

# Filamentation Regulatory Pathways Control Adhesion-Dependent Surface Responses in Yeast

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**ABSTRACT** Signaling pathways can regulate biological responses by the transcriptional regulation of target genes. In yeast, multiple signaling pathways control filamentous growth, a morphogenetic response that occurs in many species including fungal pathogens. Here, we examine the role of signaling pathways that control filamentous growth in regulating adhesion-dependent surface responses, including mat formation and colony patterning. Expression profiling and mutant phenotype analysis showed that the major pathways that regulate filamentous growth [fungal MAPK (fMAPK), RAS, retrograde (RTG), RIM101, RPD3, ELP, SNF1, and PHO85] also regulated mat formation and colony patterning. The chromatin remodeling complex, SAGA, also regulated these responses. We also show that the RAS and RTG pathways coregulated a common set of target genes, and that SAGA regulated target genes known to be controlled by the fMAPK, RAS, and RTG pathways. Analysis of surface growth-specific targets identified genes that respond to low oxygen, high temperature, and desiccation stresses. We also explore the question of why cells make adhesive contacts in colonies. Cell adhesion contacts mediated by the coregulated target, and adhesion molecule, Flo11p, deterred entry into colonies by macroscopic predators and impacted colony temperature regulation. The identification of new regulators (e.g., SAGA), and targets of surface growth, in yeast may provide insights into fungal pathogenesis in settings where surface growth and adhesion contributes to virulence.

**KEYWORDS** MAPK pathways; signaling networks; expression profiling; fungal pathogenesis; microbial predator–prey relationships; pseudohyphal growth; invasive growth; biofilm; mat; complex colony; temperature control

**F**UNGAL microorganisms exhibit a range of nutrient-related responses. Under certain conditions, fungal cells can differentiate into filamentous or hyphal cells that can expand across, and/or penetrate into, new environments (Soll and Daniels 2016). Many fungal species can also grow in communities of biofilms or mats, which are composed of interconnected cells that attach to each other and to surfaces. One property of mats is the formation of highly organized patterns that result from adhesive contacts between cells. In patho-

gens, filamentous growth (Lo *et al.* 1997) and biofilm formation (Desai *et al.* 2014) are critical determinants of virulence. For example, cells can adhere to medical devices and grow in dense mats that are resistant to antifungal medicines (Chandra *et al.* 2001; Sudbery *et al.* 2004; Kumamoto 2005; Ramage *et al.* 2005; Nett and Andes 2015).

The budding yeast *Saccharomyces cerevisiae* is a unicellular fungal microbe, and a convenient model for studying nutrient-regulated foraging responses like filamentous growth and mat formation. These responses are best studied in “wild” strain backgrounds (such as  $\Sigma$ 1278b) as the responses have been lost in certain laboratory strains due to genetic manipulation (Liu *et al.* 1996; Dowell *et al.* 2010; Chin *et al.* 2012). During filamentous growth, yeast cells differentiate into elongated and polarized filaments that remain connected in pseudohyphae (Gimeno *et al.* 1992; Cullen and Sprague 2012).

At least 600 genes have been identified by genetic screens (Lorenz and Heitman 1998; Palecek *et al.* 2000) and genome-wide

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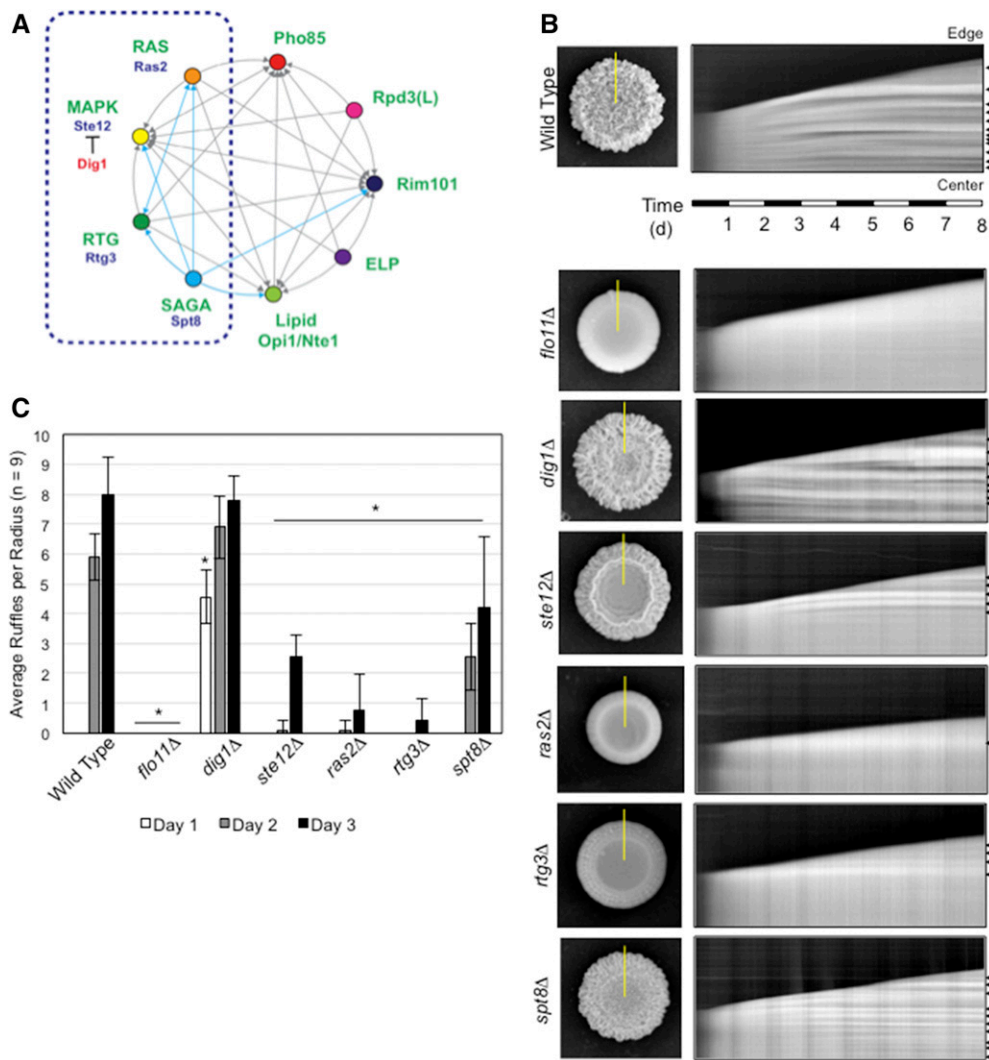
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**Figure 1** Functionally interconnected signaling pathways regulate mat expansion and colony pattern formation over time. (A) Circles represent signaling pathways or regulatory complexes that control filamentous growth. Arrows refer to target genes coregulated by pathways, as adapted from Chavel *et al.* (2014). Cyan arrows refer to functional connections identified in this study. The dashed blue box marks the part of the network on which the study is focused. (B) Time-course experiment of colony pattern formation in wild-type cells and the indicated mutants. Colony expansion was examined over an 8-day time period (Videos S1–S7). In the left panels, colonies at the 8-day time period are shown. On the right are kymographs that show colony expansion over time. The yellow lines in the left panels refer to the region selected for kymograph analysis. The panels on the right show colony patterns that developed over time in the region selected by the yellow line, where the bottom of the figure represents the colony center and the top represents the colony perimeter (edge). Arrows mark features that are characteristic of ruffle formation, which is quantitated in (C). (C) Bar graph showing the average number of ruffles in wild-type and the indicated mutant colonies at  $t = 1$  day (white), 2 days (gray), and 3 days (black). Error bars represent SD among nine radii from three biological replicates. \*  $P < 0.05$  between the mutant and wild-type for the same time point.

studies (Jin *et al.* 2008; Xu *et al.* 2010; Ryan *et al.* 2012) that play some role in filamentous growth. A subset of these genes encode signaling pathway components that include at least four major nutrient-sensing pathways [fungal MAPK (fMAPK), RAS, TOR (target of rapamycin), and SNF1], as well as pathways that regulate the response to pH (RIM101), phosphate utilization (PHO85), and mitochondrial stress [the retrograde mitochondria-to-nucleus (RTG) pathway]. In addition, proteins that control the epigenetic modification of histones to alter gene expression have also been implicated in the regulation of filamentous growth (Rpd3p).

