Global Regulation of a Differentiation MAPK Pathway in Yeast

Colin A. Chavel,[1] Lauren M. Caccamise,[1] Boyang Li, and Paul J. Cullen[2]
Department of Biological Sciences, State University of New York, Buffalo, New York 14260-1300
ORCID ID: 0000-0002-6703-1480 (P.J.C.)

ABSTRACT Cell differentiation requires different pathways to act in concert to produce a specialized cell type. The budding yeast *Saccharomyces cerevisiae* undergoes filamentous growth in response to nutrient limitation. Differentiation to the filamentous cell type requires multiple signaling pathways, including a mitogen-activated protein kinase (MAPK) pathway. To identify new regulators of the filamentous growth MAPK pathway, a genetic screen was performed with a collection of 4072 nonessential deletion mutants constructed in the filamentous (Σ1278b) strain background. The screen, in combination with directed gene-deletion analysis, uncovered 97 new regulators of the filamentous growth MAPK pathway comprising 40% of the major regulators of filamentous growth. Functional classification extended known connections to the pathway and identified new connections. One function for the extensive regulatory network was to adjust the activity of the filamentous growth MAPK pathway to the activity of other pathways that regulate the response. In support of this idea, an unregulated filamentous growth MAPK pathway led to an uncoordinated response. Many of the pathways that regulate filamentous growth also regulated each other's targets, which brings to light an integrated signaling network that regulates the differentiation response. The regulatory network characterized here provides a template for understanding MAPK-dependent differentiation that may extend to other systems, including fungal pathogens and metazoans.

Cell differentiation is the process by which cells undergo specialization to produce different cell types with different functions. Cell-type specialization can result from execution of an intrinsic developmental program and also in response to extrinsic cues. The process of cell differentiation is one of exquisite precision: cells undergo complete morphogenetic restructuring in a specific spatiotemporal context (Kholodenko *et al.* 2010). Multiple signaling pathways collaborate to control cell differentiation responses. For example, the activity of the Wnt and Hippo pathways is integrated at multiple levels to coordinate development (McNeill and Woodgett 2010). A critical problem in the field of cell differentiation is to elucidate how signals from different pathways become integrated to produce a cohesive response. This problem is relevant from the standpoint of human health, because misregulation of differentiation pathways is an underlying cause of developmental problems and diseases such as cancer (Wagner and Nebreda 2009).

Depending on ploidy and growth condition, the budding yeast *Saccharomyces cerevisiae* can differentiate into different cell types. Haploid yeast undergoes morphological changes in response to secreted pheromones to mate and form diploids (Bardwell 2005; Dohlman and Slessareva 2006; Merlini *et al.* 2013). Diploid yeast starved for carbon and nitrogen initiate a meiotic program known as sporulation (Neiman 2011). Haploid and diploid yeast starved for only carbon or nitrogen undergoes filamentous (or invasive/pseudohyphal) growth (Gimeno *et al.* 1992; Cullen and Sprague 2000, 2012). During filamentous growth, major changes occur to cell polarity (Gimeno *et al.* 1992; Roberts and Fink 1994; Pruyne and Bretscher 2000; Cullen and Sprague 2002; Bi and Park 2012), cell-cycle progression (Kron *et al.* 1994; Edgington *et al.* 1999), and cell adhesion (Lambrechts *et al.* 1996; Lo and Dranginis 1998; Guo *et al.* 2000), which results in formation of branched chains of interconnected invasive filaments. Filamentous cells form complex communities during biofilm formation (Reynolds and Fink 2001; Verstrepen and Klis 2006; Bojsen *et al.* 2012). Many fungal species undergo filamentous growth. In pathogens, differentiation to filamentous/hyphal cells in biofilms is critical for pathogenicity (Lo

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[1]These authors contributed equally to the work.
[2]Corresponding author: Department of Biological Sciences, 341 Cooke Hall, State University of New York, Buffalo, NY 14260-1300. E-mail: pjcullen@buffalo.edu

et al. 1997; Wendland 2001; Nobile *et al.* 2006; Sohn *et al.* 2006). Budding yeast therefore provides a convenient genetic system to define the pathways that regulate filamentous growth and has provided insights into the genetic basis of fungal pathogenesis and eukaryotic differentiation.

Signal transduction pathways regulate filamentous growth and control the changes that occur in response to nutrient limitation (Zhao *et al.* 2007). Among the pathways that regulate filamentous growth in yeast is a MAPK pathway called the filamentous growth MAPK pathway (Supporting Information, Figure 1A). MAPK pathways are evolutionary conserved signaling modules that regulate diverse responses in eukaryotes (Raman *et al.* 2007). The filamentous growth MAPK pathway is composed of plasma-membrane sensors (Msb2p, Sho1p, and Opy2p) (O'Rourke and Herskowitz 1998; Cullen *et al.* 2004; Wu *et al.* 2006; Yamamoto *et al*. 2010; Karunanithi and Cullen 2012) that connect to a Rho-type GTPase (Cdc42p; Bi and Park 2012) and a kinase cascade consisting of a p21-activated kinase (Ste20p; Peter *et al.* 1996; Leberer *et al.* 1997) and MAPK module (including the MAPKKK Ste11p, MAPKK Ste7p, and MAPK Kss1p; Roberts and Fink 1994). The MAP kinase Kss1p regulates the activity of two transcription factors (Ste12p and Tec1p; Madhani and Fink 1997; Madhani *et al.* 1997) that induce target genes (Madhani *et al.* 1999) by binding to well-defined promoter elements (Zeitlinger *et al.* 2003; Chou *et al.* 2006).

In addition to the MAPK pathway, other pathways also regulate filamentous growth. Major nutrient regulatory pathways include the Ras2p–cAMP–protein kinase A (PKA) pathway (Toda *et al.* 1985; Gimeno *et al.* 1992; Mosch *et al.* 1996; Colombo *et al.* 1998; Robertson and Fink 1998; Mosch *et al.* 1999; Rupp *et al.* 1999), the AMP-dependent kinase (AMPK) Snf1p and transcriptional repressors Nrg1p and Nrg2p (Celenza and Carlson 1989; Woods *et al.* 1994; Lesage *et al.* 1996; Cullen and Sprague 2000; McCartney and Schmidt 2001; Kuchin *et al.* 2002), the target of rapamycin (TOR) pathway, which responds to nitrogen availability (Beck and Hall 1999; Cardenas *et al.* 1999; Bruckner *et al.* 2011), and the mitochondrial retrograde (RTG) pathway (Sekito *et al.* 2002; Liu *et al.* 2003; Liu and Butow 2006), which senses changes in metabolic respiration (Aun *et al.* 2013). The pH sensing Rim101p pathway (Lamb *et al.* 2001; Barrales *et al.* 2008), lipid-responsive transcription factor Opi1p (White *et al.* 1991; Reynolds 2006), tRNA modification complex Elongator [ELP, (Krogan and Greenblatt 2001; Winkler *et al.* 2001; Petrakis *et al.* 2004; Li *et al.* 2007; Svejstrup 2007)], and chromatin remodeling complex Rpd3p(L) (Carrozza *et al.* 2005; Barrales *et al.* 2008; Ryan *et al.* 2012) also regulate filamentous growth. These proteins represent only a subset of a large collection of regulators identified in *S. cerevisiae* and *Candida albicans* by gene expression profiling (Madhani *et al.* 1999; Roberts *et al.* 2000; Carlisle and Kadosh 2013), genetic screens (Lorenz *et al.* 2000; Palecek *et al.* 2000; Barrales *et al.* 2008), and systematic genome-wide approaches, including large-scale deletion and overexpression studies (Jin *et al.* 2008; Bharucha *et al.* 2011; Shively *et al.* 2013), mass spectrometry (MASS SPEC) approaches (Xu *et al.* 2010; Zhang *et al.* 2013) and analysis of ordered deletion collections made in the filamentous (Σ1278b) background (Dowell *et al.* 2010; Ryan *et al.* 2012). A critical challenge is to understand how many different proteins and pathways come together to produce a new cell type.

Several of the pathways that regulate filamentous growth can regulate each other's activities. A landmark finding comes from the discovery that the Ras2p pathway regulates the filamentous growth MAPK pathway (Mosch *et al.* 1996). More recently, the ELP (Abdullah and Cullen 2009), Rim101, RTG, Rpd3p(L), and Opi1p pathways have also been shown to regulate the filamentous growth MAPK pathway (Chavel *et al.* 2010). These pathways control expression of the gene encoding one of the plasma-membrane sensors for the filamentous growth MAPK pathway, Msb2p (Chavel *et al.* 2010). It has also been shown that the major transcription factors that regulate filamentous growth regulate each other's targets, which creates hubs where signal integration events are coordinated (Borneman *et al.* 2006). One hub is the *FLO11* promoter, where multiple transcription factors converge to fine tune cell adhesion (Rupp *et al.* 1999). Likewise, the major protein kinases that regulate filamentous growth function in an interdependent network (Bharucha *et al.* 2008). Therefore, signal coordination occurs at multiple levels to regulate the filamentous growth response.

Here we examine the question of signal integration by performing a genetic screen with an ordered deletion collection in the filamentous (Σ1278b) background (Ryan *et al.* 2012). This effort, combined with hypothesis-based testing, identified 97 new regulators of the filamentous growth MAPK pathway, which map to known regulatory pathways and provide entirely new connections. Using the screen as a platform, we examin questions related to network connectivity. We show that tuning the activity of the filamentous growth MAPK pathway to the other pathways is critical to producing a coordinated response. We also show that several of the key pathways that regulate filamentous growth also regulate each other's targets. Thus, an integrated network regulates the filamentous growth response. We speculate that similarly coordinated networks coordinate cell differentiation responses in other systems.

Materials and Methods

Strains, plasmids, and microbiological techniques

Filamentous growth was evaluated in the Σ1278b strain background (Liu *et al.* 1996). The haploid gene deletion collection constructed in the Σ1278b strain background has been described (Ryan *et al.* 2012) and was generously provided by C. Boone. p*FRE–lacZ* was provided by H. Madhani (Madhani *et al.* 1997). The YCp–Cdc12–GFP was provided by J. Pringle (Fares *et al.* 1996). The p*ste12*::*URA3* plasmid was provided from G. Sprague (McCaffrey *et al.* 1987). p*8XCRE–lacZ* was provided by H. Saito (Tatebayashi *et al.* 2006).

p*CIT2–lacZ* was provided by the Liu lab and has been described (Liu and Butow 1999).

Standard laboratory conditions were used to grow yeast and bacterial cultures (Rose *et al.* 1990). *Escherichia coli* was grown in LB and 2XYT media. Yeast was grown in rich media YEPD (2% glucose) or YEP–GAL (2% galactose) or synthetic complete media at 30° unless otherwise noted. Yeast strains are listed in Table 1. Gene deletions were constructed using antibiotic resistance markers (Goldstein and McCusker 1999) or auxotrophic markers amplified by PCR and introduced into yeast by lithium acetate transformation by standard methods as described (Chavel *et al.* 2010). The plate washing (Roberts and Fink 1994) and single-cell invasive growth assays (Cullen and Sprague 2000) were used to measure filamentous growth. Colony morphology was examined by visual inspection on YEPD media (Granek and Magwene 2010; Voordeckers *et al.* 2012). Functional analysis of the MAPK regulatory genes came from SGD (http://www.yeastgenome.org).

Halo assays were performed as described (Jenness *et al.* 1987). Specifically, wild-type and mutant strains were grown to saturation in YEPD (2% glucose). Cell density was determined by OD A_{600}. Cultures were serially diluted such that ~10,000 cells were spread onto YEPD plates. After the cell suspension had dried, four spots of 1 μg/μl alpha factor were spotted (10 μl). Plates were incubated at 30° for 2 days and photographed.

Invasive growth screen of the filamentous deletion collection

The *MAT***a** Σ1278b deletion collection (Ryan *et al.* 2012) was pinned in 96-well format to YEPD media omnitrays (Thermo Scientific, Waltham, MA). Each plate was pinned independently using a pinning tool (V&P Scientific, VP408, San Diego, CA)—sterilized with a 10% bleach solution, 95% ethanol, 70% ethanol and flame—and a pinning guide tray (V&P Scientific, VP381). Plates were pinned in duplicate and incubated for 5 and 12 days. Plates were photographed, washed in a stream of water, and photographed again. Each plate was scored visually for colonies that showed changes in morphology and invasive growth. Scores were tabulated to produce a single score called the invasive growth index. The results of the screen and details of the scoring system are presented in Table S2.

Evaluating filamentous growth MAPK pathway activity

The activity of the filamentous growth MAPK pathway was evaluated with a transcriptional reporter [p*FRE–lacZ* (Madhani and Fink 1997)]. Strains that showed clear-cut invasive growth phenotypes (hyper- and hypoinvasive) were transformed with the p*FRE–lacZ* plasmid. Strains were grown in media lacking uracil to maintain selection for the plasmid and the nonpreferred carbon source galactose (S-GAL–URA) to induce pathway activity (Pitoniak *et al.* 2009). Mutants were induced in S-GAL–URA for 4 hr. Mutants were grown in batches of ~20 alongside control strains (*tec1*Δ and *dig1*Δ) to minimize batch-to-batch variation. Cell extracts were prepared, and β-galactosidase assays were performed as described (Chavel *et al.* 2010). The average values of at least two independent experiments were reported. Statistical significance was determined by comparing the difference between wild-type and experimental p*FRE–lacZ* expression averages in a *z*-test score (Freedman *et al.* 1998). The *z*-test score was converted to the *P*-value (http://www.graphpad.com). Samples with a *P*-value ≤0.0001 and ≥1.5-fold change from wild type were considered statistically significant. Raw data for the β-galactosidase assays can be found in Table S3. For some experiments, the filamentous growth MAPK pathway was evaluated with a growth reporter [*FUS1–HIS3*, (McCaffrey *et al.* 1987)]. In Σ1278b cells lacking an intact mating pathway (*ste4*Δ), growth on SD–HIS is dependent on the filamentous growth MAPK pathway (O'Rourke and Herskowitz 1998; Cullen *et al.* 2004; Pitoniak *et al.* 2009; Chavel *et al.* 2010; Karunanithi and Cullen 2012). Growth assays are shown in Figure S1. As a separate test, 26 genes identified by the screen were disrupted in a wild-type Σ1278b strain and checked for invasive growth and p*FRE–lacZ*; 77% passed a preliminary test.

