bait plasmid (pGBDUC1-*Opy2*^{CT}). Twenty-eight isolates activated the *GAL1-HIS3* reporter in the absence of pGBDUC1-*Opy2*^{CT}. Plasmid rescue and retransformation with the bait plasmid identified 11 isolates that were plasmid-dependent. Insert ORFs were sequenced by Roswell Park Sequencing facility (Roswell Park Cancer Institute, Buffalo, NY), which were identified to be *Ste50* (5 isolates), *Mig1* (5 isolates), and *Mtc1* (1 isolate). *Mig2* was not identified in the screen, which suggests that some clones might be underrepresented.

Immunoblots and protein analysis

Immunoblots were performed as described (Cullen *et al.* 2004). Proteins were separated by SDS-PAGE on 12% gels

and transferred to nitrocellulose membranes (protran BA85, VWR International Inc., Bridgeport NJ). Membranes were incubated in 25 ml of blocking buffer (5% nonfat dry milk, 10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% Tween 20) for 1 hr at 25° or for 16 hr at 4°. Preblocked membranes were incubated in blocking buffer containing primary antibodies for 1 hr at 25°. Blots were washed three times for 5 min each in TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl and 0.05% Tween 20). Blots were incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hr at 25° and washed as above. A WesternBright MCF fluorescent Western blotting kit from Advanta Inc. (Menlo Park, CA) was used to visualize the proteins. Shedding of *Flo11* protein was evaluated as described (Karunanithi *et al.* 2010). Anti-GFP (#11814460001) and anti-HA (12CA5, #11666606001) antibodies were from Roche Applied Science (Indianapolis). Anti-GST antibodies (#600-101-200) were from Rockland Immunochemicals (Gilbertsville, PA). Anti-MBP antiserum (#E8030S) was obtained from New England Biolabs (Ipswich, MA). HRP-conjugated goat anti-mouse secondary antibodies were purchased from Bio-Rad (Hercules, CA).

Phospho-Kss1/Fus3 assay

The assay for monitoring the phosphorylation of *Kss1* and *Fus3* has been described (Lee and Dohlman 2008; Takahashi and Pryciak 2008). Cultures were grown to logarithmic phase for 6 hr in YEPD or YEPGAL for basal and induced conditions, respectively. *Kss1* phosphorylation levels under induced (galactose) conditions are shown unless indicated. An equal number of cells were harvested and cell extracts were prepared by trichloroacetic acid precipitation (Cox *et al.* 1997; Lee and Dohlman 2008). Protein concentrations were normalized using a protein BCA kit (Thermo-Fisher, Waltham, MA). Equal amounts of protein were loaded onto SDS-PAGE gels. Immunoblots were performed using p44/42 MAPK antibodies (#4370s) from Cell Signaling Technology (Danvers, MA) in 5% bovine serum albumin (BSA) at 4° for 16 hr. Blots were washed at 25°. Secondary antibody incubation was performed for 1 hr at 25° with HRP-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch, Westgrove, PA). Incubations with anti-*Kss1* (rabbit, Santa Cruz Biotechnology Inc., Santa Cruz, CA; #6775) and anti-*Fus3* (goat, Santa Cruz Biotechnology; #6773) were performed in 5% BSA at 4° for 16 hr. Antibody detection for mouse and goat primary antibodies was achieved using HRP-conjugated secondary

Table 2 Plasmids used in this study

No.	Plasmid	Description	Source
PC171	pRS316	<i>AMP/CEN/URA3</i>	Sikorski and Hieter (1989)
PC5174	pRS426	<i>AMP/2μ/URA3</i>	Christianson <i>et al.</i> (1992)
PC2415	pBS34	<i>AMP/mCherry/kanMX6</i>	Shaner <i>et al.</i> (2004)
PC922	pMPY-3xMYC	<i>AMP/URA3</i>	Schneider <i>et al.</i> (1995)
PC921	pMPY-3xHA	<i>AMP/URA3</i>	Schneider <i>et al.</i> (1995)
PC1744	pGAD-C1	<i>AMP/2μ/LEU2</i>	James <i>et al.</i> (1996)
PC1743	pGBDU-C1	<i>AMP/2μ/URA3</i>	James <i>et al.</i> (1996)
PC5250	pGEX-4T1	GST-fusion	Amersham Biosciences
PC166	pMAL-C2	MBP-fusion	New England Biolabs
PC2773	pYLR042c-lacZ	<i>AMP/CEN/LEU2</i>	Roberts <i>et al.</i> (2000)
PC1308	pFRE-lacZ	<i>AMP/CEN/LEU2</i>	Madhani and Fink (1997)
PC1715	pRS316-SHO1 ^{P120L}	<i>AMP/CEN/URA3</i>	Vadaie <i>et al.</i> (2008)
PC1441	pSTE11-4	<i>AMP/CEN/URA3</i>	Stevenson <i>et al.</i> (1992)
PC1601	pRS316-SHO1-GFP	<i>AMP/CEN/URA3</i>	Marles <i>et al.</i> (2004)
PC1820	pHA-MSB2	<i>AMP/CEN/URA3</i>	Vadaie <i>et al.</i> (2008)
PC1696	pMSB2-GFP	<i>AMP/CEN/URA3</i>	Cullen <i>et al.</i> (2004)
PC5764	pGAL-OPY2	<i>AMP/2μ/URA3</i>	Gelperin <i>et al.</i> (2005)
PC5765	pGAL-ACT1	<i>AMP/2μ/URA3</i>	Gelperin <i>et al.</i> (2005)
PC5766	pGAL-MIG1	<i>AMP/2μ/URA3</i>	Gelperin <i>et al.</i> (2005)
PC5767	pGAL-MIG2	<i>AMP/2μ/URA3</i>	Gelperin <i>et al.</i> (2005)
PC5192	pBM3315	F64L, S65T variant of GFP fused to N terminus of Mig1	De Vit <i>et al.</i> (1997)
PC4370	pGAD-C1-STE50	<i>AMP/2μ/LEU2</i>	Pitoniak <i>et al.</i> , unpublished results ^a
PC4234	pGBDU-C1-MSB2	<i>AMP/2μ/URA3</i>	Pitoniak <i>et al.</i> , unpublished results ^a
PC4690	pGAD-C1-CDC24	<i>AMP/2μ/LEU2</i>	Pitoniak <i>et al.</i> , unpublished results ^a
PC4692	pGBDU-C1-CDC24	<i>AMP/2μ/URA3</i>	Pitoniak <i>et al.</i> , unpublished results ^a
PC4689	pGAD-C1-CDC42 ^{D118A}	<i>AMP/2μ/LEU2</i>	Pitoniak <i>et al.</i> , unpublished results ^a
PC4691	pGBDU-C1-CDC42 ^{D118A}	<i>AMP/2μ/URA3</i>	Pitoniak <i>et al.</i> , unpublished results ^a
PC4369	pGAD-C1-STE11	<i>AMP/2μ/LEU2</i>	Pitoniak <i>et al.</i> , unpublished results ^a
PC4366	pGBDU-C1-STE11	<i>AMP/2μ/URA3</i>	Pitoniak <i>et al.</i> , unpublished results ^a
PC4684	pGBDU-C1-STE7	<i>AMP/2μ/URA3</i>	Pitoniak <i>et al.</i> , unpublished results ^a
PC4681	pGAD-C1-KSS1	<i>AMP/2μ/LEU2</i>	Pitoniak <i>et al.</i> , unpublished results ^a
PC4683	pGBDU-C1-KSS1	<i>AMP/2μ/URA3</i>	Pitoniak <i>et al.</i> , unpublished results ^a
PC5647	pGBDU-C1-FUS3	<i>AMP/2μ/URA3</i>	This study
PC4027	pRS316-OPY2-GFP	<i>AMP/CEN/URA3</i>	This study
PC4362	pGBDU-C1-OPY2 ^{Δ1-115}	<i>AMP/2μ/URA3</i>	This study
PC4364	pGBDU-C1-OPY2 ^{G207D}	<i>AMP/2μ/URA3</i>	This study
PC5759	pRS316-OPY2 ^{G207D} -GFP	<i>AMP/CEN/URA3</i>	This study
PC5758	pRS316-OPY2 ^{C30Y} -GFP	<i>AMP/CEN/URA3</i>	This study
PC5226	pRS426-OPY2-GFP	<i>AMP/2μ/URA3</i>	This study
PC5239	pRS426-OPY2 ^{G207D} -GFP	<i>AMP/2μ/URA3</i>	This study
PC5237	pRS426-OPY2 ^{C30Y} -GFP	<i>AMP/2μ/URA3</i>	This study
PC5470	pMAL-C2-OPY2 ^{Δ1-115}	<i>E. coli</i> expression	This study
PC5488	pGBDU-C1-OPY2 ^{Δ1-245}	<i>AMP/2μ/URA3</i>	This study
PC5490	pGBDU-C1-OPY2 ^{Δ1-115,269-360}	<i>AMP/2μ/URA3</i>	This study
PC5492	pGBDU-C1-OPY2 ^{Δ1-115,324-360}	<i>AMP/2μ/URA3</i>	This study
PC4907	pGAD-C1-MIG2	<i>AMP/2μ/LEU2</i>	This study
PC5241	pGAD-C1-MIG1	<i>AMP/2μ/LEU2</i>	This study
PC5467	pGEX-4T1-MIG1	<i>E. coli</i> expression	This study
PC5614	pMAL-C2-OPY2 ^{Δ1-245}	<i>E. coli</i> expression	This study
PC5616	pMAL-C2-OPY2 ^{Δ1-115,269-360}	<i>E. coli</i> expression	This study
PC5618	pMAL-C2-OPY2 ^{Δ1-115,324-360}	<i>E. coli</i> expression	This study