Many of the pathways that regulate filamentous growth are functionally connected through their ability to coregulate common target genes (Figure 1A). In some cases, this occurs at the level of transcription. In a pioneering study, it was shown that many of the transcription factors that control filamentous growth control each other's expression (Borneman *et al.* 2006). Transcription factors can also converge at common promoter elements. One example is the

gene encoding the major cell adhesion molecule in yeast, Flo11p (Kraushaar *et al.* 2015; Chan *et al.* 2016). The *FLO11* gene contains one of the largest and most highly regulated promoters in the yeast genome, and functions as a “hub” where multiple transcription factors and chromatin remodeling enzymes bind (Robertson and Fink 1998; Rupp *et al.* 1999; Palecek *et al.* 2000; Pan and Heitman 2000; Kuchin *et al.* 2002; van Dyk *et al.* 2005; Barrales *et al.* 2008). Signaling pathways that control filamentous growth can also regulate each other's activity. The classic example comes from the discovery that the RAS pathway can regulate the activity of the fMAPK pathway (Mösch *et al.* 1996). It is now clear that many pathways regulate the activity of the fMAPK pathway (Chavel *et al.* 2010, 2014). One way this may occur is through the protein kinases of the major regulatory pathways, which can regulate each other's localization and activity (Bharucha *et al.* 2008).

187 Yeast can also undergo mat formation (Reynolds and Fink  
188 2001; Bojsen *et al.* 2012), where colonies expand radially  
189 across surfaces and form ruffled patterns (Granek and Mag-  
190 wene 2010; Granek *et al.* 2011; Tam *et al.* 2018). Mats form  
191 wheel-spoke patterns in low-percentage agar media [0.3%  
192 agar (Reynolds and Fink 2001)], and wrinkled or ruffled  
193 colonies on high-percentage agar media [1–4% (Granek  
194 and Magwene 2010; Karunanithi *et al.* 2012)]. Mat expan-  
195 sion and patterning require contacts between cells that are  
196 also mediated by Flo11p. Regulators of mat formation have  
197 been identified by direct approaches (Reynolds 2006; Sarode  
198 *et al.* 2011; Váchová *et al.* 2011), genome-wide deletion  
199 screens (Ryan *et al.* 2012; Voordeckers *et al.* 2012; Scherz  
200 *et al.* 2014), overexpression screens (Cromie *et al.* 2017), and  
201 comparative expression profiling (Traven *et al.* 2012;  
202 Maršíková *et al.* 2017). Many of the molecular pathways that  
203 control mat formation regulate FLO11 expression. In addi-  
204 tion, several regulators of mat formation, like the cell wall  
205 integrity sensor Wsc1p (Sarode *et al.* 2014) and vacuolar  
206 protein-sorting genes (Sarode *et al.* 2011), appear to regulate  
207 mat formation by mechanisms that do not involve Flo11p.  
208 Despite the identification of many proteins and pathways  
209 that regulate mat formation, the benefits that cells derive  
210 from forming these complex patterns remain unclear. Fur-  
211 thermore, the stresses encountered during colonial surface  
212 growth remain relatively unexplored.

213 As seen in other fungi, filamentous growth and mat for-  
214 mation are related responses in yeast. Both responses require  
215 Flo11p (Lo and Dranginis 1998; Reynolds and Fink 2001),  
216 and a corresponding set of regulatory proteins and pathways  
217 that control FLO11 expression (Ryan *et al.* 2012). These in-  
218 clude the TOR (Cutler *et al.* 2001; Bojsen *et al.* 2016),  
219 Rim101 (Li and Mitchell 1997; Sarode *et al.* 2011;  
220 Voordeckers *et al.* 2012), fMAPK (Roberts and Fink 1994;  
221 Reynolds and Fink 2001; Granek and Magwene 2010), and  
222 RAS pathways (Gimeno *et al.* 1992; Granek and Magwene  
223 2010; Zara *et al.* 2011; Ryan *et al.* 2012; Bojsen *et al.* 2016).  
224 The tRNA modification complex elongator (ELP) regulates all  
225 three responses (Abdullah and Cullen 2009). It has also been  
226 shown that under nutrient-limiting conditions, mats are com-  
227 posed of filamentous cells (Karunanithi *et al.* 2012). Given  
228 the abovementioned connections between filamentous  
229 growth and mat formation, we sought to further define  
230 how the network of signaling pathways that control filamen-  
231 tous growth might regulate adhesion-dependent surface  
232 growth.

233 To learn more about the regulation of surface growth in  
234 yeast, adhesion-dependent surface responses were examined  
235 from several perspectives. In one set of experiments, we de-  
236 veloped a method for recording colony pattern formation over  
237 time by kymograph analysis, which allowed us to confirm that  
238 the major signaling pathways that control filamentous growth  
239 also regulate mat formation. A new regulator of filamentous  
240 growth, the chromatin remodeling complex SAGA, was also  
241 uncovered. Comparative expression profiling identified a  
242 large number of target genes as well as new regulatory

connections between pathways. By seeking to understand  
why cells form adhesive connections during surface growth,  
we identified a role for Flo11p in protection from macroscopic  
predators. We also showed that colony ruffling aids in tem-  
perature regulation. Our findings broaden the role of fila-  
mentation regulatory pathways to include the regulation of  
adhesion-dependent surface responses. Our findings may be  
relevant to studies of fungal pathogens that must tolerate  
growth on surfaces to become virulent.

## Materials and Methods

### Media and growth conditions

Yeast strains were grown and manipulated by standard meth-  
ods (Sambrook *et al.* 1989; Rose *et al.* 1990). For most ex-  
periments, colonies were grown on standard YPD (2%) or  
YEP-galactose (Gal) semisolid agar media (Cullen 2015a).  
Some experiments were performed on low-agar (0.3%) me-  
dia. Glutamate sensitivity experiments used synthetic agar  
(dextrose, 2%; yeast nitrogen base, 6.7 g/liter; uracil,  
20 mg/liter; His, 20 mg/liter; Leu, 120 mg/liter; adenine,  
20 mg/liter; Lys, 60 mg/liter; Arg, 20 mg/liter; Trp,  
20 mg/liter; Tyr, 30 mg/liter; Thr, 200 mg/liter; Met,  
20 mg/liter; and Phe, 50 mg/liter), minimal agar (dextrose,  
2%; yeast nitrogen base, 6.7 g/liter; and uracil, 20 mg/liter),  
and minimal + Glu (dextrose, 2%; yeast nitrogen base, 6.7 g/  
liter; uracil, 20 mg/liter; and Glu 20 mg/liter) media. To  
generate low-oxygen (5–15% oxygen) and anaerobic condi-  
tions, agar plates were incubated in GasPAK EZ Campy Pouch  
System (BD 260685; Becton, Dickinson and Company, Frank-  
lin Lakes, NJ) or BD GasPAK EZ Anaerobe Pouch System (BD  
260683; Becton, Dickinson and Company) bags. To maintain  
consistent moisture levels, plates were poured and left  
unwrapped for 3 days at 22° to allow the evaporation of  
excess moisture. To generate media with reduced moisture  
levels, YPD plates were left unwrapped for 10 days at 22°.

### Strains and genetic manipulations

Yeast strains are listed in Table 1. Gene deletions were con-  
structed using auxotrophic markers amplified by PCR and  
introduced into yeast by lithium acetate transformation by  
standard methods as described (Gietz and Schiest 2007). To  
generate the *spt8Δ* mutant, homologous recombination at  
the SPT8 locus was performed using the pKIURA3  
(PC5225) cassette as a template. For some experiments,  
yeast strains were used from an ordered knockout collection  
(Ryan *et al.* 2012). *Caenorhabditis elegans* strains used in this  
study include N2 Bristol wild-type and KP4 *glr-1(n2461)*  
(Kaplan and Horvitz 1993). Strains were maintained at 20°  
under standard conditions on nematode growth media  
(NGM) agar plates seeded with OP50 *Escherichia coli* bacteria  
(Brenner 1974).