Budding pattern analysis

Patterns of bud-site selection were based on established principles (Chant and Pringle 1995). Budding pattern was determined in two ways. In one method, budding pattern was based on visual inspection of connected cells. Σ1278b cells grown in liquid YEPlowD (0.2% glucose) media undergo filamentous growth and exhibit Ste12p-dependent changes in cell length, cell–cell adhesion, and distal-unipolar budding. Cells were grown to midlog phase in YEPlowD (0.2% glucose) liquid medium for 12–14 hr and examined by microscopy at 100× magnification. Buds were assigned as proximal, equatorial, or distal depending on their position relative to mother cells. At least 150 cells were counted for each experiment.

In a separate approach, cells were stained by FITC-ConA and TRITC-ConA based on published protocols (Matheos *et al.* 2004; Gao and Bretscher 2009) with the following modifications. Cells were grown in YEPlowD for 16 hr. FITC-ConA (0.1 mg/ml) was added to 1 ml cells. Cells were incubated in the dark for 15 min, washed three times, and resuspended in YEPlowD for 4 hr. Cells (1 ml) were then stained with TRITC-ConA (0.1 mg/ml), washed three times in water, and examined by fluorescence microscopy to visualize the position of buds. At least 200 buds were recorded for each condition.

Quantitative PCR analysis

Quantitative PCR (qPCR) analysis was performed as described (Pfaffl 2001). To prepare total RNA, cells were grown in 50-ml aliquots in YEP–GAL medium to midlog phase (~6 hr). Total RNA was isolated by hot acid phenol extraction. cDNA synthesis and real-time PCR reactions were performed as described (Chavel *et al.* 2010). qPCR and melt curve data collection was performed as described (Chavel *et al.* 2010) with the following alterations to the amplification cycles: initial denaturation for 3 min at 95°, followed by 35× cycle 3 (denaturation for 30 sec at 95°, annealing for 30 sec at 60°, and extension for

Table 1 Yeast strains used in this study

Strain	Genotype	Reference
PC313[a]	*MAT***a** *ura3-52*	Liu *et al.* (1993)
PC538	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52*	Cullen *et al.* (2004)
PC539	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste12::KLURA3*	Cullen *et al.* (2004)
PC563	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bud8::KLURA3*	Cullen and Sprague (2002)
PC586	*MATα ura3-52 leu2*	Cullen *et al.* (2004)
PC622	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-SHO1*	Cullen *et al.* (2004)
PC949	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pop2::KanMX6*	This study
PC950	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ccr4::KanMX6*	This study
PC999	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA*	Cullen *et al.* (2004)
PC1083	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA GAL-MSB2::KanMX6*	Cullen *et al.* (2004)
PC1415	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bni1::KLURA3*	Cullen and Sprague (2002)
PC1516	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA$^{\Delta 100\text{-}818}$*	Cullen *et al.* (2004)
PC1558	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sho1::HYG ssk1::NAT*	Pitoniak *et al.* (2009)
PC1621	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA$^{\Delta 100\text{-}818}$ GAL-SHO1::GENT*	This study
PC1625	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-MSB2-HA::NAT GAL-SHO1::GENT*	This study
PC1895	*MAT***a** *ura3-52 leu2::HYG*	This study
PC2043	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA::KanMX6*	Karunanithi *et al.* (2010)
PC2061	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ssk1::NAT ste11::KLURA3*	Pitoniak *et al.* (2009)
PC2112	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 leu2::HYG lacZ::NAT tec1::LEU2*	Vadaie *et al.* (2008)
PC2360	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ras2::NAT*	Chavel *et al.* (2010)
PC2362	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ira1::NAT*	Chavel *et al.* (2010)
PC2511	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ras2::NAT ste12::KLURA3*	This study
PC2515	*MATα ura3-52 leu2 flo8::NAT*	Chavel *et al.* (2010)
PC2532	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 flo8::HYG*	Chavel *et al.* (2010)
PC2534	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pde2::HYG*	Chavel *et al.* (2010)
PC2535	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 gpa2::NAT*	Chavel *et al.* (2010)
PC2537[b]	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 gpr1::KLURA3*	Chavel *et al.* (2010)
PC2588	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 tpk1::NAT*	Chavel *et al.* (2010)
PC2618	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 grr1::KLURA3*	This study
PC2622	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 snf8::HYG*	This study
PC2633	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sdc25::NAT*	Chavel *et al.* (2010)
PC2688	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA ste12::KLURA3*	This study
PC2690	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA ras2::KLURA3*	This study
PC2763	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 elp2::KLURA3*	Abdullah and Cullen (2009)
PC2845	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA::KanMX6 gal11:KLURA3*	This study
PC2945	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rxt2::KLURA3*	Chavel *et al.* (2010)
PC2952	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA hda1::KLURA3*	Chavel *et al.* (2010)
PC2953	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rim101::KLURA3*	Chavel *et al.* (2010)
PC2954	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA snf2::KLURA3*	Chavel *et al.* (2010)
PC2955	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA msn1::KLURA3*	Chavel *et al.* (2010)
PC2956	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA yak1::KLURA3*	Chavel *et al.* (2010)
PC2957	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA mss11::KLURA3*	Chavel *et al.* (2010)
PC2980	*MAT***a** *ura3-52 elp2::KLURA3*	This study
PC3016	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bem4::HYG*	A. Pitoniak, C. Chavel, J. Chow, j. Smith, D. Camara, S. Karunanithi, K. Wolfe, K., and P. J. Cullen, (unpublished data)
PC3030	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA sin3::NAT*	Chavel *et al.* (2010)
PC3031	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA isw1::NAT*	Chavel *et al.* (2010)
PC3032	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA cka1::NAT*	Chavel *et al.* (2010)
PC3033	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA nhp10::NAT*	Chavel *et al.* (2010)
PC3034	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA isw2::NAT*	Chavel *et al.* (2010)
PC3035	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA mks1::NAT*	Chavel *et al.* (2010)
PC3037	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA sds3::KLURA3*	Chavel *et al.* (2010)
PC3038	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rpd3::KLURA3*	Chavel *et al.* (2010)
PC3039	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA dig1::KLURA3*	Chavel *et al.* (2010)
PC3352	*MAT***a** *ura3-52 ras2::NAT*	This study
PC3353	*MAT***a** *ura3-52 sin3::NAT*	This study
PC3362	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA fkh1::KLURA3*	Chavel *et al.* (2010)
PC3363	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA nrg1::KLURA3*	Chavel *et al.* (2010)
PC3414	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA spo14::KLURA3*	Karunanithi *et al.* (2010)

(continued)

Table 1, *continued*

Strain	Genotype	Reference
PC3415	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA dfg16::KLURA3*	Karunanithi *et al.* (2010)
PC3419	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA ash1::KLURA3*	Karunanithi *et al.* (2010)
PC3421	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA plb3::KLURA3*	Karunanithi *et al.* (2010)
PC3428	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA swi4::KLURA3*	Chavel *et al.* (2010)
PC3429	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA mga1::KLURA3*	Chavel *et al.* (2010)
PC3430	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA fkh2::NAT*	Chavel *et al.* (2010)
PC3431	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA sfl1::KLURA3*	Chavel *et al.* (2010)
PC3432	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA fkh1::KLURA3 fkh2::NAT*	Chavel *et al.* (2010)
PC3435	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA nrg1::KLURA3 nrg2::NAT*	Chavel *et al.* (2010)
PC3635	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bud3::KLURA3*	This study
PC3637	*MAT*α *ura3-52 leu2 ste12::kanMX6*	This study
PC3642	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg3::NAT*	Chavel *et al.* (2010)
PC3643	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA tco89::NAT*	Chavel *et al.* (2010)
PC3644	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA gzf3::NAT*	Chavel *et al.* (2010)
PC3652	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg2::NAT*	Chavel *et al.* (2010)
PC3654	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA tor1::NAT*	Chavel *et al.* (2010)
PC3657	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA lsc2::NAT*	This study
PC3687	*MAT*α *ura3-52 leu2 opi1::NAT*	This study
PC3688	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA opi1::NAT*	Chavel *et al.* (2010)
PC3690	*MAT*α *ura3-52 leu2 rim101::NAT*	This study
PC3691	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rim101::NAT*	This study
PC3695	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg1::NAT*	This study
PC3861	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste11::NAT*	Karunanithi and Cullen (2012)
PC3920	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA vps8::NAT*	This study
PC4006	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mdh1::KLURA3*	This study
PC4007	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 spt3::KLURA3*	This study
PC4008	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 spt8::KLURA3*	This study
PC4032	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 rim20::KLURA3*	This study
PC4035	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bmh2::KLURA3*	This study
PC4038	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 cmk2::KLURA3*	This study
PC4039	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 cmk1::HYG*	This study
PC4043	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bmh1::KLURA3*	This study
PC4141	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 tpk2::KLURA3*	This study
PC4256	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bud1::NAT*	This study
PC4468	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 rga1::KLURA3*	This study
PC5071	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pcl9::NAT*	This study
PC5072	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pcl1::KLURA3 plc9::NAT*	This study
PC5073	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pcl2::HYG plc9::NAT*	This study
PC5074	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pcl1::KLURA3 pcl2::HYG pcl9::NAT*	This study
PC5075	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pcl1::KLURA3 plc2::HYG*	This study
PC5084	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA tpk3::NAT*	This study
PC5085	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 gln3::KLURA3*	This study
PC5090	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 nte1::NAT*	This study
PC5091	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pho85::NAT*	This study
PC5095	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA sir2::NAT*	This study
PC5102	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA gcn5::KLURA3*	This study
PC5108	*MAT***a** *ura3-52 tpk2::NAT*	This study
PC5111	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 stb3::NAT*	This study
PC5113	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 stb6::NAT*	This study
PC5115	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pho4::NAT*	This study
PC5121	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pho80::NAT*	This study
PC5332	*MAT***a** *ura3-52 rtg2::NAT*	This study
PC5335	*MAT***a** *ura3-52 pho85::KLURA3*	This study
PC5340	*MAT*α *ura3-52 leu2 gcn5::LEU2*	This study
PC5351	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA ygr125w::NAT*	This study
PC5352	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA gpb2::KLURA3*	This study
PC5354	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA cup2::KLURA3*	This study
PC5360	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA aca1::KLURA3*	This study
PC5362	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA sko1::KLURA3*	This study
PC5364	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA plb1::TRP*	This study
PC5651	*MAT***a** *ura3-52 ste12::NAT*	This study

(continued)

Table 1, *continued*

Strain	Genotype	Reference
PC5822	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 vps27::KLURA3*	This study
PC5826	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 vps26::KLURA3*	This study
PC5831	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 vps35::KKLURA3*	This study
PC5856	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 hap4::KLURA3*	This study
PC5860	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mep2::KLURA3*	This study
PC5862	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 rim15::KLURA3*	This study
PC5865	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sch9::KLURA3*	This study
PC5871	*MAT***a** *ura3-52 leu2::HYG ssk1::NAT*	This study
PC5872	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 crz1::KLURA3*	This study
PC5875	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pho81::KLURA3*	This study
PC5876	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pho84::KLURA3*	This study
PC5878	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 rim21::KLURA3*	This study
PC5880	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 rim9::KLURA3*	This study
PC5881	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pho5:KLURA3*	This study
PC6016[c]	*MAT***a** *can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0*	Ryan *et al.* (2012)
PC6093	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 gpb1::NAT gpb2::KLURA3*	This study
PC6103	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA tec1::NAT*	This study
PC6135	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 rsr1::HIS3 bni1::KLURA3*	This study
PC6136	*MAT***a** *ura3-52 pde2::NAT*	This study
PC6137	*MAT***a** *ura3-52 ccr4::NAT*	This study
PC6138	*MAT***a** *ura3-52 nte1::NAT*	This study
PC6139	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ino1::KLURA3*	This study
PC6140	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 smp1::KLURA3*	This study
PC6141	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 flo1::KLURA3*	This study
PC6159	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ccr4::NAT ssk1::KLURA3*	This study
PC6161	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 nte1::NAT ssk1::KLURA3*	This study
PC6163	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pde2::NAT ssk1::KLURA3*	This study
PC6165	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pho85::NAT ssk1::KLURA3*	This study
PC6166	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ras2::NAT ssk1::KLURA3*	This study
PC6192	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 faa4::KLURA3*	This study
PC6193	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA hac1::KLURA3*	This study
PC6197	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA stb5::NAT*	This study
PC6198	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA stp1::KLURA3*	This study
PC6201	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA skn7::KLURA3*	This study
PC6202	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA yhp1::KLURA3*	This study
PC6204	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA mot3::NAT*	This study
PC6206	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA stb2::NAT*	This study
PC6208	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA ace2::KLURA3*	This study
PC6210	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA azf1::NAT*	This study
PC6212	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA phd1::NAT*	This study
PC6218	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA sok2::KLURA3*	This study
PC6222	*MAT***a** *ura3-52 ras2::NAT*	This study
PC6253	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bcy1::KLURA3*	This study
PC6258	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA mot2::KLURA3*	This study
PC6284	*MAT***a** *can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 mcy1::kanMX4 ste12::LEU2*	This study
PC6285	*MAT***a** *can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 pnc1::kanMX4 ste12::LEU2*	This study
PC6286	*MAT***a** *can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 mnl1::kanMX4 ste12::LEU2*	This study
PC6287	*MAT***a** *can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 rxt3::kanMX4 ste12::LEU2*	This study
PC6288	*MAT***a** *can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 cwc27::kanMX4 ste12::LEU2*	This study
PC6289	*MAT***a** *can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 ssn8::kanMX4 ste12::LEU2*	This study
PC6290	*MAT***a** *can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 nut1::kanMX4 ste12::LEU2*	This study
PC6291	*MAT***a** *can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 ela1::kanMX4 ste12::LEU2*	This study
PC6292	*MATα ura3-52 leu2 flo8::NAT ssk1::KLURA3*	This study
PC6293	*MATα ura3-52 leu2 gcn5::LEU2 ssk1::KLURA3*	This study
PC6294	*MATα ura3-52 leu2 opi1::NAT ssk1::KLURA3*	This study
PC6295	*MATα ura3-52 leu2 rim101::NAT ssk1::KLURA3*	This study
PC6296	*MAT***a** *ura3-52 sin3::NAT ssk1::KLURA3*	This study
PC6297	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 leu2::HYG lacZ::NAT tec1::LEU2*	This study
PC6298	*MATα ura3-52 leu2 elp2::LEU2*	This study
PC6299	*MAT***a** *ura3-52 leu2::HYG ssk1::NAT elp2::LEU2*	This study

[a] All strains are in the Σ1278b background unless otherwise indicated.
[b] KLURA3 refers to the *Kluyveromyces lactis* URA3 cassette.
[c] Σ1278b ordered deletion collection control strain *MAT***a** *can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ*.