^a Contributors to these unpublished results are: A. Pitoniak, C. Chavel, N. Vadaie, J. Smith, S. Karunanithi, D. Camara, and P. J. Cullen.

antibodies raised in goat (Bio-Rad) and donkey (Santa Cruz Biotechnology; #2020), respectively. To detect phosphorylation of *Hog1* (Lee and Dohlman 2008), log-phase cultures were induced with 1 M KCl for 5 min. p38 MAPK antibodies (Cell Signaling Technology) were used. Proteins were detected using the WesternBright MCF fluorescent Western blotting kit from Advanta Inc. (Menlo Park, CA).

Protein purification and in vitro pull-down assays

pMAL-C2-*Opy2*^{CT} and the truncated versions were expressed in *E. coli* strain [(K12) F-lacZ(am) pho(am) lon tyrT[supC(ts)] trp(am) rpsL(StrR) rpoH(am)165 zhg::Tn10)]. Cells (100 ml) were grown to an OD of 0.6 when isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a concentration of 0.3 mM and grown for 3 hr. Cell pellets

were resuspended in 4 ml lysis buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% TritonX-100) and frozen at -20° , and 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonylfluoride (PMSF) were added to the cell suspension after thawing and lysed by sonication. Batch purification was performed using 50 μ l of amylose resin (per 1 ml of lysate) (New England Biolabs). Purification was carried out as per manufacturer's protocol and eluted using lysis buffer containing 10 mM maltose.

In vitro protein binding has been described (Truckses *et al.* 2006). BL21(DE3) cells containing pGEX4T1 or pGEX4T1-Mig1 were grown to logarithmic phase in 2XYT medium (25 ml) with carbenicillin (100 μ g/ml) to select for plasmids. Cells were induced at an OD of 0.6 with 0.3 mM IPTG for 3 hr at 37° . Cell pellets were resuspended in lysis buffer and frozen. After thawing the cell suspension, 1 mM DTT and 1 mM PMSF were added and sonicated. Fifty microliters of glutathione-sepharose beads (Pierce, Rockford, IL) were added to the cell lysates and incubated at room temperature for 30 min. Beads were washed three times, and purified MBP-Opy2^{CT}/truncations or His-Msb2^{CT} (25 μ l of 1 μ g/ μ l) was added along with BSA (0.26 mg/ml) to a final volume of 300 μ l. Incubation was carried out at 4° for 1 hr followed by three washes with lysis buffer. Bound proteins were extracted in SDS-PAGE buffer by boiling the beads at 100° for 5 min.

Databases

Mig1-binding sites were determined using PatchMatch (http://gfx.cs.princeton.edu/pubs/_2011_PAF/index.php). The Saccharomyces Genome Database was used for gene annotations (<http://www.yeastgenome.org/>). ImageJ was used for densitometric analysis (<http://rsbweb.nih.gov/ij/>).

Results

Opy2 regulates the filamentous growth pathway by plasma-membrane recruitment of Ste50

Opy2 functions in the HOG pathway (Wu *et al.* 2006; Ekiel *et al.* 2009; Yang *et al.* 2009; Yamamoto *et al.* 2010; Cappell and Dohlman 2011) and may also regulate the filamentous growth pathway (Yang *et al.* 2009; Yamamoto *et al.* 2010), although this possibility has not been extensively explored (Wu *et al.* 2006; Chen and Thorner 2007). To determine whether Opy2 regulates the filamentous growth pathway, the *OPY2* gene was deleted in strains of the filamentous (Σ 1278b) background (Liu *et al.* 1996). The *opy2* Δ mutant had an invasive growth defect by the plate-washing assay (Roberts and Fink 1994) that was similar to the defect seen in mutants lacking other MAPK pathway regulators (Figure 1B, *msb2* Δ , *ste50* Δ , and *ste12* Δ). The filamentous growth pathway induces the reorganization of cell polarity [e.g., budding pattern, (Gimeno *et al.* 1992; Roberts and Fink 1994; Chant and Pringle 1995; Cullen and Sprague 2002)] and a delay in the G₂ phase of the cell cycle (Ahn *et al.* 1999; Madhani *et al.* 1999). The single-cell invasive