**Table 1 Yeast strains used in the study**

Strain <sup>a</sup>	Description	Reference
PJ69-4A <sup>b</sup>	<i>MATa trp1-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2::GALI-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	James et al. (1996)
PC313	<i>MATa ura3-52</i>	Liu et al. (1993)
PC538	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3</i>	Cullen et al. (2004)
PC549	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 ste20::URA3</i>	Cullen and Sprague (2000)
PC555	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 hsl7::URA3</i>	Cullen and Sprague (2000)
PC562	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 ras2::URA3</i>	Cullen and Sprague (2002)
PC611	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 ste11::URA3</i>	Cullen and Sprague (2002)
PC999	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 MSB2-HA</i>	Chavel et al. (2010)
PC1029	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 flo11::KanMX6</i>	Karunanithi et al. (2010)
PC1079	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 ste12::URA3</i>	Karunanithi et al. (2010)
PC2523	<i>MATa ura3-52 ste4 FUS1-lacZ -::NAT FUS1-HIS3 flo8::HYG</i>	Chavel et al. (2010)
PC2712	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 GAL-FLO11::KanMX6</i>	Karunanithi et al. (2010)
PC3030	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 MSB2-HA sin3::NAT</i>	Chavel et al. (2010)
PC3039	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 MSB2-HA dig1::NAT</i>	Chavel et al. (2010)
PC3431	<i>MATa ura3-52 ste4 FUS1-lacZ -::NAT FUS1-HIS3 MSB2-HA sfl1::KIURA3</i>	Chavel et al. (2010)
PC3642	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg3::NAT</i>	Chavel et al. (2010)
PC3652	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg2::NAT</i>	Chavel et al. (2010)
PC3695	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg1::NAT</i>	Chavel et al. (2014)
PC4005	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 gcn5::KIURA3</i>	This study
PC3688	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 opi1::NAT</i>	Chavel et al. (2010)
PC4008	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 spt8::KIURA3</i>	This study
PC5090	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 nte1::NAT</i>	Chavel et al. (2014)
PC5119	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 pho80::NAT</i>	This study
PC6733	<i>MATa ura3-52 ste4 FUS1-lacZ FUS1-HIS3 ura3-52::pTEF2-mCherry URA3 his5::NAT mCherry::GFPy-HIS</i>	Chow et al. (2019)
PC6016	<i>MATa can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0</i>	Ryan et al. (2012)
CB13A9 <sup>c</sup>	<i>MATa can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 dan1Δ</i>	Ryan et al. (2012)
CB52G2	<i>MATa can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 sip18Δ</i>	Ryan et al. (2012)
CB53F12	<i>MATa can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 sno4Δ</i>	Ryan et al. (2012)
CB60E1	<i>MATa can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 gre1Δ</i>	Ryan et al. (2012)
CB29E8	<i>MATa can1Δ::Ste2pr-spHIS5 lyp1Δ::Ste3pr-LEU2 his3::hisG leu2Δ0 ura3Δ0 hsp26Δ</i>	Ryan et al. (2012)

<sup>a</sup> Unless indicated, strains are derived from the Σ1278b strain background.

<sup>b</sup> Strain from M. Johnston's laboratory.

<sup>c</sup> Strains from an ordered deletion collection labeled with "CB" followed by the plate number and location.

### Assays for mat formation and filamentous growth

Assays for mat formation were performed as described (Reynolds and Fink 2001; Karunanithi et al. 2012). The plate-washing assay was performed as described (Cullen 2015b).

### Microscopy

Mats were examined by bright-field microscopy using an Axioplan 2 fluorescent microscope (Zeiss [Carl Zeiss], Thornwood, NY) with 5×, 10×, 20×, 40×, and 100× PLAN-APOCHROMAT 100×/1.4 (oil) (N.A. 0.17) objectives. Digital images were obtained with an Axiocam MRm camera (Zeiss). Axiovision 4.4 software (Zeiss) was used for image acquisition. Digital images were imported into ImageJ (<https://imagej.nih.gov/ij/>) in 8-bit format.

### Comparative RNA sequencing analysis

To compare the transcriptional response of wild-type cells (PC538) and the *ste12Δ* (PC1079), *dig1Δ* (PC3039), *rtg3Δ* (PC3642), *spt8Δ* (PC4008), and *ras2Δ* (PC562) mutants, cells were concentrated (OD<sub>A600</sub> = 20) and spotted in 10-μl aliquots onto YEP-Gal (2% agar) for 24 hr. Cells were spotted as six colonies per plate, equidistant to each other and the plate center. All six colonies were harvested for each trial and three separate trials were compared for each strain. The entire colony surface was scraped into 500 μl of distilled water, harvested by centrifugation, washed, and stored at -80°. RNA was harvested by hot-acid phenol-chloroform extraction as described (Adhikari and Cullen 2014). Samples were further purified using a QIAGEN RNeasy Mini Kit (catalog number 74104; QIAGEN, Valencia, CA). RNA concentration and purity was measured via NanoDrop (NanoDrop

2000C; Thermo Fisher Scientific, Waltham, MA). RNA stability was determined by running the sample on an agarose gel.

RNA sequencing (RNA-seq) was performed as described previously (Adhikari and Cullen 2014) by sequencing RNA prepared from three separate cultures. RNA-seq libraries were prepared from total RNA using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA). Library size distributions were validated using an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA). Additional library quality control, blending of pooled indexed libraries, and cluster optimization was performed using Invitrogen's Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). RNA-seq libraries were pooled (21-plex) and clustered onto a flow cell lane using an Illumina cBot. Sequencing was performed using an Illumina HiSeq 2500 in Rapid Mode employing a paired-end, 50-base read length (PE50) sequencing strategy.

Image analysis and base calling were performed using Illumina's Real Time Analysis v1.18 software, followed by "demultiplexing" of indexed reads and generation of FASTQ files, using Illumina's bcl2fastq Conversion Software v1.8.2 ([http://support.illumina.com/downloads/bcl2fastq\\_conversion\\_software\\_184.html](http://support.illumina.com/downloads/bcl2fastq_conversion_software_184.html)). For analysis of the RNA-seq data, reads of low quality were filtered out prior to alignment to the reference genome (*S. cerevisiae* assembly R64-1-1, Ensembl release 75) using TopHat v2.0.9 (Trapnell *et al.* 2009). Counts were generated from TopHat alignments for each gene using the Python package HTSeq v0.6.1 (Anders *et al.* 2015). Genes with low counts across all samples were removed.

For comparisons of mutant to wild-type colony samples, differentially expressed genes were identified using the Bioconductor package DESeq2 (Love *et al.* 2014) with the apeglm package to estimate t-prior shrinkage (Zhu *et al.* 2018). We employed the IHW package to weight hypotheses and optimize power (Ignatiadis *et al.* 2016). Differential expression was defined as  $|\log_2(\text{ratio})| \geq 0.585$  ( $\pm 1.5$  fold) with  $P < 0.01$ . Genes were categorized as "upregulated" ( $\log_2(\text{fold change}) > 0.585$ ,  $P\text{-value} < 0.01$ ), "down-regulated" ( $\log_2(\text{fold change}) < -0.585$ ,  $P\text{-value} < 0.01$ ), or "insignificant" ( $\log_2(\text{fold change}) < -0.585$  or  $\log_2(\text{fold change}) > 0.585$ ,  $P\text{-value} > 0.01$ ). Differentially expressed pathway-specific genes were visualized in principal component analysis (PCA), volcano plot, and Venn diagram figures using ggplot2. Pathway-specific gene expression was visualized using the Kyoto Encyclopedia of Genes and Genomes (Kanehisa and Goto 2000), and the Bioconductor package pathview (Luo and Brouwer 2013). Classification of targets was based on gene ontology (GO) terms and descriptions in the *Saccharomyces* Genome Database (<http://www.yeastgenome.org>). For all other comparisons, edgeR v3.18.1 (Robinson *et al.* 2010) was used. A false discovery rate (FDR) method was employed to correct for multiple testing (Reiner *et al.* 2003). Differential expression was defined as  $|\log_2(\text{ratio})| \geq 0.585$  ( $\pm 1.5$  fold) with  $P\text{-value} < 0.01$ .

## GO term analysis

GO term analysis (Ashburner *et al.* 2000) was performed using the GO enrichment analysis and visualization tool (GORilla) (Eden *et al.* 2007, 2009) using the two unranked lists mode. The Gorilla database had been last updated January 12, 2019. The background list was all of the ORFs identified during the analysis. The target list for genes regulated by all five regulators was identified as genes whose  $|\log_2\text{FC}| > 0.585$  and  $P\text{-value} < 0.01$  in all mutant sets. The target list for genes regulated by both RAS and RTG was identified as genes  $|\log_2\text{FC}| > 0.585$  and  $P\text{-value} < 0.01$  in only the *ras2* $\Delta$  and *rtg3* $\Delta$  sets. Enriched GO terms were identified by having  $P < 10^{-3}$  and  $\text{FDR} < 0.05$ . Values for background and target sets, and enriched GO terms, are in Supplemental Material, Table S2.