30 sec at 72°). Gene expression was quantified using the $\Delta\Delta C_t$ method as described (Livak and Schmittgen 2001). All reactions were performed in triplicate, and average values are reported. Primers used are as follows: *ACT1* (forward 5′-GGCTTCTTTGACTACCTTCCAACA-3′ and reverse 5′-GATGGACCACTTTCGTCGTATTC-3′), *NRG1* (forward 5′-CTAATGATGCATATAATAAGATGGC-3′ and reverse 5′-ATGACCCGATGTAGTGAATCCT-3′), *PHO5* (forward 5′-ACATCACCTTGCAGACTGTCA-3′ and reverse 5′-AAGTACTAGCGTCAGTTGAGG-3′), *INO1* (forward 5′-CTAATCAAGATGAGAGAGCCAAT-3′ and reverse 5′-ATACTTCTACGTACCTCTCAGTA-3′), and *SMP1* (forward 5′-AGTCAAGATTCCTCCAGTGTAC-3′ and reverse 5′-ATCCGCTCGTGATATTGCTC-3′).

Fluorescence microscopy

Actin staining by rhodamine phalloidin has been described (Amberg 2000). Differential interference contrast (DIC) and fluorescence microscopy using rhodamine and GFP filter sets were performed using an Axioplan 2 fluorescent microscope (Zeiss, Jena, Germany) with a PLAN-APOCHROMAT 100X/1.4 (oil) objective (N.A. 0.17). Digital images were obtained with the Axiocam MRm camera (Zeiss). Axiovision 4.4 software (Zeiss) was used for image acquisition and analysis.

Results

Identification of filamentous growth MAPK pathway regulators

An ordered collection of 4072 deletion mutants constructed in the filamentous (*MAT***a** Σ1278b) background (provided by the Boone Lab, Toronto, ON; Ryan *et al.* 2012) was screened for changes in colony morphology (Granek and Magwene 2010; Voordeckers *et al.* 2012) and invasive growth based on the plate-washing assay (Roberts and Fink 1994) to identify regulators of filamentous growth. These assays provide a readout of filamentous growth that correlate with the activity of the filamentous growth MAPK pathway (Figure 1B; Roberts and Fink 1994).

Screens were performed at two time periods (5 and 12 days), which allowed evaluation of the progression of invasive growth. The 5-day screen was better suited to identify hyperfilamentous growth mutants, and the 12-day screen enriched for hypofilamentous growth mutants. The two screens also provided independent validation of the mutants identified (Table S2). A scoring system incorporated colony morphology and agar invasion from both screens into a single value called the invasive growth index that was used to rank the mutants by strength-of-phenotype (Table S2).

Of the 4072 mutants represented in the collection, 220 showed hyperfilamentous growth and 478 showed hypofilamentous growth (Table S2). Many of these mutants have been identified in other screens (Table S1) (Lambrechts *et al.* 1996; Lo and Dranginis 1998; Pan and Heitman 1999; Lorenz *et al.* 2000; Ryan *et al.* 2012; Shively *et al.* 2013). The screen uniquely identified new regulators of filamentous growth (Table S1), which may have been due to the specific incubation times or differences in scoring systems.

To identify those regulators of filamentous growth that also regulate the filamentous growth MAPK pathway, mutants identified in the screen were examined for changes in the activity of a transcriptional reporter, p*FRE–lacZ*, which provides a readout of filamentous growth MAPK pathway activity (Madhani and Fink 1997; Pitoniak *et al.* 2009). Control strains verified that loss of negative regulators showed elevated p*FRE–lacZ* activity (Figure 1, A and C, asterisks, and Table S3), which included the transcriptional repressor Dig1p (Cook *et al.* 1996), the mating pathway MAP kinase Fus3p (Bruckner *et al.* 2004), and the HOG pathway MAP kinase kinase Pbs2p (Figure 1, A and C and Table S3; asterisks in panel C refers to pathway components). Loss of pathway components showed reduced p*FRE–lacZ* activity (Figure 1, A and D, asterisks; *msb2*Δ, *sho1*Δ, *ste50*Δ, *tec1*Δ, *ste20*Δ, *ste11*Δ, *kss1*Δ, and *ste12*Δ; Table S3).

Mutants identified by the invasive growth screens were transformed with p*FRE–lacZ* reporter and evaluated for β-galactosidase activity. For the hyperinvasive growth mutants, 41 of 110 showed elevated p*FRE–lacZ* expression (37%, Figure 1C). For the hypoinvasive growth mutants, 43 of 116 tested showed a defect in p*FRE–lacZ* expression (37%, Figure 1D). Not all candidates were examined, because mutants with weaker phenotypes showed differences that fell below the statistical cutoff employed (≥1.5-fold, *P*-value ≤ 0.0001). Thus, the screen was not saturating.

To validate the results of the screen, and/or extend connections of known pathways to the filamentous growth MAPK pathway, ∼100 genes were disrupted in a wild-type Σ1278b strain, and gene disruptants were evaluated for invasive growth and MAPK activity (Table S1 and Figure S1). The analysis was facilitated by a cross-talk reporter that in a mating-deficient strain (*ste4*Δ *FUS1–HIS3*) provides a readout of the filamentous growth MAPK pathway. The analysis eliminated ∼15% of the candidates as false positives (Table S1). The analysis also identified several new components. In total, 97 proteins were identified by the screen and gene disruption analysis that regulate the filamentous growth MAPK pathway and play a corresponding role in the regulation of filamentous growth.

Evaluating filamentous growth MAPK pathway regulators by the change in budding pattern and cell elongation

Candidate regulators were examined for morphological phenotypes that are controlled by the filamentous growth MAPK pathway. The filamentous growth MAPK pathway regulates changes in budding pattern (Gimeno *et al.* 1992; Roberts and Fink 1994; Cullen and Sprague 2000, 2002) and the cell cycle that results in an increase in cell length (Kron *et al.* 1994; Madhani *et al.* 1999). The change in budding pattern is visually striking in haploid cells that switch from axial to distal-unipolar budding (Cullen and Sprague 2002). A recent study showed an abundance of filamentous cells in MAPK pathway mutants, raising the question of whether and to what extent the MAPK pathway regulates this aspect of the

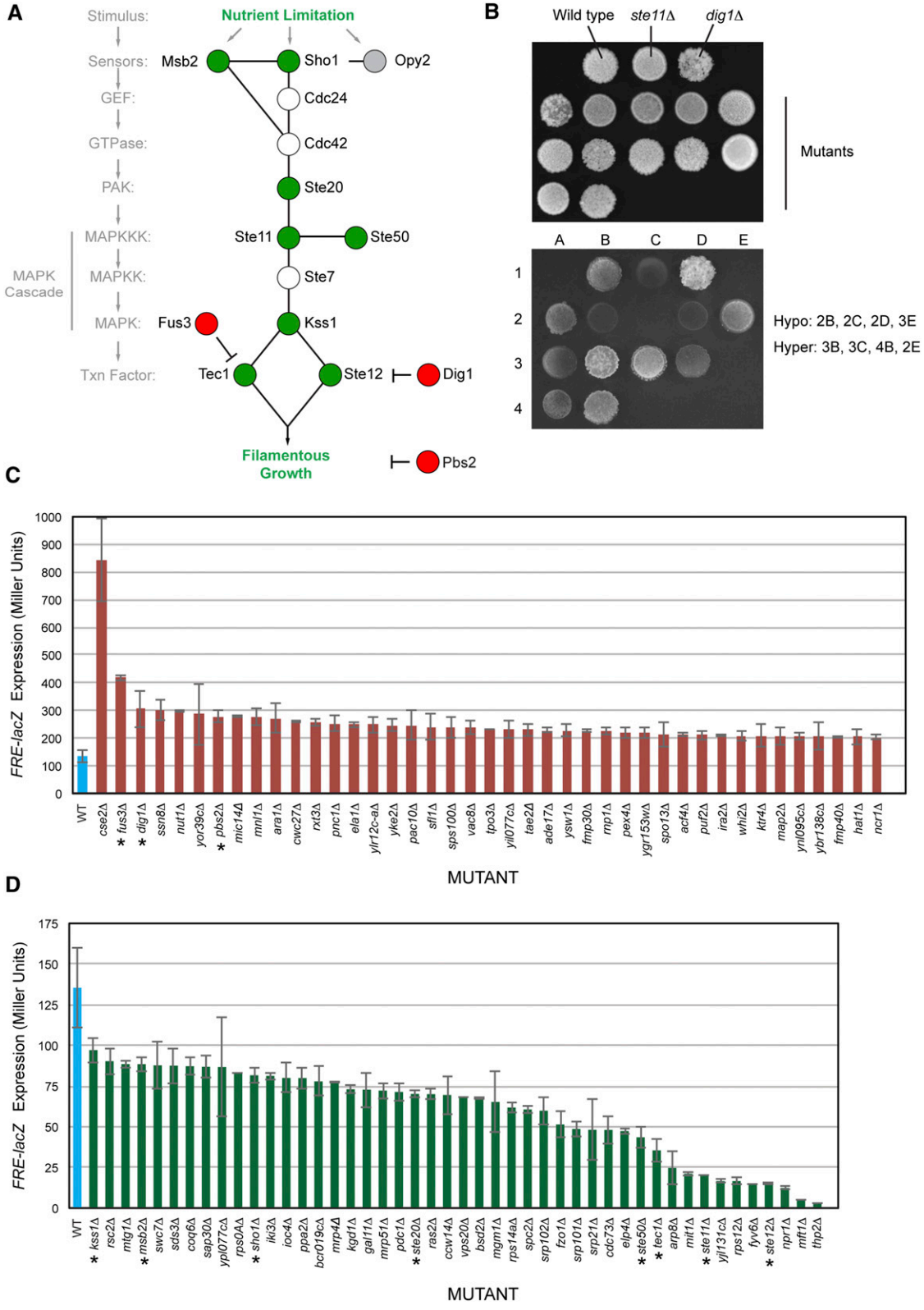

Figure 1 Positive and negative regulators of the filamentous growth MAPK pathway. (A) The filamentous growth MAPK pathway is shown with components that were identified by the screen represented as positive regulators (green circles) and negative regulators (red circles). Cdc24p and Cdc42p are essential proteins and were not tested here, and Ste7p was not present in the collection. Opy2p is an established regulator of the filamentous growth MAPK pathway (Yang *et al.* 2009, 2010; Karunanithi and Cullen 2012) and showed a defect in *FRE–lacZ* expression, but the levels fell below the range of statistical significance (Table S3). (B) Example of the plate-washing assay. Equal concentrations of cells were spotted onto YEPD media. Cells were grown for 2 days. Plates were photographed (top) and washed in a stream of water to reveal invaded cells (bottom). Examples

response (Chen and Thorner 2010). To clarify this issue, the budding pattern of filamentous cells was examined by two approaches. In one approach, filamentous growth was examined in liquid culture by microscopy (Figure 2A, left columns). In a second approach, cells were stained with FITC-ConA and TRITC-ConA at different times to visualize bud position (Figure 2A, right columns). The latter approach had the advantage of determining bud position without the assumption of which cell the parent was (Figure 2B). The approaches were in close agreement for wild-type cells and control strains lacking axial [*bud3*Δ (Chant *et al.* 1991)], distal [*bud8*Δ (Harkins *et al.* 2001; Schenkman *et al.* 2002; Kang *et al.* 2004), and core [*rsr1*Δ (Park *et al.* 1997, 2002; Kang *et al.* 2010)] bud-site-selection markers (Figure 2A).

Wild-type haploid cells showed a characteristic change in budding pattern from axial to distal-unipolar budding when grown in glucose-limited medium (Figure 2A) (Chant and Pringle 1995; Cullen and Sprague 2002)]. The *ste12*Δ mutant showed a 15% reduction in distal-unipolar budding, and the *ste20*Δ mutant showed a 20% reduction (Figure 2A). Many cells retained distal-pole budding (60%), which can account for the conclusion that the filamentous growth MAPK pathway is dispensable (Chen and Thorner 2010). Therefore, the filamentous growth MAPK pathway regulates the change in polarity during filamentous growth. Other signaling pathways probably also regulate the change in budding pattern. Under this condition, the *ras2*Δ mutant did not play a role (Figure 2A). The *rsr1*Δ mutant did not show a completely random budding pattern, but retained the propensity to bud at the distal pole. This may be due to increased polarized growth of filamentous cells, which bias bud-site-selection to the distal pole (Sheu *et al.* 2000; Cullen and Sprague 2002). Disruption of the gene encoding the formin Bni1p, which reduces the polarized growth of filamentous cells (Cullen and Sprague 2002), conferred random budding to the *rsr1*Δ mutant (Figure 2B, *bni1*Δ *rsr1*Δ).