growth assay (Cullen and Sprague 2000) showed that *opy2* Δ cells were defective for filamentous growth and distal-unipolar budding (Figure 1C, SC-GLU, arrows) and were rounder than wild-type cells (Figure 1C, SC-GLU). Phosphorylation of the MAP kinase that regulates the filamentous growth pathway, *Kss1*, by the MAP kinase kinase *Ste7* is required for pathway activation (Courchesne *et al.* 1989; Ma *et al.* 1995; Cook *et al.* 1997; Madhani *et al.* 1997) and provides a readout of pathway activity. The *opy2* mutant had reduced levels of phosphorylated *Kss1* (Figure 1D). Activated *Kss1* induces transcriptional activators and inactivates transcriptional repressors (Liu *et al.* 1993; Roberts and Fink 1994; Cook *et al.* 1997; Bardwell *et al.* 1998), which results in the expression of genes that regulate filamentous growth. Transcriptional reporters of the filamentous growth pathway, *FRE-LacZ* and *YLR042c-LacZ* (Roberts *et al.* 2000), were dependent on Opy2 for expression (Figure 1E). Like other pathway regulators, Opy2 was required for mat formation on low-agar (Figure 1C, YEPD 0.3% agar) and high-agar media (Figure 1C, YEPD 4% agar; Supporting Information, Figure S1). Opy2 was required for cell differentiation into pseudohyphae at mat perimeters, which contain densely aggregated pseudohyphal cells (Figure 1C, YEP 0.3% agar) (Karunanithi *et al.* 2012). Flo11 is the major adhesion molecule that regulates biofilm/mat formation (Reynolds and Fink 2001; Karunanithi *et al.* 2010) and filamentous/invasive growth (Lambrechts *et al.* 1996; Lo and Dranginis 1998; Guo *et al.* 2000). *FLO11* expression is regulated by the filamentous growth pathway (Lo and Dranginis 1998) and other pathways (Rupp *et al.* 1999; Barrales *et al.* 2008; Vinod *et al.* 2008) and provides another measure of pathway activity. The *opy2* mutant, like other pathway mutants (e.g., *ste12* Δ), showed reduced Flo11 levels (Figure 1F, "P"). Shedding of Flo11 from cells, which regulates mat expansion (Karunanithi *et al.* 2010), was reduced in the *opy2* Δ mutant (Figure 1F, "S"). Together, these results demonstrate that Opy2 regulates the filamentous growth pathway.

Opy2 is a single-pass transmembrane protein with an N-terminal extracellular domain and a cytosolic C-terminal domain. Opy2-GFP localized to the cell periphery and vacuoles (Wu *et al.* 2006; data not shown). Colocalization of Opy2-mCherry with Msb2-GFP and Sho1-GFP, two plasma membrane regulators of the filamentous growth pathway (O'Rourke and Herskowitz 1998; Cullen *et al.* 2000, 2004), showed that these proteins all localized to the plasma membrane (data not shown). To determine how Opy2 regulates the filamentous growth pathway in the context of these plasma-membrane regulators, genetic suppression analysis was performed. Hyperactive versions of Msb2*, which lacks the inhibitory N-terminal mucin-homology domain (Cullen *et al.* 2004), and Sho1^{P120L} (Tatebayashi *et al.* 2007; Vadaie *et al.* 2008), did not bypass the signaling defect of the *opy2* Δ mutant (Figure 2A). Thus, Msb2 and Sho1 may function through Opy2 in the pathway. Msb2 and Sho1 connect to the polarity regulatory GTPase

Figure 2 Opy2 is required for plasma-membrane recruitment of Ste50. (A) Genetic suppression analysis showing phosphorylated Kss1 in an *opy2Δ* mutant with a control plasmid (CTL) or plasmids containing hyperactive alleles of *MSB2*, *SHO1*, and *STE11*. For *rga1Δ*, an *rga1Δ opy2Δ* strain was examined. Blots were performed as in Figure 1D. (B) Levels of phosphorylated Kss1 in wild-type and *opy2Δ* mutant strains that expressed Myr-Ste50 under the control of its own promoter or the *GAL1* promoter (*P_{GAL}*-Myr-Ste50). (C) Strains in B were spotted onto YEPGAL media for 48 hr and examined by the plate-washing assay to measure agar invasion. (D) Phosphorylation of Kss1 in cells overexpressing a wild-type (*P_{GAL}*-*OPY2*) or membrane-anchored version of the cytosolic domain of Opy2 (*P_{GAL}*-Myr-*Opy2^{CT}*). (E) Phosphorylation of Kss1 in an *opy2Δ* mutant containing a control plasmid (Ctl) and wild-type (WT) Opy2, Opy2^{C30Y}, or Opy2^{G207D}. (F) Two-hybrid analysis of the interaction between pGADC1-*STE50* (Ste50), pGBDUC1-*OPY2* (Opy2), or pGBDUC1-*OPY2^{G207D}* (G207D). Interactions are represented by growth on synthetic medium lacking adenine (SD-Ura-Leu-Ade, bottom panel). Growth of strains on control media is also shown (SD-Ura-Leu, top panel).

Cdc42 (Peter *et al.* 1996; Leberer *et al.* 1997; Park and Bi 2007). Deletion of *RG1*, which encodes the GTPase-activating protein for *Cdc42* in the filamentous growth pathway (Stevenson *et al.* 1995; Smith *et al.* 2002), failed to restore signaling to the *opy2Δ* mutant (Figure 2A, *rga1Δ*), which indicates that Opy2 functions below *Cdc42* in the pathway. A hyperactive allele of the gene encoding the MAP kinase kinase kinase Ste11, *STE11-4* (Stevenson *et al.* 1992), restored signaling in the *opy2Δ* mutant (Figure 2A, *STE11-4*), indicating that Opy2 functions above Ste11 in the pathway.

Several proteins function between *Cdc42* and Ste11 in the filamentous growth pathway (including Ste20 and Ste50; Figure 1A). In the HOG pathway, Opy2 functions to recruit Ste50 to the plasma membrane (Wu *et al.* 2006; Yamamoto *et al.* 2010). Ste50 is a general adaptor that associates with Ste11 (Posas *et al.* 1998; Ramezani Rad *et al.* 1998). Like Opy2, Ste50 functions in the filamentous growth pathway (Figure 1, B and D, and Figure S1) (Ramezani Rad *et al.* 1998; Ramezani-Rad 2003; Truckses *et al.* 2006). To determine whether Opy2 regulates the filamentous growth pathway by plasma-membrane recruitment of Ste50,

the Ste50 protein was tethered to the plasma membrane by an N-terminal myristoylation signal (Myr-Ste50) (Gillen *et al.* 1998; Jansen *et al.* 2005). Myr-Ste50 partially restored MAPK signaling to an *opy2Δ* mutant (Figure 2B, Myr-Ste50 *opy2Δ*). Overproduction of Myr-Ste50 caused a more effective bypass than Myr-Ste50 expressed under its own promoter (Figure 2B, *P_{GAL}*-Myr-Ste50 *opy2Δ*). Myr-Ste50 and *P_{GAL}*-Myr-Ste50 also bypassed the invasive growth defect of the *opy2Δ* mutant (Figure 2C and Figure S2, A and B).