## Quantitative PCR analysis

Differential gene expression was confirmed by quantitative (q) real-time PCR analysis as described previously (Chavel *et al.* 2014). cDNA libraries from RNA samples were generated using iScript Reverse Transcriptase Supermix (catalog number 1708840; Bio-Rad, Hercules, CA). qPCR was performed using iTaq Universal SYBR Green Supermix (catalog number 1725120; Bio-Rad) on the Bio-Rad CFX384 Real-Time System. Primers were ordered from Sigma ([Sigma Chemical], St. Louis, MO) and are listed in Table S3. FCs in expression were determined by calculating  $\Delta\Delta\text{Ct}$  (Livak and Schmittgen 2001) using *ACT1* mRNA as the housekeeping gene for each sample. RNA was prepared from at least three samples and the average of at least three biological replicates was recorded. Statistical significance was determined by the Student's *t*-test.

## Time-lapse photography

Cells were grown on YEP-Gal semisolid agar media at 22° for 8 days. Photographs were taken using a Nikon D3000 (Nikon, Garden City, NY) digital camera at 30-min intervals using automatic exposure without flash. Graph paper (0.25 cm) was glued to the plate bottom for scaling and later image stabilization; these were cropped out in the final images. Images were imported into ImageJ as an image stack for image stabilization using Kang Li's image stabilizer plug-in ([http://www.cs.cmu.edu/~kangli/code/Image\\_Stabilizer.html](http://www.cs.cmu.edu/~kangli/code/Image_Stabilizer.html)). Stabilized image series were saved in video format. Kymographs were generated using the reslice tool in ImageJ (<https://imagej.nih.gov/ij/>).

Ruffles were identified as light bands flanked by dark bands. The number of ruffles per time point was counted manually at 1-day intervals for each kymograph. Statistical analysis was carried out for each time point using an unpaired Student's *t*-test.

## Evaluation of phosphorylated Kss1 levels

Samples were harvested at specific distances from the colony perimeter at  $t = 0$ . To determine P-Kss1p levels at the

growing edge of a colony, the outermost millimeters of representative colonies were harvested at 1, 2, 3, and 4 days. To determine the changes in P-Kss1p levels before and after the colony ruffles, samples were harvested from regions of the colony 2 mm from the starting colony edge from representative colonies before and after ruffling.

Samples were evaluated by SDS-PAGE analysis. Immunoblots were performed as described (Cullen 2015a). P-Kss1p was detected with phospho-p44/42 primary antibodies (catalog number 4370S; Cell Signaling Technology, Danvers, MA) and anti-rabbit HRP secondary antibodies (catalog number 111-035-144; Jackson ImmunoResearch, West Grove, PA). Total Kss1p was detected by anti-Kss1p antibodies (SC-6775-R; Santa Cruz Biotechnology, Dallas, TX) and with anti-rabbit HRP secondary antibodies (catalog number 111-035-144; Jackson ImmunoResearch). Loading control Pgk1p was identified by anti-Pgk1p primary antibodies (catalog number 459250; Invitrogen) and with anti-mouse HRP secondary antibodies (catalog number 1706516; Bio-Rad).

### **Effect of *S. cerevisiae* cell-cell adhesion on pharyngeal uptake by *C. elegans***

Wild-type yeast cells expressing GFP (PC6733) were grown in synthetic or YPD liquid media at 30° for 16 hr. Cells were washed twice in M9 buffer (KH<sub>2</sub>PO<sub>4</sub>, 3 g/liter; Na<sub>2</sub>HPO<sub>4</sub>, 6 g/liter; NaCl, 5 g/liter; and 1 mM MgSO<sub>4</sub>). As previously described (Bois *et al.* 2013), adult *C. elegans* were transferred into suspensions of *S. cerevisiae*. After 45 min, *C. elegans* were removed and mounted onto 2% agarose pads, and immobilized with 10 mM sodium azide. Slides were examined with an Axioplan 2 fluorescent microscope (Zeiss) at 40×. Individual *S. cerevisiae* cells were counted. Independent replicates were performed on three separate days.

### **Experiments involving yeast colonies exposed to entry by *C. elegans***

Yeast cells were grown in YPD liquid media at 30° for 16 hr. For interactions between *C. elegans* and *S. cerevisiae* colonies, 200-μl aliquots of cells were dispensed onto NGM (Wood 1988) agar media and grown as colonies at 22° for 72 hr. Under this condition, yeast colonies formed Flo11p-dependent patterns. OP50 *E. coli* (Brenner 1974) were taken from stock cultures, and 200 μl aliquots were dispensed onto NGM agar media and grown at 22° for 3 days.

To examine colony penetration, adult and fourth-stage larva (L4) *C. elegans* were transferred from stock OP50 plates to experimental plates around the yeast or *E. coli*. Mat penetration times were determined by measuring the time from a worm nose first contacting the colony to the tail fully entering the mat, up to 100 sec. The number of stalls and reversals was determined by counting the number of incidents when a worm's tail would stop forward movement or reverse. The percent of time moving forward was determined by recording the amount of time that a worm was moving forward divided by the total time required to penetrate a mat. Independent replicates were performed on at least three separate days.

To examine the pharyngeal uptake of *S. cerevisiae* by *C. elegans*, wild-type (PC538) and *flo11Δ* (PC1029) yeast were transformed with a plasmid containing GFP-2xPH (PC2560) [CS189 (Stefan *et al.* 2005) provided by the Emr laboratory (Cornell University)]. Cells were grown in YPD liquid media at 30° for 16 hr and 200-μl aliquots were spotted onto NGM agar media. Colonies were grown at 22° for 3 days. Worms were transferred directly into colonies and left for 45 min before mounting and imaging. Independent replicates were performed on three separate days.

Video was captured using an AmScope MD35 camera and AmScope image capture software. The camera was inserted into the eyepiece of a Zeiss SteREO Discovery V8. Dot plots for worm entry times were generated in ggplot2 (Wickham 2016). Statistical analysis was performed using the Student's *t*-test.

### **Infrared imaging**

Infrared images were taken using FLIR A325sc (FLIR Systems, Wilsonville, OR) and captured using FLIR ResearchIRMax4 (FLIR Systems), provided by the Sustainable Manufacturing And Robotic Technology center at the University at Buffalo. Average and coolest temperatures for each colony were measured using FLIR ResearchIRMax4 (FLIR Systems).

An insulated housing unit was built to mount the thermal imaging camera above a stage for agar plates. Yeast cells were grown in YPD liquid media at 30° for 16 hr. Next, 10-μl aliquots were spotted onto YEP-Gal agar media. Colonies were grown for 72 hr at 30°. For imaging, plates were transferred from the incubator to the mount in a photographing area with an ambient temperature of 22°. Plate lids were removed to allow imaging. Images were taken immediately after removing the lid.

### **Data availability**

All strains are available upon request. The Gene Expression Omnibus (GEO) accession number for raw sequencing data is GSE115657. A comparison with previously published expression profiling data sets for cells grown in liquid culture has been described [GEO accession number GSE61783 (Adhikari and Cullen 2014)]. Supplemental material available at FigShare: <https://doi.org/10.25386/genetics.8066615>.

## **Results**

### **A signaling network regulates adhesion-dependent surface responses in yeast**

Multiple signal transduction pathways regulate filamentous growth (Figure 1A) (Chavel *et al.* 2010, 2014). A large number of genes that control invasive growth, pseudohyphal formation, and mat formation show significant overlap based on a genome-wide analysis using deletion mutants in the filamentous strain background (Ryan *et al.* 2012). Thus, we examined and compared how the signaling network that regulates filamentous growth impacts adhesion-dependent

635 surface responses in yeast. We sought to directly compare key  
636 pathways in the network (MAPK and RAS) to less well-char-  
637 acterized pathways (RTG) and to a newly identified chroma-  
638 tin remodeling complex (SAGA). All of the pathways were  
639 studied in reference to a key target adhesion molecule,  
640 Flo11p.


641 Some strains (e.g.,  $\Sigma$ 1278b) exhibit ruffled colony mor-  
642 phology when grown on surfaces. This phenotype is depen-  
643 dent on cell adhesion contacts mediated by Flo11p (Reynolds  
644 and Fink 2001; Karunanithi *et al.* 2012). To evaluate colony  
645 pattern formation, we performed a time-course experiment  
646 followed by kymograph analysis, which can reveal changes in  
647 local features over time (Kaksonen *et al.* 2003). Wild-type  
648 cells were spotted on 2% agar media (YEP-Gal), and ruffle  
649 formation was examined by photographing colonies at  
650 30-min intervals over an 8-day period (Video S1). In a series  
651 of experiments, kymographs were generated along multiple  
652 radii of expanding colonies to measure the extent of ruffle  
653 formation [Figure 1B, wild-type (PC538), yellow line corre-  
654 sponds to one kymograph at right]. Ruffles were identified as  
655 light horizontal bands flanked by dark bands and could be  
656 counted manually by this method (Figure 1B, wild-type,  
657 black arrows). Measuring the banding pattern from separate  
658 sections of the colony allowed us to assess the extent of ruffle  
659 formation over time (Figure 1C, wild-type). As has been pre-  
660 viously demonstrated (Granek and Magwene 2010), colonies  
661 lacking the adhesion molecule Flo11p did not form ruffles  
662 (Video S2), which was also evident by kymograph analysis  
663 [Figure 1, B and C and Figure S1, *flo11 $\Delta$*  (PC1029)]. There-  
664 fore, kymograph analysis permitted the numerical assess-  
665 ment of pattern formation in yeast colonies. In principle,  
666 kymograph analysis may allow quantification of colony pat-  
667 tern formation in other microbial systems.