The single-cell invasive-growth assay provides a convenient measure of the changes in budding pattern and cell length that occur during filamentous growth (Cullen and Sprague 2000) and was used to examine mutants identified in the screen. Most hyperinvasive growth mutants showed hyperelongated morphology by the single-cell assay. Glucose suppresses the filamentous morphology (Cullen and Sprague 2000) and effectively suppressed hyperelongated morphology and distal-pole budding pattern in all but two hyperinvasive growth mutants, *dig1*Δ and *fus3*Δ (Figure 2C). These mutants regulate the filamentous growth MAPK pathway; thus, a hyperactive filamentous growth MAPK pathway can bypass the inhibition of cell elongation and distal-pole budding induced by growth of cells in high-glucose environments.

Other hyperinvasive growth mutants showed hyperelongated cell morphology (Figure 2D and Figure S2). A subset of these were dependent on Ste12p for invasive growth and morphology (Figure S3, A and B). Not all mutants showed Ste12p dependence (Figure S3), which might reflect a role for these proteins in regulating filamentous growth outside the MAPK pathway. Hypoinvasive growth mutants were similarly examined. Most hypoinvasive growth mutants, like the *ste12*Δ mutant, showed a defect in unipolar budding and cell elongation (Figure 2E and Figure S2). Similarly, a subset of mutants showed defects in distal-pole budding (Figure 2F). Whereas no mutant was completely defective, several (like the *ste12*Δ mutant) showed minor differences. It is possible that distal-pole budding during filamentous growth results from the additive contribution of multiple pathways. Therefore, the budding pattern and single-cell analysis corroborated a role for many of these proteins in regulating the filamentous growth MAPK pathway.

Functional analysis of candidate genes and pathways

The genes identified by the screen and directed gene deletion analysis were classified by GO annotation terms for biological process, cellular compartment, and molecular function (Ashburner *et al.* 2000). Genes were also overlaid onto known protein and genetic interaction maps (Uetz *et al.* 2000; Drees *et al.* 2001; Ho *et al.* 2002; Miller *et al.* 2005; Costanzo *et al.* 2010). As a result, the genes were found to comprise functional categories that were explored in detail below (Figure 3).

A subset of Ras2p pathway regulators was found to regulate the filamentous growth MAPK pathway. These include Ras2p (Mosch *et al.* 1996), the alternative GEF Sdc25p (Damak *et al.* 1991; Jones *et al.* 1991), the phosphodiesterase Pde2p, and PKA subunit Tpk2p (Figure 3A; Chavel *et al.* 2010). Tpk2p regulates the transcription factor Flo8p (Robertson and Fink 1998) and negatively regulates the transcriptional repressor Sfl1p (Conlan and Tzamarias 2001; Pan and Heitman 2002). Flo8p and Sfl1p were identified by the screen (Figure 3A). Sfl1p-interacting proteins Ssn8p and Nut1p are required for Sfl1p to carry out its role as a transcriptional repressor (Conlan and Tzamarias 2001) and were identified by the screen (Figure 3A). Ccr4p, a component of the Ccr4p–NOT deadenylase complex, which is an effector of Tpk2p (Lenssen *et al.* 2002) and target of the MAP kinase Kss1p (Fasolo *et al.* 2011) was also identified.

The screen uncovered components of the chromatin remodeling complex Rpd3p(L) (Figure 3B, Ash1p, Sap30p, Ume1p,

of hyper- and hypoinvasive growth mutants are listed at right in reference to strains lacking a positive (*ste11*Δ) and negative (*dig1*Δ) regulator. (C) Mutants showing elevated p*FRE–lacZ* expression and hyperinvasive growth. β-Galactosidase assays were performed in at least duplicate. Blue bar, wild-type control strain; red bars, mutants tested. Values are expressed in Miller units (U). Error bars represent standard deviation between independent trials. *P*-values and raw data provided in Table S3. (D) Mutants showing reduced p*FRE–lacZ* expression and reduced invasive growth. Blue bar, wild-type control strain; green bars, mutants tested. See C for details. The p*FRE–lacZ* activity of the *kss1*Δ did not fall within the 1.5-fold cutoff but is shown as a reference.

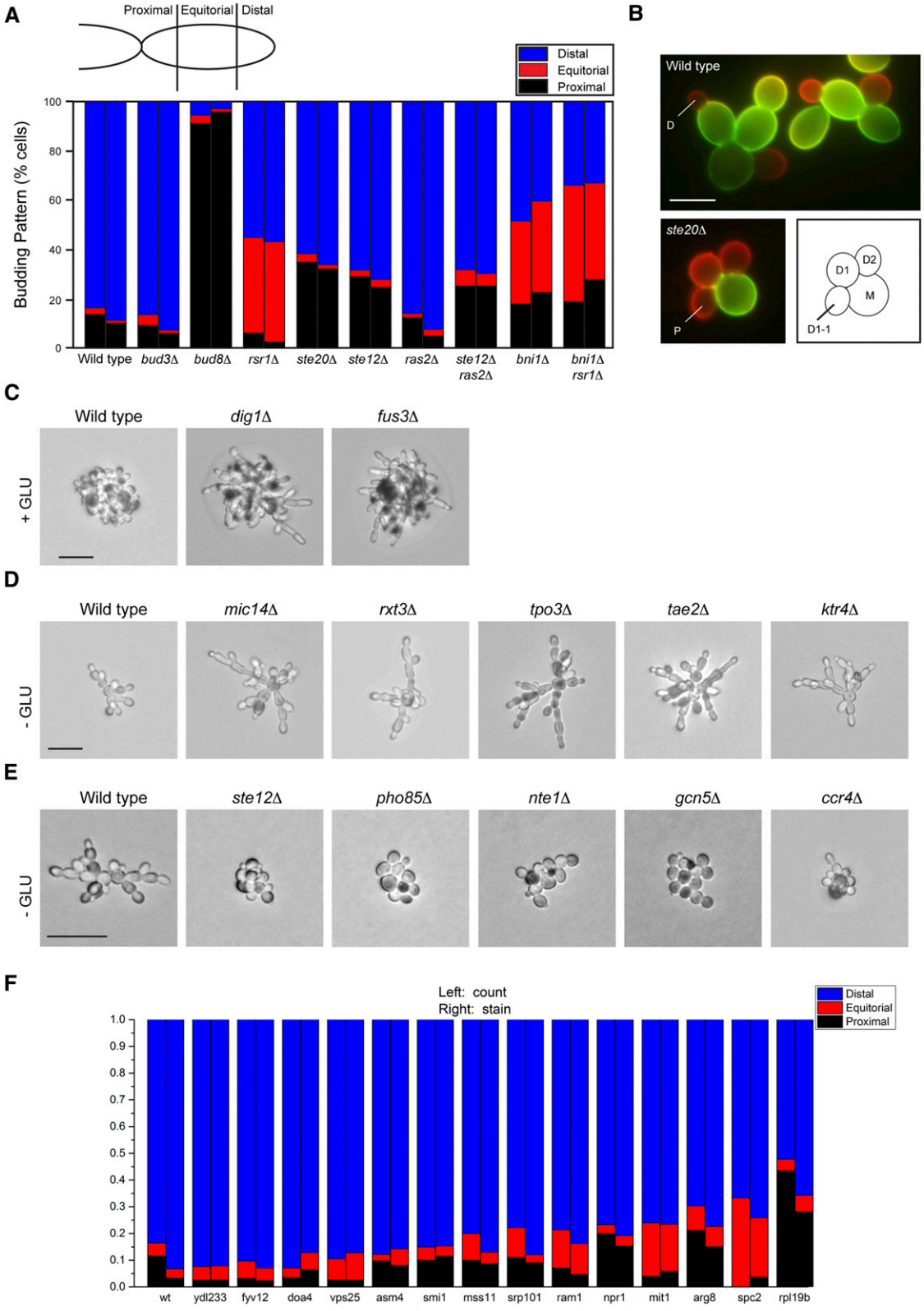

Figure 2 Role of filamentous growth MAPK pathway regulators in bud-site selection and filament formation. (A) Bar graph, the percentage of cells exhibiting distal (blue), equatorial (red), or proximal (black) budding pattern. Left column: assignment based on visual inspection. Right column: assignment based on fluorescence microscopy. (B) Example of cells costained with FITC/TRITC-ConA. Top: wild-type cells. Bottom left: the *ste20*Δ mutant. Bar, 5 μm. Bottom right: cartoon representing budding cells. D, distal; P, proximal. (C) Single-cell assay shows hyperpolarized growth and distal-pole budding pattern of the *fus3*Δ and *dig1*Δ mutants in medium containing 2% glucose (HIGH GLU). Bar, 20 μm. (D) Single-cell assay of select hyperinvasive growth mutants. Cells were grown on S-GLU (-GLU) medium at low density for 16 hr and photographed at 100×. Bar, 20 μm.

Cti6p, Pho23p, and Rxt3p; Rpd3p, Sin3p, Rxt2p, and Sds3p were previously identified; Chavel *et al.* 2010). The ELP complex regulates the filamentous growth MAPK pathway (Figure 3C; Abdullah and Cullen 2009; Elp2p, Elp6p, Iki3p, Kti12p). The screen identified members of the ELP complex (Figure 3C, Elp3p, Iki3p, and Elp4p). Thus, the screen was effective at identifying regulatory connections to the filamentous growth MAPK pathway and supports the idea that the Ras2p pathway, Rpd3p(L), and ELP are major regulators of the filamentous growth MAPK pathway.

The screen identified the cyclin-dependent kinase Pho85p (Figure 3, D and I–K and Figure S1) (Measday *et al.* 1997; Huang *et al.* 2002, 2007; Shemer *et al.* 2002; Moffat and Andrews 2004). Loss of cyclins Pcl1p, Pcl2p, and Pcl9p, and the triple *pcl1*Δ *pcl2*Δ *pcl9*Δ mutant, showed no defect in filamentous growth MAPK pathway activity (Figure 3D and Figure S1), whereas loss of Pho80p, the cyclin responsible for environmental responses controlled by Pho85p (Liu *et al.* 2000), showed reduced filamentous growth MAPK pathway activity (Figure 3D). Deletion of the transcription factor *PHO2* (Kaffman *et al.* 1994; O'Neill *et al.* 1996; Liu *et al.* 2000) showed reduced filamentous growth MAPK pathway activity (Figure 3D).

Several proteins and pathways were not identified by the screen but were shown to regulate the filamentous growth MAPK pathway by direct testing. These may have been missed by the screen for several reasons. One is that they were not represented in the collection. This was true for components of Rpd3p(L), including *tod6*Δ and *ume6*Δ. A second reason is that the pathways may play a conditional role in regulating the filamentous growth MAPK pathway. For example, the Ras2p pathway does not constitutively regulate the filamentous growth MAPK pathway (Figure S4), which complicated assessment of the roles of Flo8p and Tpk2p. A third reason is that phenotypes may have fallen below threshold of statistical significance applied to the data (*e.g.*, *tpk2*Δ, *rim101*Δ, *kss1*Δ, *opy2*Δ, *rim9*Δ, *dfg16*Δ, *rim13*Δ, *rim8*Δ, *ume1*Δ; Table S3).

Components of the Rim101p pathway were found to regulate the filamentous growth MAPK pathway (Figure 3E, Chavel *et al.* 2010). Nrg1p and Nrg2p (Lamb and Mitchell 2003) did not regulate the filamentous growth MAPK pathway (Figure 3E). The lipid regulatory transcription factor Opi1p (Greenberg *et al.* 1982) regulates the filamentous growth MAPK pathway (Chavel *et al.* 2010). The serine esterase Nte1p, which serves as the phospholipase B of yeast (Fernandez-Murray *et al.* 2009) was also found to regulate the filamentous growth MAPK pathway (Figure 3F). Ncr1p, which regulates sphingolipid biosynthesis (Malathi *et al.* 2004) and has not been shown to interact with Opi1p or Nte1p genetically or physically, negatively regulated the filamentous growth MAPK pathway.

The RTG pathway regulates the filamentous growth MAPK pathway (Figure 3G, Chavel *et al.* 2010). The screen did not identify this pathway but did uncover proteins that influence mitochondrial function (Figure 3G). A main target of RTG is aconitase (Liu and Butow 1999), an enzymatic component of the TCA cycle necessary to generate α-ketoglutarate, a precursor in glutamate biosynthesis (Magasanik and Kaiser 2002). Aco1p positively regulated the filamentous growth MAPK pathway (Figure 3G). Rtg2p is also incorporated into the histone-acetyl transferase (HAT) complex SLIK, with the HAT Gcn5p as its catalytic component (Pray-Grant *et al.* 2002). Deletion of *GCN5* reduced filamentous growth MAPK pathway activity (Figure 3G). Npr1p, a kinase that stabilizes amino acid transporters at the membrane (De Craene *et al.* 2001) and is negatively regulated by the TORC1 complex (Schmidt *et al.* 1998), was found to positively regulate the filamentous growth MAPK pathway.

The above analysis accounted for nearly half the proteins identified by the screen and involved the Ras2p, Rpd3p(L), ELP, Opi1p, Rim101p, RTG, and Pho85p pathways. The remaining proteins represent new connections to the filamentous growth MAPK pathway. These included proteins that regulate transcription [including components of the THO complex and chromatin remodeling proteins that are separate from Rpd3p(L)], protein transport and trafficking (including components of the signal recognition complex, SRP), protein translation, prefoldin, metabolism, sporulation, the cytoskeleton, post-translational modification, and genes whose functions remain to be characterized (Figure 3H).

Although it is not clear how these pathways regulate the filamentous growth MAPK pathway, the majority of regulators tested did not influence the activity of the mating pathway (Figure S5) or the HOG pathway (Figure S6A), although a HOG pathway reporter p8X*CRE–lacZ* was modestly induced in some hyperinvasive growth mutants (Figure S6B). These pathways share components with the filamentous growth MAPK pathway (Chen and Thorner 2007; Saito 2010; Saito and Posas 2012). Thus, it would appear that these factors by and large play a specific role in regulating the filamentous growth MAPK pathway. We previously showed that many pathways converge on the expression of the *MSB2* promoter (Chavel *et al.* 2010). Perhaps these regulators regulate the filamentous growth MAPK pathway in a similar manner.