Overexpression of a membrane-anchored version of the cytosolic domain of Opy2 was sufficient to activate the filamentous growth pathway (Figure 2D, *P_{GAL}*-Myr-*Opy2^{CT}*), which demonstrates that the cytosolic domain of Opy2 plays the major role in pathway regulation. Chemical mutagenesis was used to identify loss-of-function mutations that reduce Opy2 function in the filamentous growth pathway. Two alleles were identified: *OPY2^{G207D}* was partially defective for pathway activity (Figure 2E, P~Kss1). *OPY2^{G207D}* contains a mutation in a Ste50-binding site (Figure 3A) (Ekiel *et al.* 2009; Yamamoto *et al.* 2010) and was partially

Figure 3 *Opy2* interacts with the transcriptional repressor Mig1. (A) The *Opy2* ORF and deletions used in the study. Yellow vertical lines, conserved cysteines; red bars, Ste50-binding sites; green bars, Mig1-binding sites. (B) *Opy2* associates with Ste50, Mtc1, Mig1, and Mig2 by two-hybrid analysis. (Left) Growth of strains on control media (SD-URA-LEU). (Right) Growth of strains represents protein interactions (SD-URA-LEU-HIS+3-ATA). (C) Two-hybrid deletion mapping. Two-hybrid analysis of versions of *Opy2* lacking specified deletions in its cytoplasmic tail with Mig1. (D) Pull-down assay. GST (~26 kDa) or GST-Mig1 (~81.5 kDa) were purified (Input, anti-GST) and bound to glutathione-sepharose beads (Pull Down, anti-GST). Beads were incubated with purified MBP-*Opy2^{CT}* (Input, anti-MBP, ~69.4 kDa). Coprecipitated MBP-*Opy2^{CT}* is shown (Pull Down, anti-MBP).

defective for binding to Ste50 by two-hybrid analysis (Figure 2F).

A second allele, *OPY2^{C30Y}*, also showed reduced filamentous growth pathway activity (Figure 2E), which contained a substitution of a conserved cysteine residue in the extracellular domain (Figure 3A). *Opy2^{G207D}*-GFP and *Opy2^{C30Y}*-GFP were expressed at wild-type levels (Figure 2E) and were localized in a similar manner as wild-type *Opy2*-GFP (data not shown). A lower band was also seen in *Opy2^{C30Y}*-GFP (Figure 2E, asterisk). Thus, the extracellular domain of *Opy2* may participate in regulating the filamentous growth pathway. The *Opy2^{G207D}* and *Opy2^{C30Y}* proteins were also defective for HOG pathway signaling, based on the salt-dependent phosphorylation of the MAPK that functions in the HOG pathway, *Hog1* (Figure S2C). Together, our results support and extend the conclusion put forward in Yang *et al.* (2009) that *Opy2* is a general adaptor for the HOG and filamentous growth pathways that functions by plasma-membrane recruitment of Ste50.

Mig1 and Mig2 proteins interact with *Opy2*

Three observations suggest that *Opy2* has a function in regulating the filamentous growth pathway separate from Ste50:

(1) the *opy2Δ* mutant had a more severe invasive growth defect than the *ste50Δ* mutant (Figure 1B); (2) Myr-Ste50, even when overexpressed, did not fully bypass the signaling defect of the *opy2Δ* mutant (Figure 2B); and (3) the *opy2Δ ste50Δ* double mutant had a more severe invasive growth defect than the *ste50Δ* single mutant (Figure S2B). An additional role for *Opy2* in regulating the filamentous growth pathway might result from *Opy2* regulating a component of the pathway in addition to Ste50. The cytosolic domain of *Opy2* (*Opy2^{CT}*) was tested for interactions with pathway regulators. *Opy2^{CT}* did not associate with the cytosolic domain of *Msb2* (*Msb2^{CT}*), *Cdc24*, *Cdc42*, *Ste11*, *Ste7*, or *Kss1* by two-hybrid analysis (data not shown). *Cdc24*, *Cdc42*, and *Ste11* (A. Pitoniak, C. Chavel, N. Vadaie, J. Smith, S. Karunanithi, D. Camara, and P. J. Cullen, unpublished results) and *Msb2^{CT}*, *Ste7*, and *Kss1* (see below) mediated two-hybrid interactions with control proteins. *Opy2* did not interact with *Ras2*, which also regulates the filamentous growth pathway (Mosch *et al.* 1999; Chavel *et al.* 2010) or with *Bem4*, a newly identified regulator of the filamentous growth pathway (A. Pitoniak, C. Chavel, N. Vadaie, J. Smith, S. Karunanithi, D. Camara, and P. J. Cullen, unpublished results).

Opy2 might regulate the pathway by associating with a pathway regulator that has yet to be identified. A two-hybrid screen with the cytosolic domain of *Opy2* using a pooled ORF library (Uetz *et al.* 2000) identified *Ste50*, which was expected, *Mig1*, and *Mtc1* (Figure 3B). Specifically, five isolates of *Ste50*, five isolates of *Mig1*, and one isolate of *Mtc1* were identified. *Mig2* is a homolog of *Mig1* (Lutfiyya and Johnston 1996; Lutfiyya *et al.* 1998; Westholm *et al.* 2008). *Mig2* was not identified in the screen but associated with the cytosolic domain of *Opy2* by two-hybrid analysis (Figure 3B). These interactions were not identified in comprehensive screens (Uetz *et al.* 2000; Ho *et al.* 2002), possibly because full-length versions of *Opy2* were used, whereas we used only the cytosolic domain of *Opy2*. Deletion of *MTC1*, which encodes a protein of unknown function (Fleischer *et al.* 2006), did not affect the filamentous growth pathway and was not explored further.

Mig1 is a transcriptional repressor of genes required for growth on nonfermentable carbon sources (Nehlin and Ronne 1990; Carlson 1999). Two-hybrid deletion mapping showed that *Mig1* associated weakly with the cytosolic domain of *Opy2* that lacked amino acids 116–245 (*Opy2*^{CTA116–245}), 269–360 (*Opy2*^{CTA1–115,269–360}), and 324–360 (*Opy2*^{CTA1–115,324–360}) (Figure 3C). The sites that showed interaction with *Mig1* also contained the *Ste50*-binding sites (Figure 3A) (Yamamoto *et al.* 2010). *Opy2*^{G207D}, which was partially defective for binding to *Ste50* (Figure 2F), was also partially defective for binding to *Mig1* (Figure 3C; see Figure 3A for deletions). To verify the interaction between *Mig1* and *Opy2*, epitope fusions of *Mig1* (GST-*Mig1*) and the cytosolic domain of *Opy2* (MBP-*Opy2*^{CT}) were overexpressed in *E. coli* and purified by affinity chromatography. Beads coated with GST-*Mig1* pulled down MBP-*Opy2*^{CT} *in vitro* (Figure 3D). Thus, *Mig1* interacts directly with the cytosolic domain of *Opy2* *in vivo* and *in vitro*.

***Mig1* and *Mig2* regulate the filamentous growth pathway in response to glucose depletion**

The identification of *Mig1* and *Mig2* as interacting partners with *Opy2* suggested a connection between the AMPK pathway and the filamentous growth pathway. To test whether *Mig1* and/or *Mig2* regulate the filamentous growth pathway, the *MIG1* and *MIG2* genes were disrupted in strains of the filamentous background. The *mig1Δ* and *mig2Δ* single mutants were not defective for pathway activity, but the *mig1Δ mig2Δ* double mutant was defective (Figure 4A). Specifically, the *mig1Δ mig2Δ* double mutant failed to show induction of the pathway in response to glucose limitation (Figure 4A, P~*Kss1*; compare GLU to GAL). The *mig1Δ mig2Δ* double mutant was also defective for invasive growth (Figure 4B). *Mig3* is a poorly defined transcriptional repressor that is thought to function separately from *Mig1* and *Mig2* (Lutfiyya *et al.* 1998). Disruption of the *MIG3* gene in wild-type cells or cells lacking *Mig1* and/or *Mig2* did not cause a further reduction in pathway activity (Figure

S5), which indicates that *Mig1* and *Mig2* primarily coregulate the filamentous growth pathway.