668 Kymograph analysis was next applied to mutants lacking  
669 key components of the major signaling pathways that regulate  
670 filamentous growth. As expected (Roberts and Fink 1994;  
671 Rupp *et al.* 1999; Reynolds and Fink 2001; Granek and Mag-  
672 wene 2010), the fMAPK pathway was required for colony  
673 ruffling. Specifically, kymograph analysis showed that col-  
674 onies lacking a negative regulator of the fMAPK pathway,  
675 Dig1p (Cook *et al.* 1996; Tedford *et al.* 1997; Bardwell  
676 *et al.* 1998; Olson *et al.* 2000; Breikreutz *et al.* 2003;  
677 Kusari *et al.* 2004; Chou *et al.* 2006; van der Felden *et al.*  
678 2014), formed more ruffles than wild-type after 1 day of  
679 growth [Figure 1, B and C, *dig1 $\Delta$*  (PC3039), Figure S1, and  
680 Video S3], though this difference was less apparent over  
681 longer time periods. Conversely, loss of the fMAPK transcrip-  
682 tion factor, Ste12p, led to a defect in colony ruffling at the  
683 examined time points [Figure 1, B and C, *ste12 $\Delta$*  (PC1079),  
684 Figure S1, and Video S4]. The fMAPK pathway was also re-  
685 quired for mat formation on 0.3% agar (Figure S1; *ste12 $\Delta$*   
686 and *dig1 $\Delta$* ; Figure S2 provides examples of other fMAPK reg-  
687 ulators; *ste11 $\Delta$*  and *ste20 $\Delta$* ). Also as expected (Gimeno *et al.*  
688 1992; Rupp *et al.* 1999; Granek and Magwene 2010; Zara  
689 *et al.* 2011; Ryan *et al.* 2012), the RAS pathway was required  
690 for colony ruffling [Figure 1, B and C, *ras2 $\Delta$*  (PC562) and

691 Video S5] and mat formation (Figures S1 and S2). Thus,  
692 kymograph analysis can be used to evaluate the roles of sig-  
693 naling pathways in regulating aspects of mat and colony pat-  
694 tern formation.

695 We next tested whether other pathways that are known to  
696 regulate filamentous growth also regulated adhesion-depen-  
697 dent colonial responses. The mitochondria-to-nucleus RTG  
698 pathway regulates invasive growth (Chavel *et al.* 2010;  
699 González *et al.* 2017). We also found that the RTG pathway  
700 was also required for colony ruffling [Figure 1, B and C, *rtg3 $\Delta$*   
701 (PC3642), Figure S3, and Video S6] and mat formation on  
702 0.3% agar (Figures S1 and S2).

703 We next tested the role of other proteins that control  
704 filamentous growth. Pho85p regulates filamentous growth  
705 (Chavel *et al.* 2010) and was also required for mat formation  
706 (Figure S1 and S2). Rpd3p (Chavel *et al.* 2010; Voordeckers  
707 *et al.* 2012), lipid regulators Opi1p and Nte1p (Chavel *et al.*  
708 2014), and Snf1p (Cullen and Sprague 2000; Voordeckers  
709 *et al.* 2012) were also required for mat formation (Figures S1  
710 and S2). Based on these results, we conclude that many of the  
711 major signaling pathways that regulate filamentous growth  
712 also regulate mat formation and colony patterning.


713 We hypothesized that the chromatin remodeling complex  
714 SAGA (Koutelou *et al.* 2010) might also regulate adhesion-  
715 dependent surface growth. The hypothesis was based on the  
716 fact that a component of SAGA, Gcn5p (Georgakopoulos and  
717 Thireos 1992; Sterner and Berger 2000), is required for fila-  
718 mentous growth (Chavel *et al.* 2014). Moreover, SAGA com-  
719 ponents have previously been shown to control aspects of  
720 colonial patterning (Voordeckers *et al.* 2012). Spt8p, a SAGA  
721 component (Winston *et al.* 1987), also regulates filamentous  
722 growth (Voordeckers *et al.*, personal communication). Kymograph  analysis of the *spt8 $\Delta$*   
723 mutant showed a defect in ruffle forma-  
724 tion at 2 and 3 days [Figure 1, B and C, *spt8 $\Delta$*  (PC4008),  
725 Figure S3, and Video S7]. The defect was subtle by kymo-  
726 graph analysis but was obvious in the time-lapse analysis. The  
727 *spt8 $\Delta$*  mutant was also defective for colony ruffling and mat  
728 pattern formation (Figure S1). Therefore, we include SAGA  
729 as a regulator of filamentous surface responses in yeast (Fig-  
730 ure 1A, cyan).

### 731 Expression profiling of colony surface growth identifies 732 new targets

733 To explore how filamentation signaling pathways might reg-  
734 ulate surface growth, comparative RNA-seq was performed in  
735 several mutants that disrupt the main pathways that regulate  
736 filamentous growth (dashed blue box in Figure 1A, pathway  
737 diagrams can be found in Figure S4, A–D). RNA was prepared  
738 from wild-type and mutant colonies with the following geno-  
739 types: *ste12 $\Delta$*  (fMAPK), *dig1 $\Delta$*  (fMAPK), *ras2 $\Delta$*  (RAS), *rtg3 $\Delta$*   
740 (RTG), and *spt8 $\Delta$*  (SAGA). RNA was prepared from colonies  
741 under conditions that favored pattern formation (YEP-Gal).  
742 Each mutant showed the expected colony patterning. Specif-  
743 ically, the *ras2 $\Delta$*  (PC562), *rtg3 $\Delta$*  (PC3642), *ste12 $\Delta$*  (PC1079),  
744 and *spt8 $\Delta$*  (PC4008) mutants were less ruffled than wild-type  
745 colonies (PC538), and the *dig1 $\Delta$*  (PC3039) mutant was more  
746

747 ruffled than wild-type colonies (Figure S4E). After sequenc- 803  
748 ing, PCA of the RNA-seq data showed close clustering of 804  
749 strain replicates, while strains differentiated into their repli- 805  
750 cate clusters (Table S1). 806

751 Comparative RNA-seq analysis between the wild-type col- 807  
752 onies and each mutant was performed with the DESeq2 808  
753 package in R (Love *et al.* 2014). Differential gene expression 809  
754 was defined by  $|\log_2FC| > 0.585$  and  $P\text{-value} < 0.01$ . By this 810  
755 method, 1833 genes were differentially expressed in at least 811  
756 one of the mutants tested (Figure S4F) and represented 29% 812  
757 of the ORFs in the genome (Lin *et al.* 2013). The annotated 813  
758 data set can be found in Table S1. To identify the most dif- 814  
759 ferentially regulated targets between the mutant and wild- 815  
760 type colonies, volcano plots showing all differences in gene 816  
761 expression were generated for each mutant. Individual genes 817  
762 were distributed by change in expression ( $x\text{-axis}$ ,  $\log_2FC$ ) and 818  
763 significance of change in expression [ $y\text{-axis}$ ,  $-\log_{10}(P\text{-value})$ ] 819  
764 (Figure 2A). Many of the genes whose expression is 820  
765 known to be induced during filamentous growth were un- 821  
766 covered during the initial analyses (Figure 2A and Table 822  
767 S1). These included targets of the fMAPK pathway: *FLO11* 823  
768 (Rupp *et al.* 1999) (Figure 2A, *dig1Δ* and *ste12Δ*; Table S1, 824  
769 *dig1Δ* and *ste12Δ*), *YLR042C* (Roberts *et al.* 2000) (Figure 825  
770 2A, *ste12Δ*; Table S1, *dig1Δ* and *ste12Δ*), *CLN1* (Madhani 826  
771 *et al.* 1999) (Table S1, *dig1Δ* and *ste12Δ*), *PGU1* (Madhani 827  
772 *et al.* 1999; Roberts *et al.* 2000) (Figure 2A, *dig1Δ* and 828  
773 *ste12Δ*; Table S1, *dig1Δ* and *ste12Δ*), *SVS1* (Roberts *et al.* 829  
774 2000) (Figure 2A, *dig1Δ* and *ste12Δ*; Table S1, *dig1Δ* and 830  
775 *ste12Δ*), *KSS1* (Table S1, *dig1Δ* and *ste12Δ*) (Roberts *et al.* 831  
776 2000), and *MSB2* (Cullen *et al.* 2004) (Table S1, *dig1Δ* and 832  
777 *ste12Δ*). The abovementioned fMAPK pathway targets 833  
778 *YLR042C*, *PGU1* (Roberts *et al.* 2000), *FLO11* (Rupp *et al.* 834  
779 1999) are also *Ras2p*-dependent (Table S1, *ras2Δ*), poten- 835  
780 tially through its regulation of the fMAPK pathway (Mösch 836  
781 *et al.* 1996, 1999; Chavel *et al.* 2010). Similarly, targets of the 837  
782 RTG pathway were identified in the wild-type-*rtg3Δ* data set: 838  
783 *CIT2*, *CIT1*, *IDH1*, *IDH2* (Liu and Butow 1999) (Figure 2A, 839  
784 *rtg3Δ*; Table S1, *rtg3Δ*), and *DLD3* (Liu and Butow 2006) 840  
785 (Table S1, *rtg3Δ*). Genes regulated by SAGA were identified 841  
786 in the wild-type-*spt8Δ* data set [*ADH1*, *ARG1*, *BDF2*, *CTT1*, 842  
787 *FBA1*, *GRE2*, *PGK1*, *TDH3*, and *PHO84* (Basehoar *et al.* 2004; 843  
788 Huisinga and Pugh 2004)] (Table S1, *spt8Δ*). 844