Unregulated filamentous growth MAPK pathway activity is detrimental to invasive growth and proper morphogenesis

It is not entirely clear why the regulation of the filamentous growth MAPK pathway is so extensive. One possibility is that the activity of the filamentous growth MAPK pathway may be adjusted to that of other pathways that regulate filamentous growth. Coordination of morphogenetic pathways that regulate cell-cycle progression and cell polarity might be critical,

Representative microcolonies are shown; other examples are in Figure S2. (E) Single-cell assay of select hypoinvasive growth mutants. Bar, 20 μm. Other mutants are shown in Figure S2. (F) Budding pattern analysis of hypofilamentous growth mutants indicated. Scoring system is the same as in A.

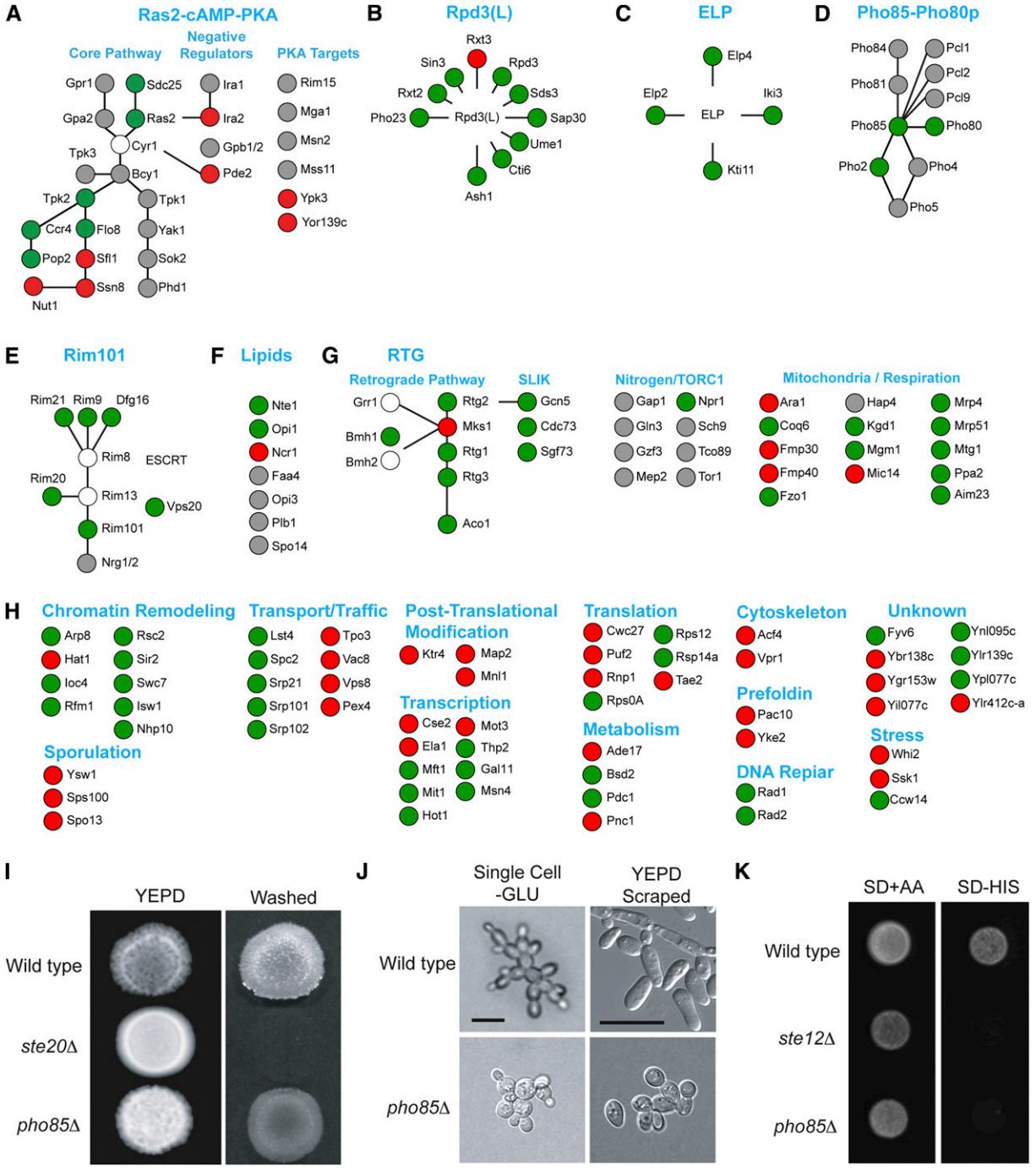

Figure 3 Functional classification of filamentous growth MAPK pathway regulators. Genes identified by the screen (Figure 1, C and D) or by hypothesis-based testing (Figure S1) are shown according to their established roles in pathways or protein complexes. Lines refer to functional connections. Green, positive regulator; red, negative regulator; gray, no phenotype; and white, not tested. (A) The Ras2p–cAMP–PKA pathway; (B) Rpd3p(L) chromatin remodeling complex (Table S3); (C) the ELP complex; (D) the Pho85p–Pho80p pathway; (E) the Rim101 pathway. (F) Lipid biosynthesis; (G) the RTG pathway and proteins associated with mitochondrial function; and (H) Functional classification of other proteins. (I) Plate-washing assay for *pho85*Δ mutant alongside controls. (J) Single-cell assay for the *pho85*Δ mutant alongside controls. Scraped refers to cells scraped from an invasive scar. (K) *FUS1–HIS3* reporter activity for the *pho85*Δ mutant alongside controls.

for example, for proper growth. To test this possibility, the activity of the filamentous growth MAPK pathway was genetically separated from other regulatory pathways using gain-of-function alleles and by driving expression of pathway regulators with inducible promoters. Hyperactive alleles of *MSB2* (Cullen *et al.* 2004), *SHO1* (Vadaie *et al.* 2008), and *STE11* (Stevenson *et al.* 1992) were examined. In addition, overexpression of pathway components was assessed with

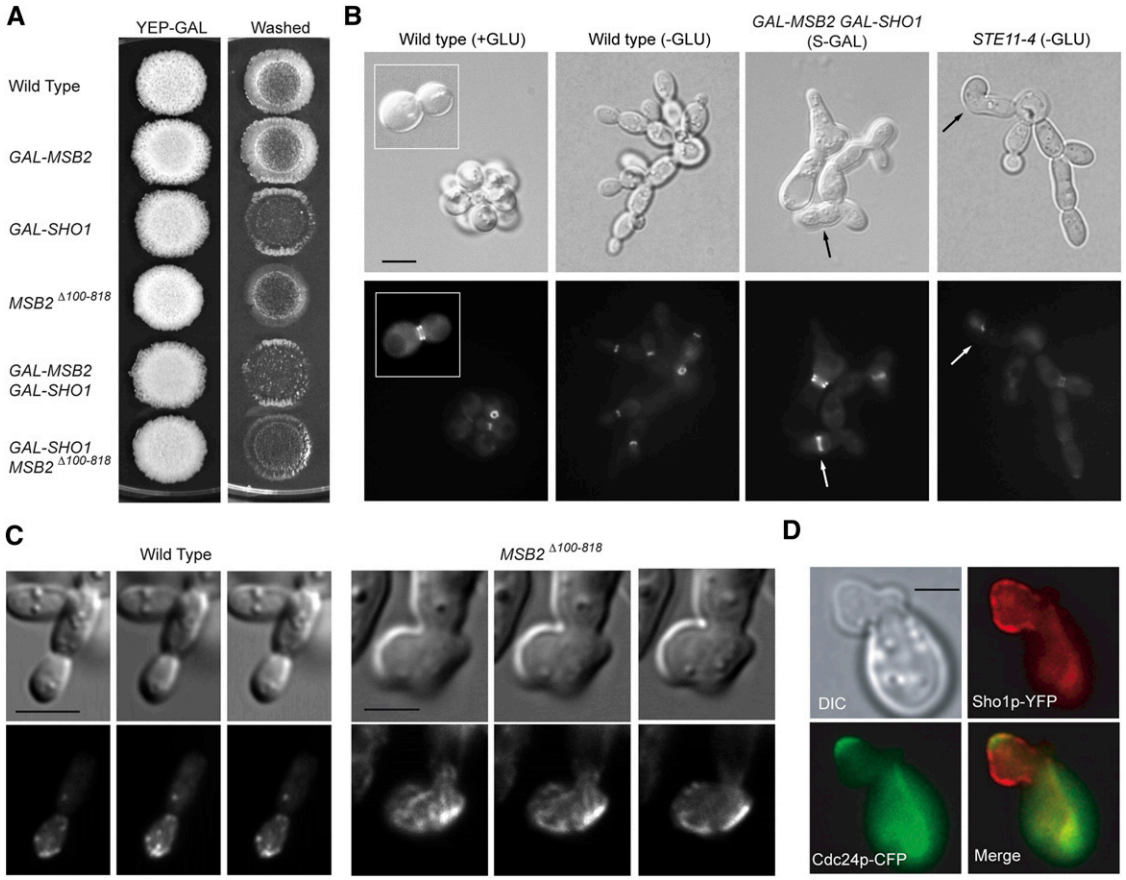

Figure 4 Overexpression/hyperactivation of pathway components does not enhance filamentous growth. (A) Plate-washing assay was performed for the indicated strains on YEP–GAL. *GAL–MSB2* and *GAL–SHO1* strains where the *GAL1* promoter has replaced the native promoter. *MSB2*$^{\Delta 100–818}$ is a hyperactive allele of *MSB2*. (B) Septin staining. Strains containing the pCdc12p–GFP plasmid were grown on SCD or S medium lacking glucose. Bar, 5 μm. (C) Indicated strains were stained with rhodamine phalloidin. Bar, 5 μm. (D) Colocalization of Sho1p and Cdc24p in cells carrying a hyperactive allele of *MSB2*. Bar, 5 μm.

the strong inducible promoter (p*GAL*; Longtine *et al.* 1998). Preliminary observations with these strains showed that hyperactivation or overexpression of Sho1p or Msb2p did not induce hyperinvasive growth but rather caused a reduction in invasive growth (Figure 4A). Microscopic examination showed that the cells had morphological defects (Figure 4, B–D).

To further explore this question, cell-polarity markers for the mother-bud neck (with the septin Cdc12p–GFP) and the cytoskeleton (with rhodamine phalloidin, which stains actin) were examined in these mutants. Septin staining showed defects in cytokinesis (Figure 4B). Actin staining showed irregular patterns, with polymerized actin at multiple surface sites on the plasma membrane (Figure 4C, File S1, and File S2). Moreover, the localization of polarity control proteins, Sho1p and Cdc24p, were localized to aberrant structures in these mutants (Figure 4D). The prevalence of these phenotypes varied among the mutants tested and ranged from ~10% irregular morphologies for the *STE11-4* mutant to >90% irregular cells in the *GAL–MSB2 GAL–SHO1* mutant. Prolonged overexpression of *SHO1* resulted in growth defects (not shown). Equivalent morphologies were observed in other mutants that exhibited filamentous growth MAPK pathway hyperactivation (not shown). However, these phenotypes stood out from most of the hyperfilamentous growth mutants identified in the screen, possibly because *pGAL*-driven and hyperactive proteins have higher pathway activity. Therefore, hyperactivation of the filamentous growth MAPK pathway causes problems with normal cell morphogenesis. A likely explanation for this phenotype is that it results from that pathway's critical roles in cell-polarity and cell-cycle control. Therefore, coordination of the activity of the filamentous growth MAPK pathway may be necessary not only to promote a coherent filamentation response but also to maintain proper cell growth.