To define how *Mig1* and *Mig2* regulate the filamentous growth pathway, genetic suppression analysis was performed. Unlike *opy2Δ* (Figure 2A), the signaling defect of the *mig1Δ mig2Δ* double mutant was suppressed by an activated version of *Sho1* (data not shown), which indicates that *Mig1* and *Mig2* regulate the filamentous growth pathway at/above the level of *Sho1* in the pathway (Figure 1A). To determine how *Mig1* and *Mig2* regulate the pathway at this level, two-hybrid analysis was performed against known regulators of the pathway. In addition to the cytosolic domain of *Opy2*, *Mig1* and *Mig2* also associated with the cytosolic domain of *Msb2* (Figure 4C). The cytosolic domain of *Msb2* has been shown to associate with only a single other protein, *Cdc42*, by two-hybrid analysis (Cullen *et al.* 2004), which suggests that the interaction with *Mig1* and *Mig2* is highly specific. The fact that *Mig1* and *Mig2* associate with the cytosolic domain of *Msb2* might explain how it regulates the pathway at/above the level of *Sho1* in the pathway. The *Mig* proteins may also modulate the *Opy2/Ste50* interaction, given that *Opy2* associates with *Mig1* and *Ste50*.

Mig1 shuttles between the cytoplasm and the nucleus in a glucose-dependent manner (De Vit *et al.* 1997; DeVit and Johnston 1999). In response to glucose limitation, *Mig1* is exported from the nucleus, which attenuates its transcriptional repression function (De Vit *et al.* 1997). The regulation of *Mig2* function is less clear (Lutfiyya and Johnston 1996). *Mig1* may regulate the filamentous growth pathway in the cytosol, under conditions in which it is exported from the nucleus. In cells undergoing filamentous growth, a functional N-terminally tagged GFP-*Mig1* protein was predominately in the cytosol (data not shown), which supports the possibility that *Mig1* associates with and may regulate *Msb2* and *Opy2* at the plasma membrane.

***Snf1* regulates starvation-dependent induction of the filamentous growth pathway**

Nucleo-cytoplasmic shuttling of *Mig1* is regulated by *Snf1* (De Vit *et al.* 1997; DeVit and Johnston 1999), the yeast AMPK (Mantovani and Roy 2011). *Snf1* is required for filamentous growth (Cullen and Sprague 2000) by regulating the activity of another set of transcriptional repressors, *Nrg1* and *Nrg2* (Kuchin *et al.* 2002). Whether *Snf1* also regulates the filamentous growth pathway is not clear. Like the *mig1Δ mig2Δ* double mutant, the *snf1Δ* mutant was defective for induction of the filamentous growth pathway in response to glucose depletion (Figure 5A). One possibility is that, in the *snf1* mutant, *Mig1* cannot be phosphorylated and exit the nucleus to regulate *Msb2* and *Opy2* at the cytosol-plasma membrane interface.

In response to glucose, *Mig1* translocates into the nucleus, where it represses the expression of genes involved in gluconeogenesis and poor carbon source utilization (De Vit *et al.* 1997; data not shown). If *Mig1* regulates the filamentous growth pathway in the cytosol, then its nuclear

Figure 4 The Mig1 and Mig2 proteins regulate starvation-dependent induction of the filamentous growth pathway. (A) Phospho-Kss1 blots of wild type, *opy2Δ*, *mig1Δ*, *mig2Δ*, and *mig1Δ mig2Δ* strains grown to mid-log phase in YEPD or YEPGAL media. (B) Strains in A were grown on YEPD media for 48 hr and assessed by the plate-washing assay. (C) Two-hybrid analysis of Mig1 and Mig2 with Msb2^{CT}.

entry might result in attenuation of the filamentous growth pathway. The addition of glucose to filamentous cells causes a rapid switch in budding pattern (to the vegetative or axial pattern) and a round-cell morphology within one cell division cycle (Cullen and Sprague 2000). The effect of glucose addition on the activity of the MAPK pathway has not been tested. Within 15 min of glucose addition, the activity of the filamentous growth pathway dropped to basal levels (Figure 5B). The attenuation of the filamentous growth pathway in response to glucose was slower than the kinetics of the nuclear entry of Mig1, which occurs in 1–2 min (De Vit *et al.* 1997; DeVit and Johnston 1999). This difference might be explained by the fact that Mig2 is regulated differently from Mig1 in terms of its localization and turnover in the cytosol (Lutfiyya *et al.* 1998; Fernandez-Cid *et al.* 2012). We also cannot exclude the possibility that other proteins might also regulate the pathway under this condition.

The above results support the idea that Mig1 functions in the cytosol to regulate the filamentous growth pathway, acting as a cytosolic indicator of low glucose levels. In support of this possibility, overexpression of MIG1 (*P_{GAL}-MIG1*) under conditions that favor its cytosolic localization (2% galactose) caused hyper-invasive growth (Figure S6A), in line with a previous report (Jin *et al.* 2008). Co-overexpression of MIG1 and MIG2 caused an additive hyper-invasive growth phenotype (Figure S6A). Although the cytosolic domains of Msb2 and/or Opy2 might enter the nucleus to modulate the

transcriptional repression function of Mig1 and Mig2, we failed to detect the cytosolic domains of these proteins in the nucleus under any condition tested (data not shown).

The AMPK pathway also regulates the response to environmental stresses such as salt, elevated pH, and hydrogen peroxide (Hong and Carlson 2007). Snf1 is phosphorylated in response to these stresses and is required for growth under these conditions (Hong and Carlson 2007). The *mig1Δ mig2Δ* double mutant shows elevated expression of some HOG pathway targets, including the gene encoding the Na⁺ transporter *ENA1* (Proft and Serrano 1999). Thus, we considered whether the HOG pathway might be hyperactive in the *mig1Δ mig2Δ* double mutant. Given that the HOG pathway can inhibit the filamentous growth pathway (Saito 2010), a hyperactive HOG pathway might attenuate the filamentous growth pathway. To test this possibility, the activity of the HOG pathway was examined in wild-type cells and in the *mig1Δ mig2Δ* double mutant. Under normal growth conditions (no salt), neither wild-type cells nor the *mig1Δ mig2Δ* mutant showed HOG pathway activity, based on the levels of phosphorylated Hog1 (Figure 5C, left panels). In response to salt (0.5 M KCl), wild-type cells and the *mig1Δ mig2Δ* mutant showed similar HOG pathway activity (Figure 5C, right panels). In the *pbs2Δ* mutant, which lacks the MAP kinase kinase for the HOG pathway, no phosphorylated Hog1 was detected (Figure 5C). Therefore, the HOG pathway is not hyperactive in the *mig1Δ mig2Δ* mutant.