789 Confirmation of other targets by qPCR analysis has been 845  
790 performed in related studies including: targets of fMAPK 846  
791 related to the fungal cell wall, *OCH1*, *PRY2*, *FLO11* and 847  
792  *TIP1* (Chow *et al.* 2018); *SUC2* and *YLR042C* (Chow *et al.*, 848  
793 unpublished data); and *GIC2* (Prabhakar *et al.*, personal com- 849  
794 munication). Therefore, comparative RNA-seq analysis iden- 850  
795 tified many of the genes expected based on previous or 851  
796 parallel studies. 852

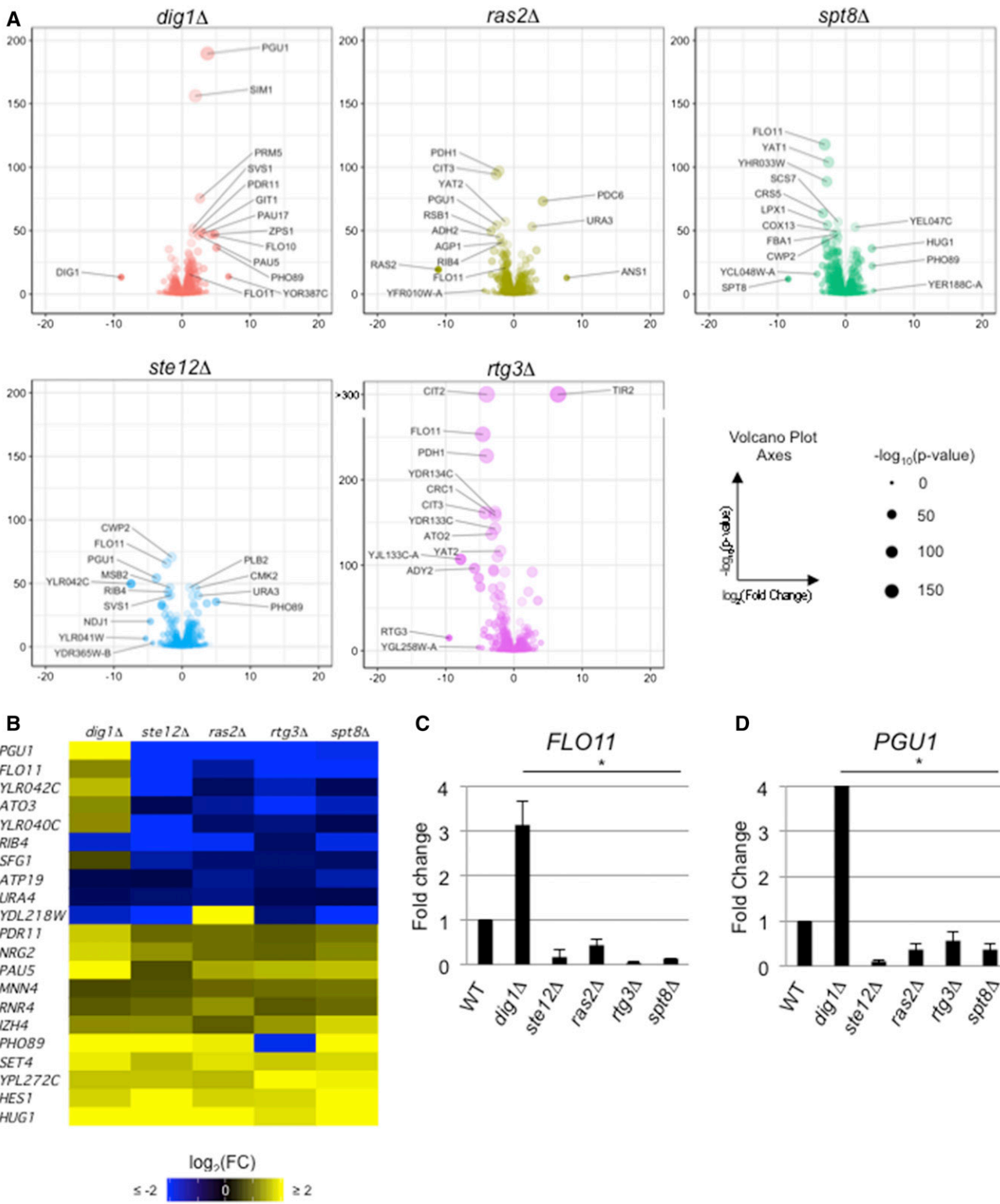
797 One question that we sought to address was the extent of 853  
798 target gene overlap among the signaling pathways that reg- 854  
799 ulate filamentous growth. We found that while most genes 855  
800 were regulated by one pathway (Figure S4G, 742/1833 or 856  
801 59%), coregulated targets were seen in almost every combi- 857  
802 nation of regulators, including targets coregulated by all five 858

regulators (Figure S4F). This result also demonstrated that 803  
the SAGA complex coregulated targets with the fMAPK, RAS, 804  
and RTG pathways (Figure S4F), which further validates it as a 805  
major regulator of filamentous growth (Figure 1A, cyan ar- 806  
rows). Additionally, *Spt8p* regulated a target of the *RIM101* 807  
pathway, *NRG1* (Chavel *et al.* 2014) (Figure 1A, cyan arrow), 808  
and a target of lipid biosynthesis, *INO1* (Chavel *et al.* 2014) 809  
(Figure 1A, cyan arrow). SAGA did not coregulate targets of 810  
the *PHO85*, *RPD3(L)*, or ELP pathways. Taken together, these 811  
data suggest that one function of this network of signaling 812  
pathways is to regulate a large number of genes (the more 813  
nodes in the network, the greater the number of differentially 814  
expressed genes); a second function is to coregulate key tar- 815  
gets to amplify target gene expression. 816

817 One possible explanation for the regulatory overlap is cross 817  
818 feedback among the pathways. To explore this aspect of 819  
820 signaling network connectivity, we examined whether genes 821  
822 encoding pathway components were themselves targets of 823  
824 other pathways that regulate filamentous growth. The RTG 825  
826 pathway regulated the expression of components of fMAPK 827  
828 (*MSB2*), RAS (*TPK2*), and SAGA (*SFG73*). fMAPK regulated 829  
830 genes encoding components of its own pathway (*MSB2*, 831  
832 *TEC1*, and *KSS1*) and the RAS pathway (*BCY1*). The RAS 833  
834 pathway regulated one of its own effectors (*TPK1*) and the 835  
836 fMAPK pathway (*TEC1*). SAGA regulated the fMAPK path- 837  
838 way (*MSB2* and *TEC1*) and itself (*SUS1*) (Figure S5, A and B). 839  
840 These results support and extend a previous study, which 841  
842 showed that the activity of the fMAPK is subject to regulation 843  
844 by other filamentation regulatory pathways (Chavel *et al.* 845  
846 2010). Interestingly, not all of the interactions would be 847  
848 expected to result in positive feedback. For example, the 849  
850 *rtg3Δ* mutant showed a  $\sim 1.9$ -fold increase in *TPK2* gene 851  
852 expression (Table S1), which is a PKA subunit and compo- 853  
854 nent of the RAS pathway. These data indicate that coregula- 855  
856 tion of targets may be the result of feedback among the 857  
858 pathways that regulate filamentous growth. 859