Pathways that regulate filamentous growth control each other's targets

The filamentous growth MAPK pathway may be the terminal pathway at which many pathways converge. An alternative possibility that has not been explored is that many of the pathways that regulate filamentous growth may also regulate each other's activities. To test this possibility, transcriptional targets of several of the major pathways that regulate filamentous growth were evaluated in a panel of pathway mutants by qPCR analysis and/or transcriptional reporters. *NRG1* and *SMP1*

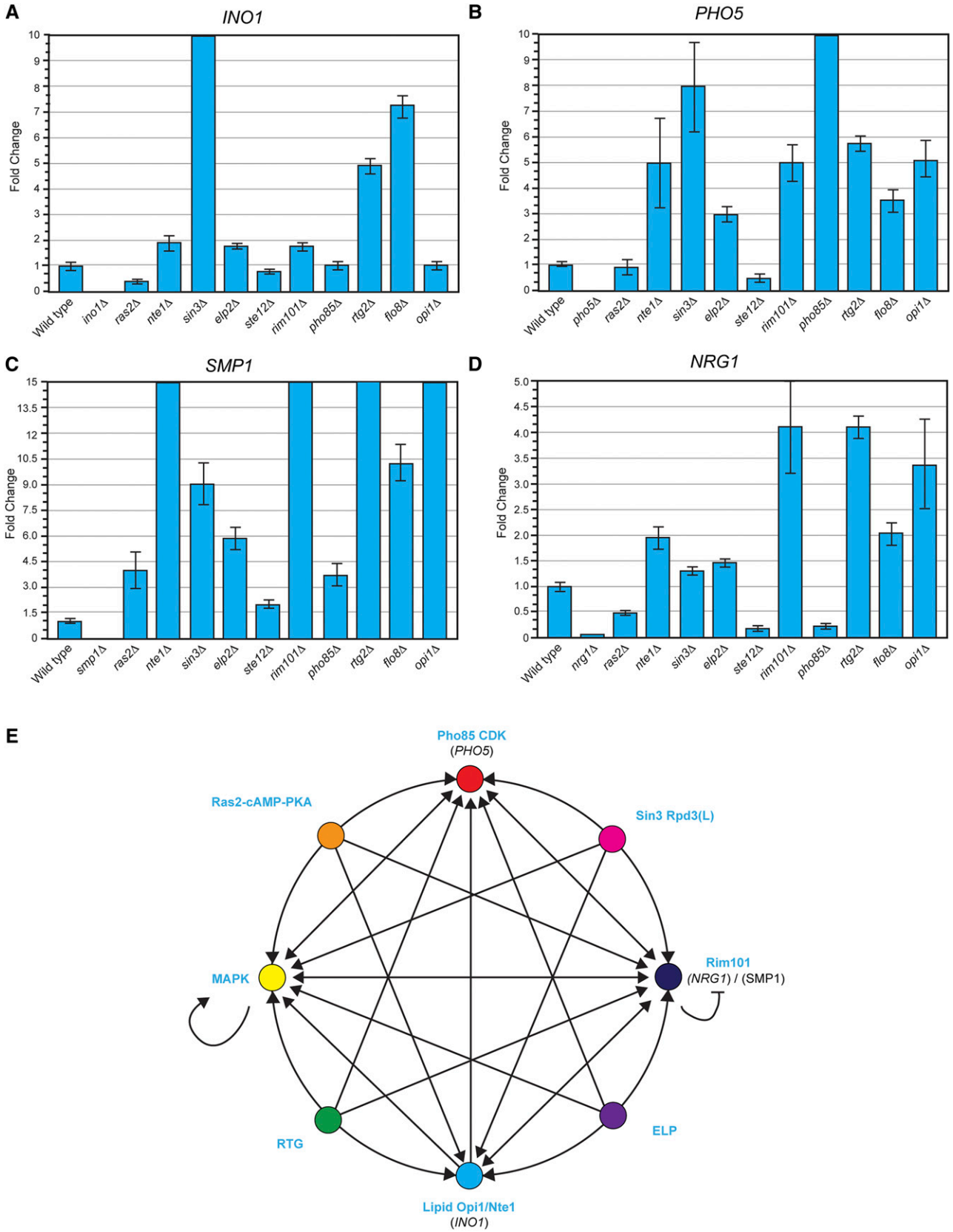

Figure 5 Signal integration between pathways during filamentous growth. (A–D) Quantitative PCR analysis of labeled genes in the indicated mutants. The bar indicates change in expression using the $\Delta\Delta C_t$ quantitation method. Wild-type expression levels were set to 1. The error bars represent standard

were selected as targets of the Rim101p pathway, which are downregulated by that pathway and upregulated in that mutant (Lamb and Mitchell 2003); *PHO5*, which is downregulated by the Pho85p pathway and upregulated in the mutant (Kaffman *et al.* 1994); *CIT2*, which is a target of the RTG pathway (Rothermel *et al.* 1995); and *INO1*, which is downregulated by the lipid/Opi1p pathway (White *et al.* 1991). qPCR and/or *lacZ* analysis was performed in mutants lacking the major filamentation control pathways (*ras2*Δ, *nte1*Δ, *sin3*Δ, *elp2*Δ, *ste12*Δ, *rim101*Δ, *pho85*Δ, *rtg2*Δ, *flo8*Δ, and *opi1*Δ). With the exception of Opi1p/*INO1*, each pathway regulated the expression of its own target (Figure 5, A–D). Many pathways similarly influenced the expression of each other' targets. For example, *INO1's* expression was upregulated in the *nte1*Δ, *sin3*Δ, *elp2*Δ, *rim101*Δ, *rtg2*Δ, and *flo8*Δ mutants (Figure 5A). Thus, in some manner, the Nte1p, Sin3p, Epl2p, Rim101p, Rtg2p, and Flo8p proteins contribute to the coregulation of a lipid pathway target. Similar results were found for *PHO5* (Figure 5B), *SMP1* (Figure 5C), and *NRG1* (Figure 5D). Several pathways were also found to regulate the RTG pathway, including Ras2p and ELP, based on *CIT2–lacZ* reporter (Figure S7). DNA microarray analysis previously identified a major transcriptional target of the filamentous growth MAPK pathway as the gene encoding Rim8p, a component of the Rim101p pathway (Chavel *et al.* 2010), and we confirmed that the filamentous growth MAPK pathway contributed to *RIM8* expression by qPCR analysis (data not shown). These results indicate that a subset of the major pathways that regulate filamentous growth regulate at least one of each other's key targets.

The mechanisms by which such regulation occurs is not clear and may occur through diverse means such as pathway-to-pathway connections or the modulation of transcription factors that serve as master regulators of signaling outputs. Moreover, not all of the possible regulatory connections were observed. For example, the filamentous growth MAPK pathway does not appear to regulate Ras2p–cAMP–PKA (Chavel *et al.* 2010) or the RTG pathways (Figure S7). Nevertheless, the results are striking from the perspective that each arrow represents in principle a regulatory connection that occurs between two pathways (Figure 5E).

Discussion

Cell differentiation involves the combined action of many different proteins and pathways. How multiple signals become integrated into a cohesive response is an important biological problem that in many cases remains unclear. Here, we explore the question of signal integration by identifying, from a global perspective, regulators of the MAPK pathway responsible for controlling differentiation to the filamentous cell type. Using a genetic screen and direct testing, we identify >95 proteins that when absent influence the activity of the filamentous growth MAPK pathway. This number likely represents an underestimate because the screen was not saturating and because a rigorous statistical cutoff was used to establish regulators. In addition, the screen was performed under a single condition where some pathways and complexes may not be required. A conservative estimate is that >35% of the major regulators of filamentous growth regulate the activity of the filamentous growth MAPK pathway.

One consequence of these regulatory connections is to sensitize MAPK activity to different stimuli. Many of the major nutrient-regulatory pathways in yeast, such as TOR (Bruckner *et al.* 2011), Snf1p (Karunanithi and Cullen 2012), Ras2p (this study; Mosch *et al.* 1996; Chavel *et al.* 2010), and RTG (this study; Chavel *et al.* 2010) impinge on the activity of the filamentous growth MAPK pathway. We also show that pathways that sense and respond to diverse stimuli, such as pH (Rim101p) and other environmental stimuli (Pho85p) also regulate the filamentous growth MAPK pathway. The connection between Pho85p and the filamentous growth MAPK pathway is particularly relevant as Pho85p has been shown in *C. albicans* to be required for temperature-dependent filamentation (Shapiro *et al.* 2012).

A second reason that the filamentous growth MAPK pathway is extensively regulated might be to coordinate its activity with other pathways that regulate the same response. In this way, the MAPK-dependent changes to budding pattern, the cell cycle, and cell adhesion can be tuned to the global network. Hence, multiple pathways can tap into these major regulatory events (instead of each pathway making changes directly). The filamentous growth MAPK pathway regulates the change in budding that occurs during filamentous growth. Mutants that reduce pathway activity (*ste20* and *ste12*) show a decrease in distal-pole budding, and mutants with elevated pathway activity (*dig1* and *fus3*) show an increase in distal-pole budding, even under high-glucose conditions. Given that multiple pathways regulate distal-pole budding, it is likely that the role of the filamentous growth MAPK pathway may be as significant as the contributions of other pathways. We show that unregulated filamentous growth MAPK pathway activity is detrimental to proper morphogenesis and cell growth.

We also show that extensive cross-regulation occurs among several of the pathways that regulate filamentous growth. Our study highlights a degree of signal integration that has not been previously appreciated. The qPCR performed here (Figure 5) was under conditions in which the filamentous growth MAPK pathway is activated (*e.g.*, poor carbon sources, like galactose). The network described in Figure 5A was examined under a single growth condition. It is possible that other connections between pathways were missed if they occur under a condition that was not tested. Genetic buffering between the pathways may obscure connections. Similarly, loss of one pathway may or may not induce loss of the filamentous growth phenotype. The ways

deviation between experiments. Cells were grown to midlog phase in YEP–GAL media for 6 hr. (E) Diagram showing connections between pathways that regulate filamentous growth. Arrows refer to positive regulation. Bars, negative regulation.

by which this regulation is accomplished is not clear. Multiple pathways could feed into a central metabolite or small molecule (cAMP) that regulates a master regulatory transcription factor, from which multiple filamentation targets are controlled. Indeed, transcriptional "hub" proteins globally regulate filamentous growth (Borneman *et al.* 2006). At least one connection may be direct. *RIM8* is a major target of the filamentous growth MAPK pathway (Chavel *et al.* 2010). As not all pathways regulate each other's targets, the connections between the pathways are presumably specific.

In conclusion, filamentous growth results from a highly coordinated and integrated signaling network. A single MAPK pathway regulates filamentous growth, which is controlled by many different pathways to integrate various signals and coordinate the response. Signal integration is commonly seen in similar differentiation responses in higher eukaryotes, where multiple stimuli activate an interconnected set of signaling pathways (Cuenda and Rousseau 2007; Katz *et al.* 2007; Raman *et al.* 2007). Perhaps the connections identified here extend to related pathways in other systems.

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Global Regulation of a Differentiation MAPK Pathway in Yeast

Colin A. Chavel, Lauren M. Caccamise, Boyang Li, and Paul J. Cullen

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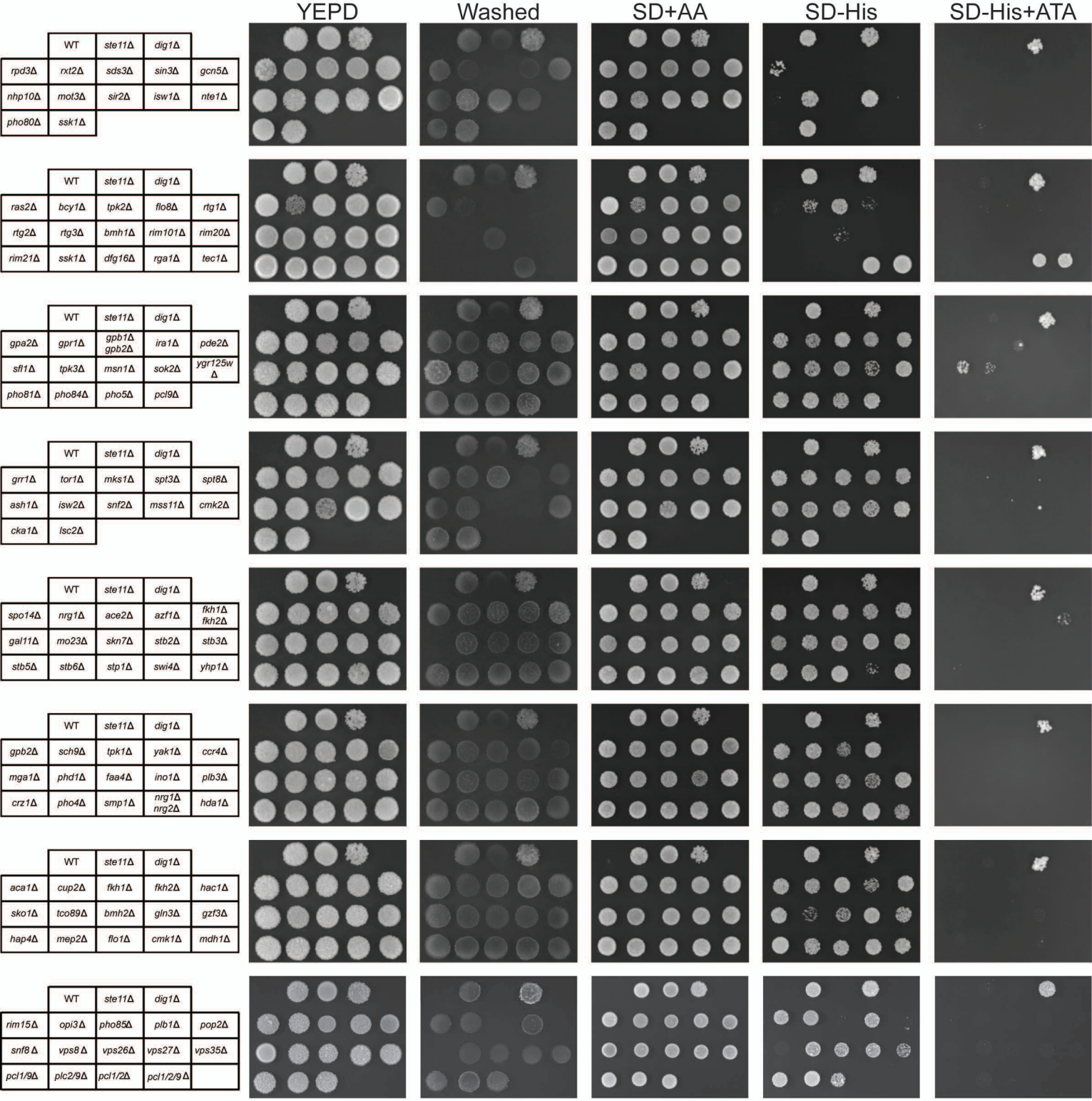


Figure S1. Evaluation of candidate regulators of the filamentous growth MAPK pathway by the plate-washing assay and cross-talk reporter (*FUS1-HIS3*). Wild type and control strains and the indicated mutants were spotted on to YEPD, SD-HIS, and SD-HIS + 2.5 mM ATA and incubated for 2d. No growth on SD-HIS indicates a defect in filamentous growth MAPK pathway activity. Growth on SD-HIS + 2.5 mM ATA indicates elevated filamentous growth MAPK pathway activity. YEPD plates were photographed, washed in a stream of water to reveal invaded cells, and photographed again (Washed).