Figure 5 Snf1 regulates the filamentous growth pathway independently of the HOG pathway. (A) Phospho-Kss1 levels for wild type, *opy2Δ*, and *snf1Δ* strains grown in YEPD (GLU) and YEPGAL (GAL) media. (B) Wild-type cells were grown in YEPD (GLU) or YEPGAL (GAL) for 6 hr. After 6 hr in galactose, cells harvested by centrifugation and transferred to YEPD media (GLU). Equal numbers of cells were withdrawn at 5, 15, and 30 min, and cell extracts were prepared and probed by immunoblot analysis. (C) Hog1 phosphorylation analysis. Phospho-blot of the MAP kinase Hog1 in response to 0.5 M KCl for 5 min was performed in the indicated strains in YPD media.

Mig1 has been implicated in regulating cell-wall integrity (Krause *et al.* 2008), which is controlled by the protein kinase C (PKC) pathway (Levin 2005). In some settings, the PKC pathway can stimulate the filamentous growth pathway (Yashar *et al.* 1995; Birkaya *et al.* 2009). The activity of the PKC pathway, as measured by phosphorylation of the MAPK *Slt2*, *Slt2*~P, did not correlate with filamentous growth pathway activity in the *snf1Δ* or *mig1Δ mig2Δ* double mutants (data not shown). Thus, *Mig1* and *Mig2* do not regulate the filamentous growth pathway through the PKC pathway. We also tested whether *Mig1* and *Mig2* were required to stabilize cell-surface components of the filamentous growth pathway. Loss of *Mig1* and *Mig2* did not affect the levels of *Msb2*, *Sho1*, or *Opy2* in the cell (Figure S3). Deletion or overexpression of *MIG* genes might alter cellular growth rate, which might lead to altered MAPK activity. No differences were observed in growth of the *mig1Δ mig2Δ* mutant or the *P_{GAL}-MIG1* strain compared to wild type

(Figure S6B). To summarize, *Snf1* and the *Mig1* and *Mig2* proteins are required for full activation of the filamentous growth pathway, where they sensitize the pathway to cellular nutrient levels.

Mig1 and *Opy2* contribute to a pathway-specific response

The filamentous growth pathway shares components with the pheromone response (Chen and Thorner 2007) and HOG (Saito 2010) pathways. *Mig1* and *Mig2* might also regulate these pathways. The *mig1Δ mig2Δ* double mutant responded to pheromone (Figure S4A). The *mig1Δ mig2Δ* *ssk1Δ* triple mutant, which lacks the *Sln1* branch of the HOG pathway (Ota and Varshavsky 1993; Chang and Meyerowitz 1994), was not sensitive to osmotic stress (Figure S4B). Thus, *Mig1* and *Mig2* specifically regulate the filamentous growth pathway and are not required to activate the pheromone response or HOG pathways.

Opy2 (overproduction induced pheromone-resistant yeast, or Opy) was previously identified in a screen for genes that, when overexpressed, overcome pheromone-induced growth arrest (Edwards *et al.* 1997). We also saw that overexpression of *OPY2* reduced sensitivity to mating pheromone (Figure 6A). Likewise, overexpression of *OPY2* dampened the activity of a mating pathway growth reporter (Figure 6B, *FUS1-HIS3*) while causing hyper-invasive growth (Figure 6C). Overexpression of *MIG1* similarly dampened the sensitivity to mating pheromone (Figure 6A) and caused a reduction in *FUS1-HIS3* reporter expression (Figure 6B), while stimulating filamentous growth (Figure 6C). The hyper-invasive growth induced by overexpression of *MIG1* was partially independent of the MAPK pathway (data not shown), suggesting that *Mig1* may have additional roles in regulating filamentous growth in addition to regulating the MAPK pathway. Inhibition of the mating pathway by overexpression of *OPY2* and *MIG1* was not seen in cells overexpressing other pathway regulators, including *Msb2* and *Sho1* (Cullen *et al.* 2004). Thus, *Opy2* and *Mig1* have a specific function in regulating signal discrimination between the mating and filamentous growth pathways.

Different MAP kinases regulate the filamentous growth (*Kss1*) and mating (*Fus3*) pathways. We found that *Mig1* and *Mig2* associated with the MAP kinase that regulates the filamentous growth pathway (Figure 6D, *Kss1*) but not with the MAP kinase that regulates the mating pathway (Figure 6D, *Fus3*). *Mig1* and *Mig2* also associated with the filamentation/mating MAP kinase kinase, *Ste7*, but not with *Cdc24*, *Cdc42*, or *Ste11* (Figure 6D, shown for *Ste7*). Thus, *Mig1* and *Mig2* may specifically regulate the filamentation MAPK. The inhibitory effect of *Opy2/Mig1* might occur through *Ste50*, which is a target of *Kss1* (Jansen *et al.* 2001; Ramezani-Rad 2003; Ekiel *et al.* 2009; Yamamoto *et al.* 2010). Indeed, overexpression of *Ste50* also dampened *FUS1-HIS3* expression (Figure 6B). When anchored to the plasma membrane (*P_{GAL}-Myr-Ste50*), reporter expression was restored to wild-type levels (Figure 6B) in an *Opy2*-independent manner

(Figure 6B). Thus, *Opy2* and *Mig1* may differentially activate the filamentous growth pathway at the levels of *Ste50* and/or *Kss1*.

MAPK pathway does not regulate the transcriptional repression function of *Mig1* and *Mig2*

The filamentous growth pathway might regulate the glucose repression function of *Mig1* and *Mig2*. In medium containing glucose and galactose, wild-type cells do not express genes under the control of the *GAL* promoter (Figure 7, wild-type *GAL* + *GLU*) (Nehlin *et al.* 1991; Lutfiyya *et al.* 1998). In cells lacking *Mig1* and *Mig2*, *GAL*-driven genes are expressed (Figure 7, *GAL* + *GLU*, *mig1* Δ *mig2* Δ). Deletion of pathway regulators *Opy2* or *Ste12* did not alter gene expression under this condition (Figure 7, *GAL* + *GLU*, *opy2* Δ and *ste12* Δ). Thus, the filamentous growth pathway does not appear to regulate glucose repression mediated by *Mig1* and *Mig2*. In support of this possibility, whole-genome DNA microarray analysis of genes induced by *MSB2* overexpression or hyperactivation did not show induction or repression of *Mig* targets (Chavel *et al.* 2010), and the expression of *GAL*-dependent genes was not affected by the MAPK pathway under inducing conditions (Figure 7, *GAL*). Furthermore, cells lacking or expressing hyperactive MAPK regulators did not influence the ability of cells to grow on medium in which galactose was the preferred carbon source.