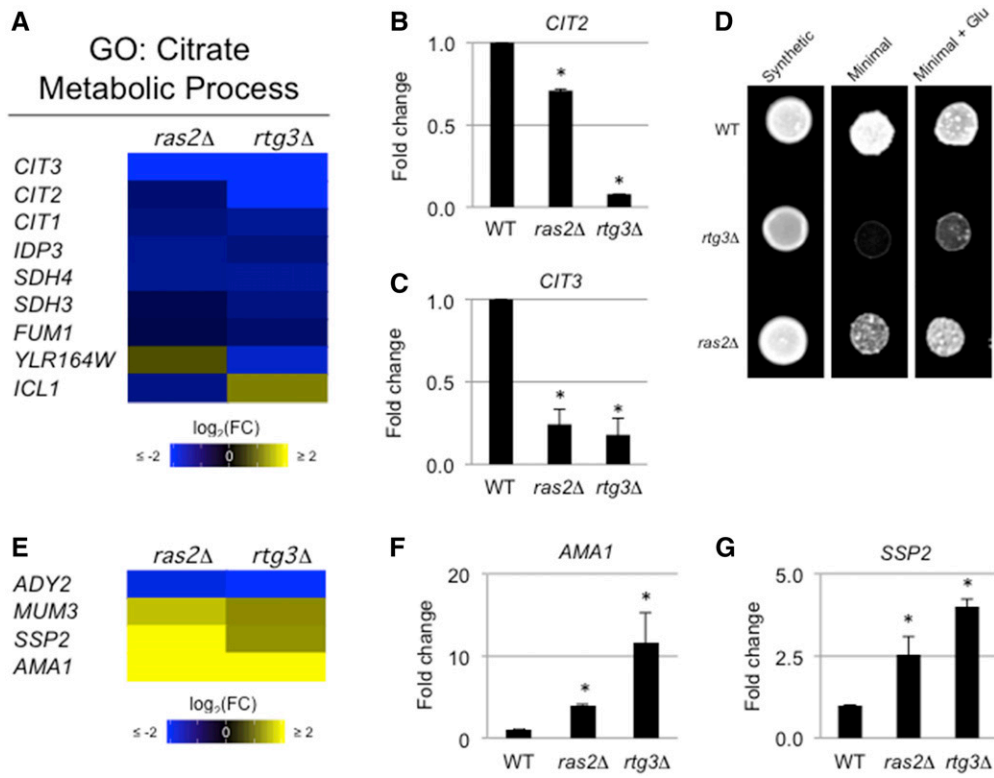
859 To identify functionally relevant targets whose expression 860  
861 was amplified by multiple pathways that regulate filamentous 862  
863 growth, we focused on genes that were differentially 864  
865 expressed in all regulatory mutants (Figure 2B and Table 866  
867 S2, all mutants). GO term analysis (Ashburner *et al.* 2000) 868  
869 of the coregulated genes showed significant enrichment in 869  
870 cell adhesion, cell wall constituents, and fungal cell wall pro- 871  
872 teins (Table S2, all mutants). One of the targets that was 873  
874 coregulated by all five regulators, and that was included in 875  
876 many of the above enriched GO terms, was the gene that 877  
878 encodes the major cell adhesion molecule *Flo11p* (Figure 879  
880 2B and Table S2, all mutants). *FLO11* is known to be regu- 881  
882 lated by a large number of proteins and pathways (Rupp *et al.* 883  
884 1999; Barrales *et al.* 2008). We confirmed that fMAPK, RAS, 885  
886 RTG, and *Spt8p* controlled *FLO11* expression by qPCR (Fig- 887  
888 ure 2C). These data fit with the key role that *Flo11p* plays in 889  
890 regulating adhesion-dependent responses in yeast. Another 891  
892 target regulated by all five mutants, which also had the larg- 893  
894 est net change in regulation, was *PGU1*, the gene that en- 895  
896 codes a secreted pectinase that is also expressed during 897  
898 899 900





**Figure 2** Comparative RNA-seq analysis identifies common and unique targets of signaling pathways that regulate filamentous growth. (A) Volcano plots showing comparative RNA-seq between the indicated mutant and WT. Dot size is  $-\log_{10}(P\text{-value})$ . Volcano plot x-axis is  $\log_2(\text{FC})$ ; y-axis is  $-\log_{10}(P\text{-value})$ . Labeled dots are 10 targets with lowest  $P$ -value or five targets with highest lfold change. *FLO11* has also been labeled on *dig1Δ* and *ras2Δ* plots. *CIT2* and *TIR2* have calculated  $P$ -values  $< 10^{-300}$  in the *rtg3Δ* mutant, as reflected in the break in the y-axis. (B) Heat map showing targets with  $|\log_2(\text{FC})| > 0.585$  and  $P\text{-value} < 10^{-3}$ . (C) Bar graph showing fold change in *FLO11* mRNA levels, normalized to *ACT1* with WT values set to 1, in the indicated mutants by qPCR analysis by the  $\Delta\Delta\text{Ct}$  quantitation method. The experiment was performed in triplicate and error bars represent the SD between experiments. \*  $P < 0.05$  for all differences compared to WT. (D) Bar graph showing fold change in *PGU1* mRNA levels. See (C) for details.  $\log_2(\text{FC})$ ,  $\log_2(\text{Fold Change})$ ; qPCR, quantitative PCR; RNA-seq, RNA sequencing; WT, wild-type.

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**Figure 3** Common and unique target genes of the RAS and RTG pathways. (A) Heat map of targets coregulated by the RAS and RTG pathways with the citrate metabolic process GO term. (B and C) Bar graph showing fold change in (B) *CIT2* and (C) *CIT3* mRNAs in *ras2Δ* and *rtg3Δ* relative to WT. See Figure 2C for details. (D) Strains spotted onto synthetic media, minimal media, or minimal media with glutamate. Cells were grown for 48 hr. (E) Heat map of targets coregulated by RAS and RTG associated with sporulation. (F and G) Bar graphs showing fold change in (F) *AMA1* and (G) *SSP2* mRNAs in *ras2Δ* and *rtg3Δ* relative to WT. See Figure 2C for details. Glu, glutamate; GO, gene ontology; log<sub>2</sub>(FC), log<sub>2</sub>(Fold Change); WT, wild-type.

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filamentous growth (Madhani *et al.* 1999; Cullen 2015a). This result was confirmed by qPCR analysis (Figure 2D). Taken together, our results are consistent with the idea that multiple signaling pathways and protein complexes coordinately regulate target gene expression during colonial surface growth (Figure 2A). One function of the network is to control the expression of *FLO11*, a major regulator of adhesion-dependent responses in yeast (Figure 2, B and C).