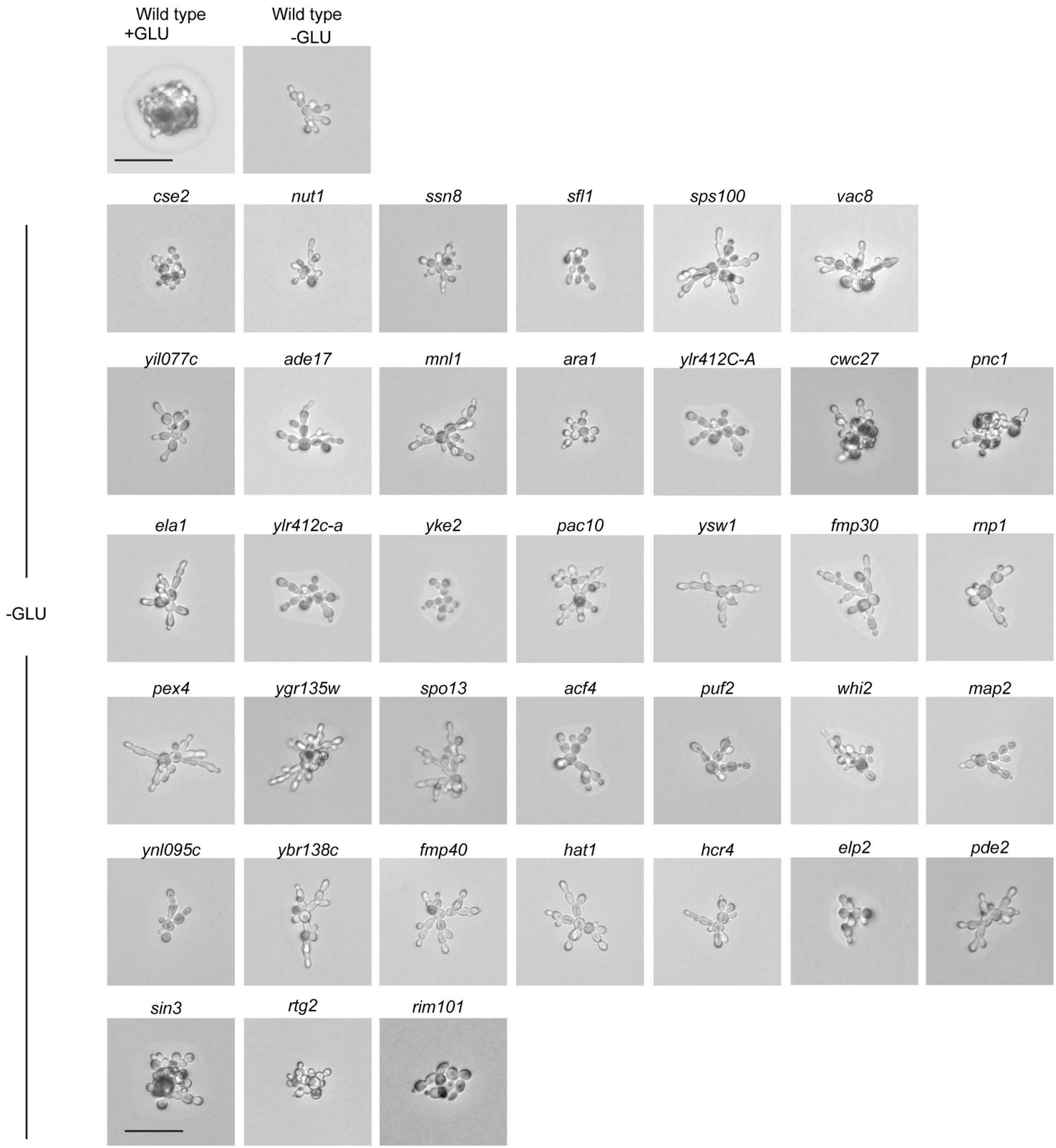


Figure S2. The role of filamentous growth MAPK pathway regulators in filament formation by the single cell invasive growth assay. Wild-type strain and the indicated mutants were grown on S-GLU medium for 16 hr and photographed at 100X. Bar, 20 microns.

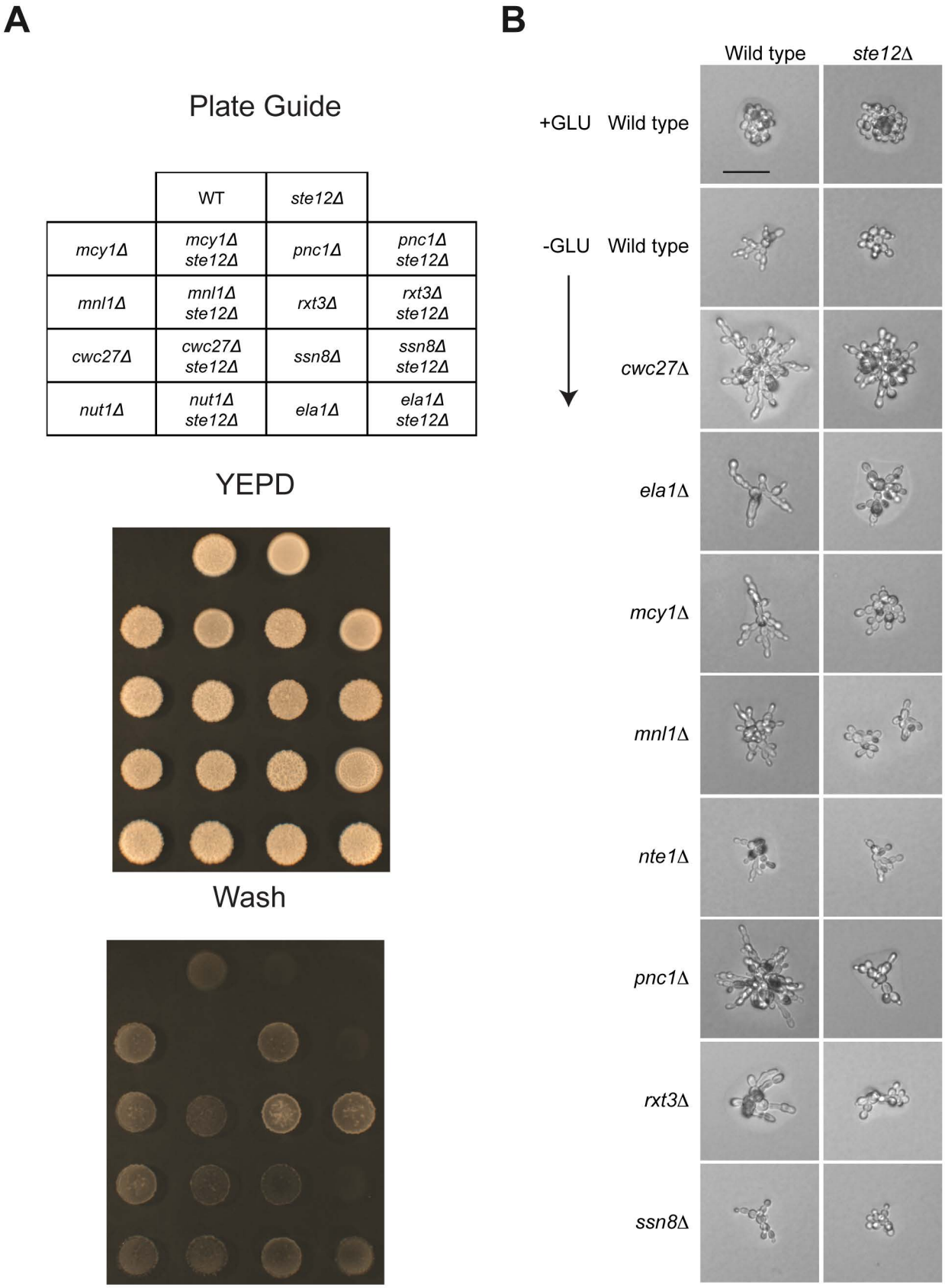


Fig. S3. Suppression of hyper-invasive growth phenotypes of mutants identified in the screen by deletion of *STE12*. A) Wild-type and *ste12*Δ mutant combinations as indicated were examined by the plate-washing assay, or in **B)** by the single cell assay. Bar, 30 microns. The *mcy1* mutant may contain a second mutation based on retesting.

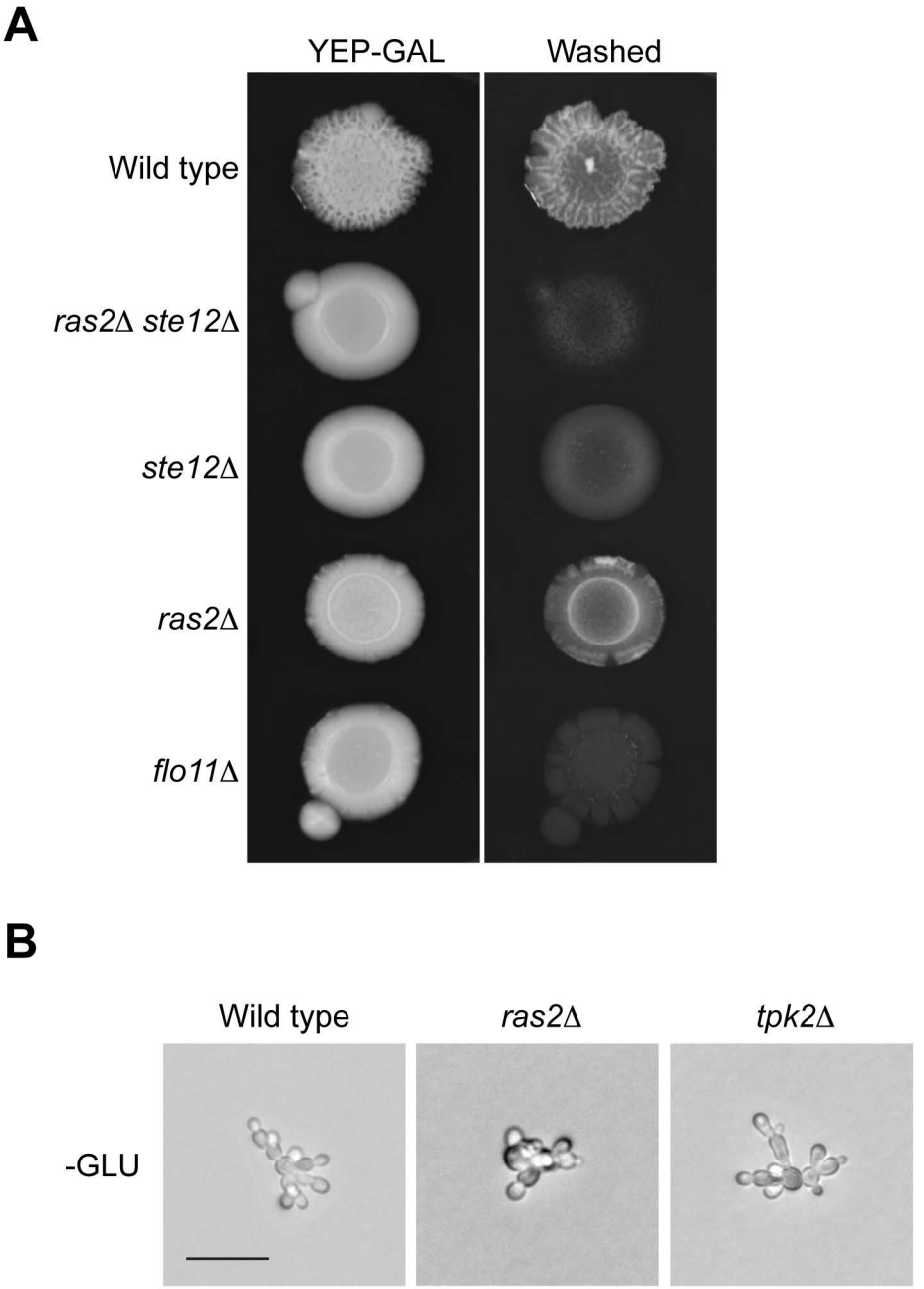


Figure S4. Role of Ras2p and Tpk2p in conditional regulation of the filamentous growth MAPK pathway. A) The plate washing assay on YEP-GAL medium of wild-type cells, and the *ste12*Δ, *ras2*Δ, and *ste12*Δ *ras2*Δ double mutants. **B)** Single cell assay of the *ras2*Δ and *tpk2*Δ mutant. Bar, 20 microns.