Discussion

Opy2 regulates the filamentous growth pathway

The identification of proteins that regulate signal transduction pathways is an important step in understanding what pathways sense and how they function. *Opy2* had previously been implicated in regulating the filamentous growth and HOG pathways (Yang *et al.* 2009; Yamamoto *et al.* 2010). Here, we confirm and extend these findings by showing that *Opy2* is a major regulator of the filamentous growth pathway. *Opy2* is required for invasive growth, filament formation, and biofilm/mat expansion. Previously, our lab and other labs have shown that the mucin-like glycoprotein *Msb2*

Figure 6 Different roles for *Opy2* and *Mig1/Mig2* in regulating the mating and filamentous growth pathways. (A) Halo assay. Wild type, $P_{GAL-OPY2}$, and $P_{GAL-MIG1}$ were spread on YEPGAL media, onto which was spotted 2, 10, and 20 μ l of 1 mg/ml α -factor. Plates were incubated for 48 hr and photographed. Bar, 1 cm. Inset portraying the loss in pheromone sensitivity is also shown. (B) Overexpression of *OPY2*, *MIG1*, and *STE50* suppresses expression of the *FUS1-HIS3* reporter. Strains were spotted onto synthetic medium with galactose (SC-GAL) or SC-GAL lacking histidine (SC-GAL-HIS), grown for 24 hr, and photographed. In the bottom panels, Myr-Ste50 was expressed in wild-type cells or in an *opy2* Δ mutant, as indicated. (C) Overexpression of *OPY2* and *MIG1* induces hyper-filamentous growth. Cells were grown in YEPGAL and examined by DIC microscopy at $\times 100$; representative images are shown. Bar, 5 μ m. (D) *Mig1* and *Mig2* associate with *Ste7* and *Kss1* but not with *Fus3* by two-hybrid analysis.

Figure 7 The filamentous growth pathway does not regulate the glucose repression function of the Mig proteins. Immunoblots with antibodies to HA-tagged Act1, expressed from a GAL promoter (P_{GAL}), $P_{GAL}\text{-ACT1}$ was obtained from Gelperin *et al.* (2005). Wild-type, *mig1Δ mig2Δ*, *opy2Δ*, and *ste12Δ* strains were grown in YEP media supplemented with glucose and galactose (GLU + GAL) or galactose (GAL). Pgk1, loading control.

(Cullen *et al.* 2004) and tetraspan protein *Sho1* (O'Rourke and Herskowitz 1998; Cullen *et al.* 2000, 2004) regulate the filamentous growth pathway. Thus, three transmembrane proteins regulate the filamentous growth pathway. Our findings show that *Msb2* and *Sho1* function through *Opy2*. Our data also corroborate the idea that *Opy2* is a cortical tether for the adaptor protein *Ste50* (Wu *et al.* 2006; Yamamoto *et al.* 2010). In principle, cortical recruitment of *Ste50* might promote pathway activation through the interacting partners *Cdc42* (Truckses *et al.* 2006) or *Ste11* (Wu *et al.* 2006). Genetic suppression analysis indicates that *Opy2* regulates the filamentous growth pathway at the level of *Ste11*. Although the cytosolic domain of *Opy2* plays the major role in *Ste50* recruitment and pathway activation, the extracellular domain of *Opy2*, which contains eight cysteine residues that may form a cysteine knot (Craik *et al.* 2001; Cemazar *et al.* 2008), may also play a minor role in filamentous growth pathway regulation.

***Mig1* and *Mig2* proteins connect glucose signaling to MAPK regulation**

We also show that the transcriptional repressors *Mig1* and *Mig2* regulate the filamentous growth pathway. Hints that *Mig1* and *Mig2* regulate the pathway have come from genome-wide screens conducted in our lab and other labs. The *mig1* mutant was identified among an ordered collection of nonessential deletion mutants (Winzeler *et al.* 1999) as defective for *Msb2*-HA secretion (Chavel *et al.* 2010), an indicator of MAPK activity (Vadaie *et al.* 2008). *MIG1* and *MIG2* were identified in a whole-genome screen for genes that, when overexpressed, induce *Msb2*-HA secretion (Chavel *et al.* 2010). Overexpression of *MIG1* (and *OPY2*) came out of a genomic screen for genes that, when overexpressed, dampen the activity of the mating pathway *FUS1-HIS3* reporter (H. S. Dionne and P. J. Cullen, unpublished data). *MIG1* was also identified in a large-scale survey for genes that, when overexpressed, induce filamentous growth (Jin *et al.* 2008).

The *Mig1* and *Mig2* proteins function in the nucleus as transcriptional repressors that bind to specific DNA elements (Nehlin and Ronne 1990; Lutfiyya and Johnston 1996; Ozcan and Johnston 1996). Our findings support the idea that *Mig1* and *Mig2* regulate the filamentous growth path-

way in the cytosol. This possibility is supported by the following data: (1) *Mig1* is found mainly in the cytosol under conditions when it regulates the filamentous growth pathway; (2) *Mig1* and *Mig2* associate with the cytosolic domains of transmembrane proteins *Msb2* and *Opy2*, which are not found in the nucleus; and (3) *Mig1* and *Mig2* associate with other regulators of the filamentation pathway such as *Ste7* and *Kss1*, which are primarily cytosolic. Given that *Mig1* and *Mig2* associate with the cytosolic domains of both *Msb2* and *Opy2*, they may play a role in clustering the cell-surface proteins into a complex at the plasma membrane. *Mig1* may also modulate the *Opy2*-*Ste50* interaction because *Mig1* and *Ste50* bind to overlapping sites in the cytosolic domain of *Opy2*. Finally, *Mig1* and *Mig2* associate with the MAP kinase that regulates the filamentous growth pathway, *Kss1*, but not with the mating pathway MAP kinase *Fus3*. Thus, *Opy2* and *Mig1* may promote pathway discrimination at the level of *Kss1*.

A growing list of transcription factors moonlight in other cellular compartments to regulate cellular functions in diverse ways. The major tumor suppressor and transcription factor p53 regulates apoptosis at the mitochondria (Murray-Zmijewski *et al.* 2008). β -Catenin is both a transcription factor and a structural component of adherens junctions (Logan and Nusse 2004). Transcription factors TFII-I (Park and Dolmetsch 2006) and the tumor suppressor PML (Giorgi *et al.* 2010) regulate calcium signaling outside the nucleus. Indeed, *Mig2* may regulate cellular respiration in the mitochondria under glucose-limiting conditions (Fernandez-Cid *et al.* 2012). Likewise, noncanonical transcription factors can function as coincidence detectors in the nucleus: the glycolytic enzyme hexokinase 2 (*Hxk2*) coregulates *Mig1*-dependent gene repression in the nucleus (Moreno *et al.* 2005; Pelaez *et al.* 2012). Together, these findings reveal a high diversity in the molecular connections that integrate nutrition, gene expression, and cellular signaling.

Mig1 and *Mig2* may alternatively regulate the filamentous growth pathway through transcriptional regulation. One possibility is that the proteins function as activators of filamentation-specific genes. *Mig1* can activate gene expression in some contexts, particularly in cells lacking the chromatin-remodeling proteins *Tup1/Ssn6* (Treitel and Carlson 1995). Recently, *Tup1* has been shown to exit the nucleus

and associate with internal membranes, resulting in modification of its gene expression function (Han and Emr 2011). It is possible that *Mig1* and *Mig2* are modified in some way by associating with *Msb2*, *Opy2*, *Ste7*, or *Kss1*, which results in alteration of their normal function. *Mig1*-binding sites (AATGCGGGGK or SYGGGG) are not found upstream of the filamentation target genes *MSB2*, *KSS1*, *PGU1*, *SVS1*, *YLRO42C*, *STE12*, and *TEC1*, which might suggest that simply switching these proteins from repressors to activators cannot explain the change in the function of these proteins. DNA microarray data on genes upregulated by *Mig1* and *Mig2* does not include filamentous growth pathway regulators (Westholm *et al.* 2008). Nevertheless, *Mig1* and *Mig2* may modulate nutritional scavenging by repression of *GAL/SUC* and other genes in the nucleus. *Mig1* and *Mig2* may also repress a subset of filamentation genes in high glucose, which is relieved under glucose-limiting conditions. At least one gene, *YLRO42c* (Roberts *et al.* 2000), falls into this category (Lutfiyya *et al.* 1998).

Cellular glucose control involves the coordination of multiple proteins and pathways. A role for *Mig1* and *Mig2* in regulating the filamentous growth pathway provides the first regulatory connection between the AMPK pathway and a MAPK pathway in yeast. Previously, the AMPK pathway and MAPK pathway have been shown to coordinate filamentation responses by converging at the *Flo11* promoter, at the level of the *Nrg1* and *Nrg2* (Kuchin *et al.* 2002, 2003). The regulatory connection that we describe brings to light an additional layer of signal integration, which may benefit the overall precision and timing of the differentiation response. The critical hole that this discovery fills is to help explain how the filamentous growth pathway becomes sensitized to nutrient levels. We show that the activity of the MAPK pathway is induced by glucose starvation and reduced by glucose addition through the action of *Mig1*, *Mig2*, and *Snf1*. These proteins might also sensitize MAPK activity to other nutrient levels. In particular, filamentous growth can be regulated by limiting nitrogen (Gimeno *et al.* 1992), which is sensed by the *Snf1*-activating kinase *Sak1* (Orlova *et al.* 2010). A link between the AMPK and MAPK pathways has been actively sought in higher eukaryotes, and several connections have been reported. In skeletal muscle, contraction-induced AMPK activation leads to MAPK-dependent transcriptional induction of glucose transporters (Tremblay *et al.* 2003). AMPK can also induce MAPK-dependent induction of diverse targets, including MUC5A (Bae *et al.* 2011).

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Supporting Information

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The Filamentous Growth MAPK Pathway Responds to Glucose Starvation Through the Mig1/2 Transcriptional Repressors in *Saccharomyces cerevisiae*

Sheelarani Karunanithi and Paul J. Cullen

Figure S1 Biological assays of mutants in filamentous growth pathway regulators. **A)** Single cell assay was performed for the indicated strains spread in SC media containing glucose (+GLU) or lacking glucose (-GLU). Indicated strains were grown for 16h and representative images of the microcolonies are shown. Bar, 15 μ m. Axial budding shown by arrows. **B)** Indicated strains were spotted on YEPD media containing 4% agar. Plates were incubated for 14 days and photographed. Bar = 1 cm. **C)** Indicated strains were spotted on YEPD media containing 4% agar atop a nitrocellulose filter. Plates were incubated for 7 days and photographed. Bar = 1 cm.

Figure S2 Analysis of Opy2 function in the filamentous growth and HOG pathways. **A)** Phospho-Kss1 blots were performed on cell extracts prepared from the indicated strains grown in YEPD for 6h. **B)** Strains in panel S2A were spotted on YEPD media and incubated for 48h. Plates were washed under running water and photographs were taken before and after washing. **C)** Phospho-blot of the MAP kinase Hog1 in response to 1M KCl for the above constructs in an *opy2*Δ *ssk1*Δ mutant.

Figure S3 Mig proteins do not affect the stability of cell-surface regulators: **A)** Msb2 levels were compared in the indicated strains by immunoblotting against pHA-Msb2. Cell lysates were prepared from logarithmic phase cultures containing glucose or galactose and antibodies to HA were used to immunoblot for Msb2 levels. Pgk1 levels are shown as loading control. **B)** C-terminal GFP tagged Msb2 (pMsb2-GFP, ~55 kDa) was used to assess if the processing of Msb2 is affected in a *mig1Δ mig2Δ* mutant. Pgk1 levels are as shown for the two blots. **C & D)** Stability and levels of Sho1 (C) and Opy2 (D) were measured in wild type and *mig1Δ mig2Δ* mutant by growing in media containing glucose or galactose for 6h. Antibodies to GFP-tagged plasmid-borne versions of Sho1 (~68.1 kDa) and Opy2 (~65.9 kDa) were used in immunoblots. Pgk1 levels are used as loading control.

Figure S4 Mig proteins are not required to activate the mating and HOG pathways. **A)** Halo assay. Wild type and *mig* mutants were spread uniformly on YEPD plates. 2μl, 10μl and 20μl of 1mg/ml α-factor were spotted on to the plates. Plates were incubated for 48h. **B)** Osmosensitivity of wildtype, *opy2Δ* and *mig* mutants when combined with a *ssk1Δ* mutant, in response to 1M KCl containing media is shown. Plates were incubated for 48h and photographs were taken.

Figure S5 Phospho-Kss1 blots of *mig* combinations. Immunoblots of wild type, *opy2Δ*, *mig1Δ*, *mig2Δ*, *mig3Δ*, *mig1Δ mig2Δ*, *mig1Δ mig3Δ*, *mig2Δ mig3Δ* and *mig1Δ mig2Δ mig3Δ* strains grown to mid-log phase in YEP-GAL media. The levels of phosphorylated Kss1 was determined using anti-p44/42 MAPK antibodies, and protein levels were assessed by probing with Pgk1 antibodies. For the graph, ImageJ analysis was used to quantify band intensity. Images were inverted, and densitometry was assessed after background subtraction. The Y-axis represents the ratio of P~Kss1 levels to Pgk1 levels, which was set to 1 for wild-type and adjusted for the other values accordingly.

A

B

Figure S6 Analysis of Mig1 and Mig2 function in the filamentous growth regulation. **A)** Plate washing assay: Wild type, *P_{GAL}-MIG1* transformed with a plasmid expressing *P_{GAL}-MIG2* were spotted on galactose-containing media and incubated for 2 days. Plates were photographed before and after washing under a stream of water. 20X magnified images of the washed colonies are also shown. **B)** Growth curve analysis of wild-type cells, the *mig1 mig2* double mutant, and the *P_{GAL}-MIG1* expressing strains grown in YEP-GAL media for the indicated time points. Strains were grown in duplicate and the average absorbances are shown for two trials. Less than 10% variation was observed between trials.

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A **B** **C**

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A

B

Figure S6 Analysis of Mig1 and Mig2 function in the filamentous growth regulation. **A)** Plate washing assay: Wild type, $P_{GAL-MIG1}$ transformed with a plasmid expressing $P_{GAL-MIG2}$ were spotted on galactose-containing media and incubated for 2 days. Plates were photographed before and after washing under a stream of water. 20X magnified images of the washed colonies are also shown. **B)** Growth curve analysis of wild-type cells, the *mig1 mig2* double mutant, and the $P_{GAL-MIG1}$ expressing strains grown in YEP-GAL media for the indicated time points. Strains were grown in duplicate and the average absorbances are shown for two trials. Less than 10% variation was observed between trials.