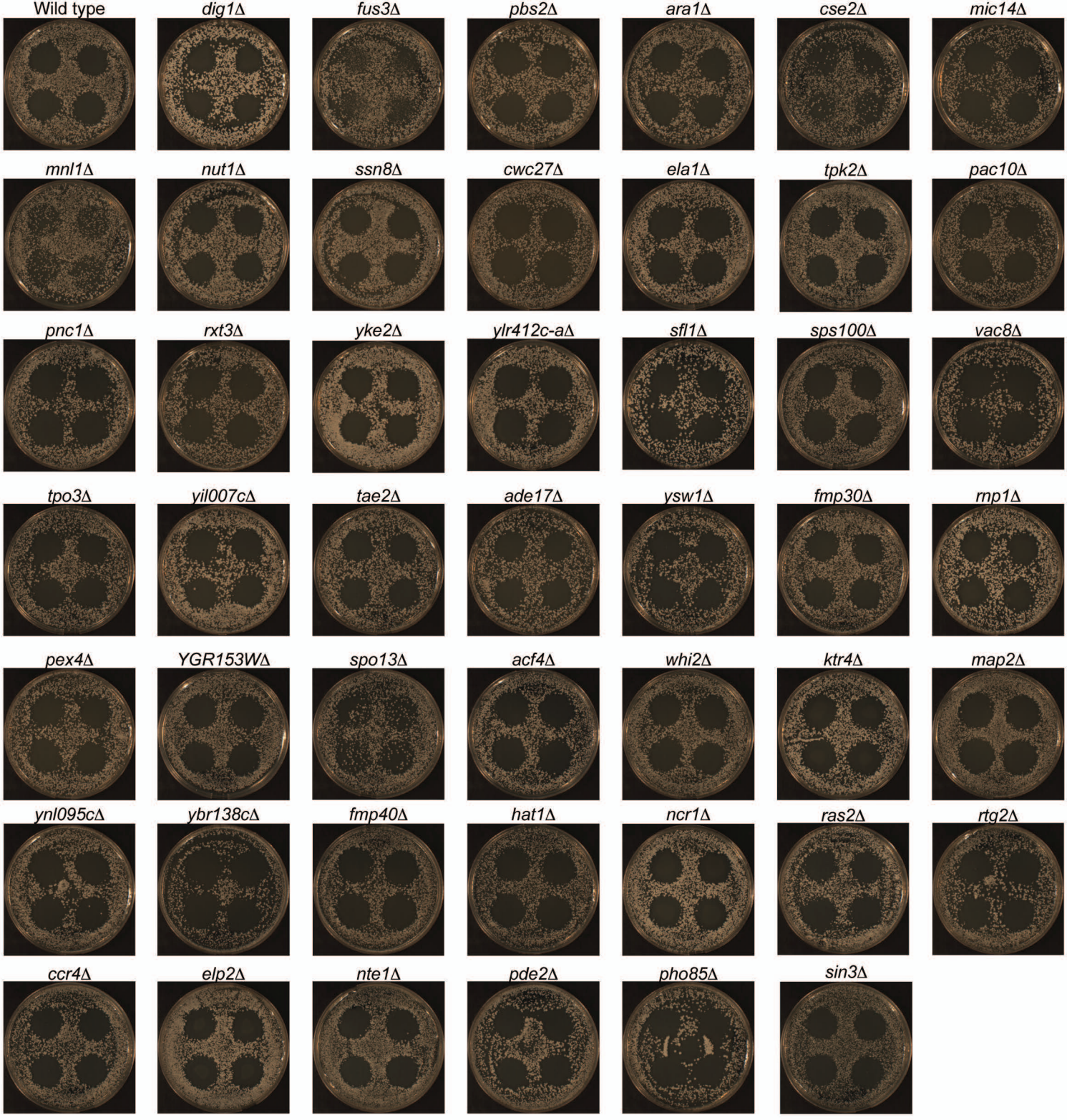


Figure S5. Specificity of filamentous growth MAPK pathway regulators in the mating pathway. Wild type and mutants were grown to saturation, diluted and top spread onto YEPD plates and 10 µl of 1µg/µl α-factor was added to four separate locations on the plate. Changes in halo size reflect altered mating pathway activity.

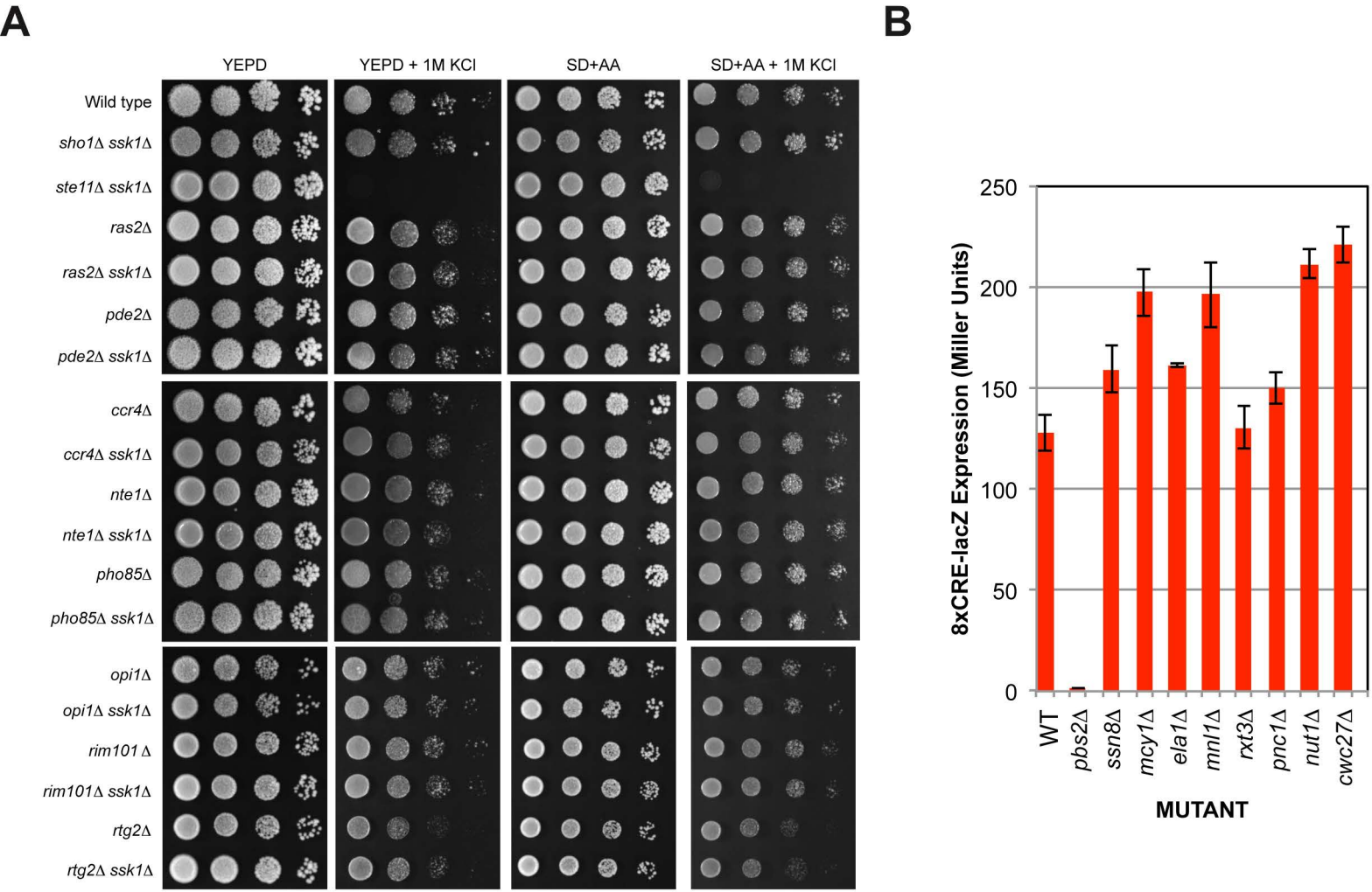
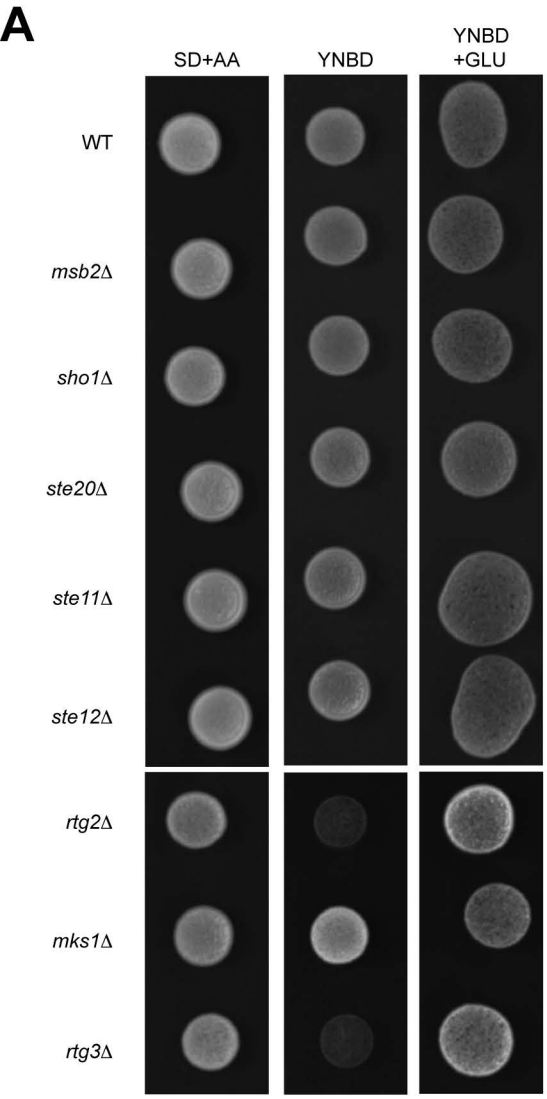


Figure S6. Specificity of filamentous growth MAPK pathway regulators in the HOG pathway. A) Wild type and indicated mutants were spotted on YEPD, YEPD + 1M KCl, SD + AA, and SD + AA + 1M KCl and grown for 2d. **B)** Level of p*8X-CRE-lacZ* activity in selected mutants that show filamentous growth MAPK pathway hyper-activation. Strains were grown in YEPD for 6 h then shifted to YEPD + .4M KCl for 30 min.



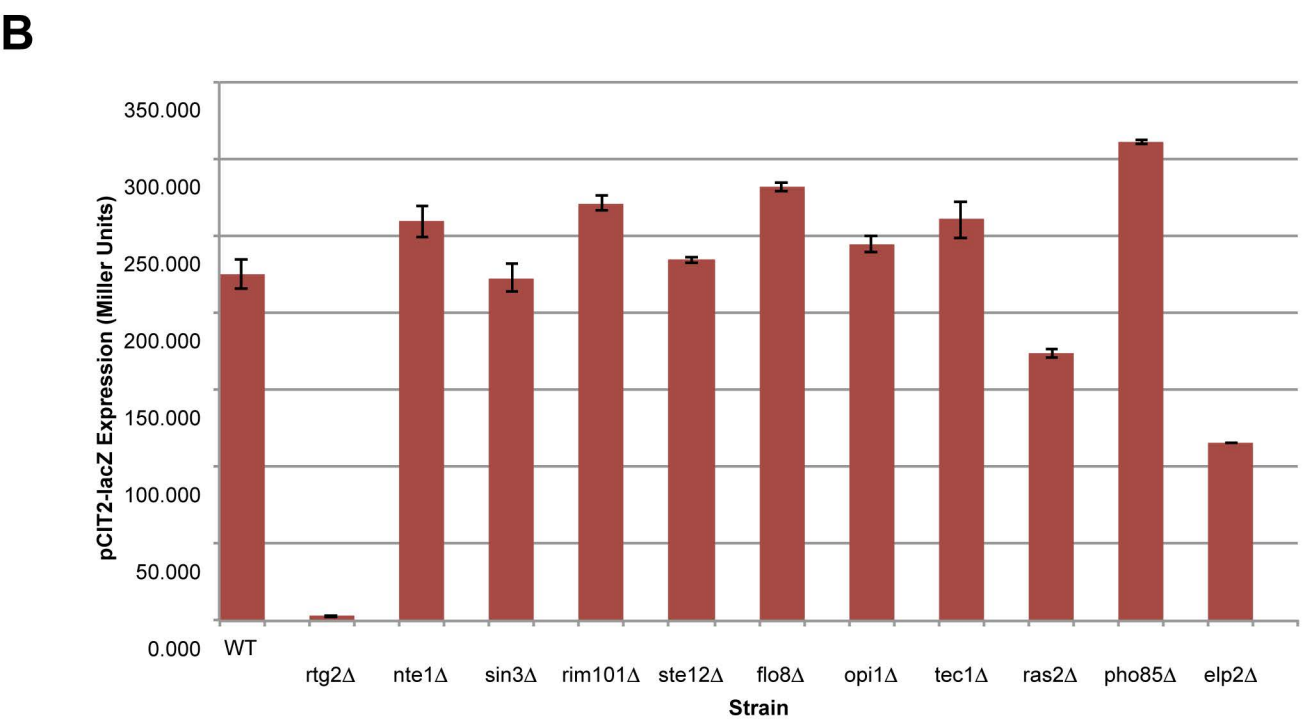


Figure S7. The filamentous growth MAPK pathway does not regulate the RTG response. A) Strains were spotted onto plates containing 6.7% Yeast Nitrogen Base without amino acids and supplemented with uracil with or without glutamate. Glutamate auxotrophy reflects a defective RTG pathway. **B)** *CIT2-lacZ* analysis of selected mutants. ß-galactosidase assays were performed in duplicate; error bars represent standard deviation between samples.

Files S1-S2

Available for download as .mov files at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.168252/-/DC1

File S1 Serial Z-stack images of rhodamine phalloidin stained wild-type cells grown for 16h in S-GLU.

File S2 Serial Z-stack images of rhodamine phalloidin stained *MSB2 cells grown for 16h in S-GLU.**

Tables S1-S3

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Table S1 Analysis of invasive growth mutants for a role in filamentous growth MAPK pathway regulation. References for previously identified regulators of filamentous growth not identified in the text are as follows: (Liu *et al.* 1993; Gimeno and Fink 1994; Stevenson *et al.* 1995; Ward *et al.* 1995; Gavrias *et al.* 1996; Lorenz and Heitman 1997; Mosch and Fink 1997; Tedford *et al.* 1997; Ramezani Rad *et al.* 1998; Entian *et al.* 1999; Gagiano *et al.* 1999; Johnson 1999; Kobayashi *et al.* 1999; Conte and Curcio 2000; Pan and Heitman 2000; Harashima and Heitman 2002; Kohler *et al.* 2002; Laprade *et al.* 2002; Smith *et al.* 2002; Breitkreutz *et al.* 2003; Bao *et al.* 2004; Wu and Jiang 2005; Bester *et al.* 2006; Bhattacharyya *et al.* 2006; Ishigami *et al.* 2006; Frydlova *et al.* 2007; Tiedje *et al.* 2007; Valerius *et al.* 2007; Fidalgo *et al.* 2008; Kim and Siede 2011; Laxman and Tu 2011; Lo *et al.* 2012; Vandenbosch *et al.* 2013).

Table S2 Analysis of invasive growth and colony morphology.

Table S3A Activity of the *FRE-lacZ* reporter in mutants that show hyper-filamentous growth. (See sheet 2 for *FRE-lacZ* analysis of hypo- filamentous growth mutants).

Table S3B Activity of the *FRE-lacZ* reporter in mutants that show hypo-invasvie growth. (See sheet 1 for *FRE-lacZ* analysis of hyper-filamentous growth mutants).

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