**A regulatory connection between the RAS and RTG pathways**

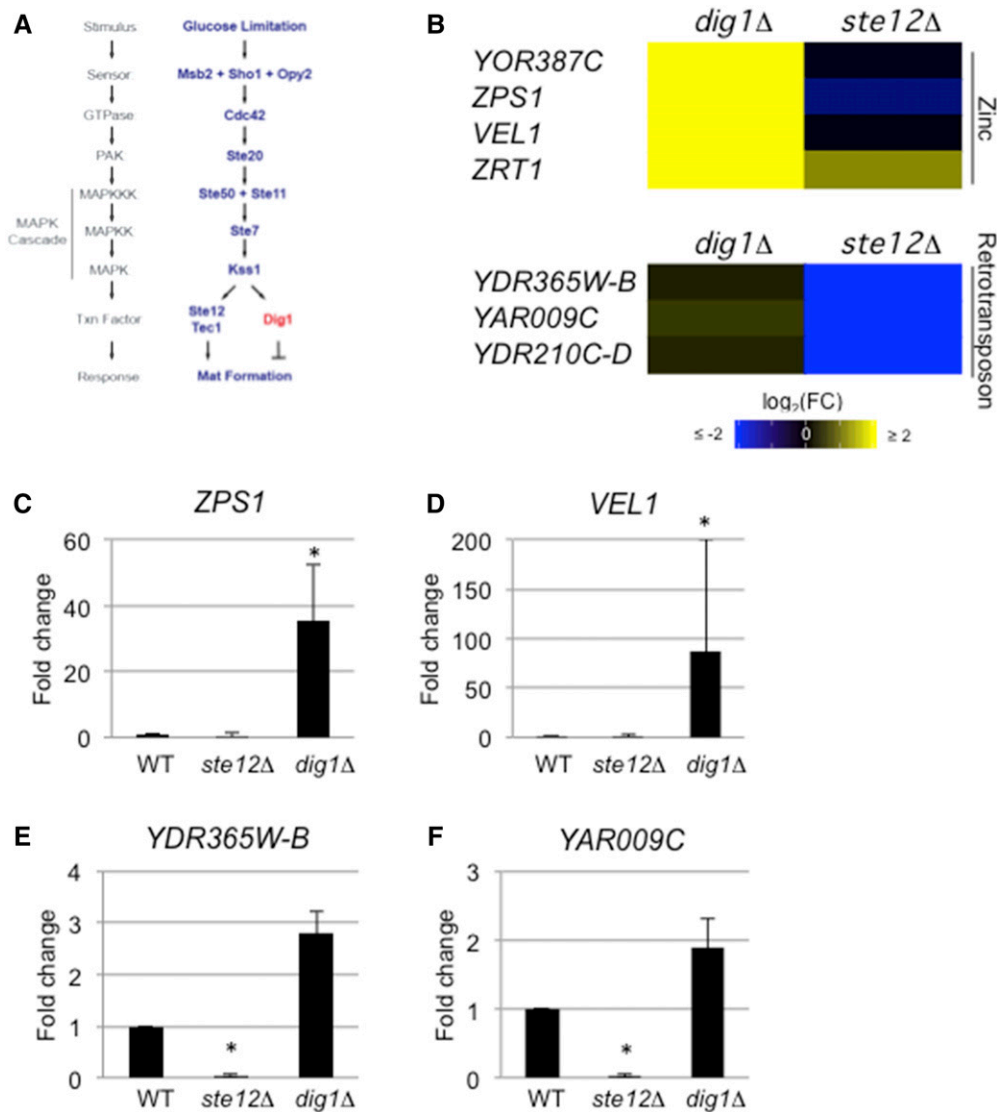
In an analysis of highly significant changes in expression in the *ras2Δ* and *rtg3Δ* sets ( $P < 10^{-12}$ ) (Figure S6), 41 targets were in common and 93% of these targets (39/41) showed the same regulation (both up or down) (Figure S6). For example, citrate synthase, *CIT3*, was among the significant, differentially expressed genes in both *ras2Δ* and *rtg3Δ* (Figure 2A). Other citrate synthases, *CIT1* and *CIT2*, were also coregulated by both the *ras2Δ* and *rtg3Δ* mutants (Figure 3A and Table S1). We confirmed that RAS and RTG coregulated *CIT2* and *CIT3* by qPCR analysis (Figure 3, B and C). Because of the extent of coregulation, especially the coregulation of all citrate synthase isoforms (Graybill *et al.* 2007), the full set of RAS and RTG coregulated targets was examined further.

As expected, the RTG pathway regulated mitochondrial gene targets (Table S1). Many of the genes were also regulated by the RAS pathway (Table S1; *PDH1*, *YAT1*, and *YAT2*) (Schmalix and Bandlow 1993; Epstein *et al.* 2001; Swiegers *et al.* 2001). Moreover, several hallmark RTG targets were also regulated by the RAS pathway (Table S1; *CIT1*, *CIT2*,

*CIT3*, *ATO2*, and *ATO3*) (Suissa *et al.* 1984; Kim *et al.* 1986). In addition to coregulation of cell wall components (*i.e.*, *FLO11*), GO term analysis identified significant enrichment of the citrate metabolic process by the overlapping RAS and RTG targets (Figure 3A and Table S2), and the citric acid cycle (Figure S7). Because RAS and RTG coregulated genes that are involved in the citric acid cycle, we hypothesized that RAS might be required to support that mitochondrial role. The mitochondria are critical for glutamate biosynthesis (Liu and Butow 1999; Magasanik and Kaiser 2002) and RTG is required for growth in medium lacking glutamate [Figure 3D, *rtg3Δ* (Liao and Butow 1993; Small *et al.* 1995; Chavel *et al.* 2014)]. More specifically, a loss of citrate synthases results in glutamate auxotrophy in *S. cerevisiae* (Kim *et al.* 1986), so we hypothesized that a RAS pathway mutant would be sensitive to limiting glutamate. Like RTG, the RAS pathway was also required for growth in this condition (Figure 3D, *ras2Δ*). The growth defects of cells lacking an intact RAS or RTG pathway were bypassed by the addition of glutamate (Figure 3D). Therefore, the RAS pathway plays a role in this mitochondrial process.

Similarly, the RAS pathway regulated genes that are required for sporulation (Table S1) and a subset of these genes were coregulated by the RTG pathway [Figure 3E, *ADY2* (Rabitsch *et al.* 2001), *AMA1* (Coluccio *et al.* 2004), *SSP2* (Sarkar *et al.* 2002), and *MUM3* (Engbrecht *et al.* 1998)]. Several of the genes were confirmed by qPCR analysis (Figure 3, F and G). Therefore, the RAS and RTG pathways coregulate functionally related genes and may be more functionally connected than has been previously thought.

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**Figure 4** Common and unique genes regulated by Ste12p and Dig1p. (A) Model of the fMAPK pathway showing Ste12p and Dig1p. (B) Heat map of indicated targets regulated in the *dig1Δ* or *ste12Δ* mutants. (C–F) Bar graphs showing fold change in target mRNA levels for (C) *ZPS1*, (D) *VEL1*, (E) *YDR365W-B*, and (F) *YAR009C*. See Figure 2C for details. fMAPK, fungal MAPK;  $\log_2(\text{FC})$ ,  $\log_2(\text{Fold Change})$ ; WT, wild-type.

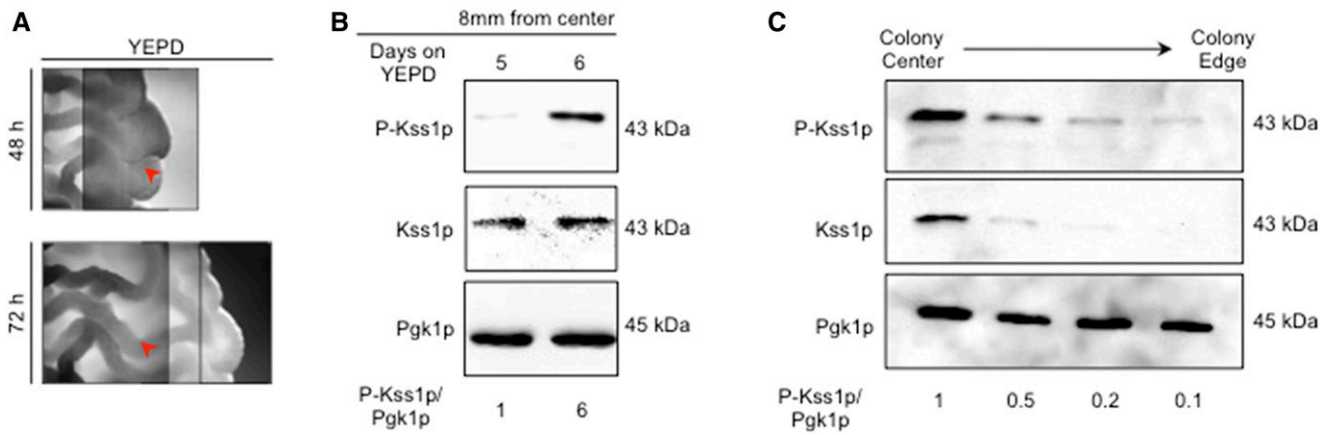
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**Nonreciprocal target genes are regulated by opposing transcriptional regulators of the fMAPK pathway**

*Ste12p* is one of the transcription factors that regulates the fMAPK pathway (Liu *et al.* 1993). The transcriptional repressor *Dig1p* inhibits *Ste12p*-dependent transcription (Cook *et al.* 1996; Tedford *et al.* 1997; Bardwell *et al.* 1998; Olson *et al.* 2000; Breitzkreutz *et al.* 2003; Kusari *et al.* 2004; Chou *et al.* 2006; van der Felden *et al.* 2014). Although the transcriptional outputs of the pathway can be complex resulting in nonuniform target gene regulation (Breitzkreutz *et al.* 2003), *Ste12p* and *Dig1p* were expected to regulate a common set of targets in a reciprocal manner (Figure 4A). We unexpectedly found that the pattern of gene regulation by the two proteins could not be solely explained by reciprocal regulation. In an analysis of the most significant changes in expression in the *dig1Δ* and *ste12Δ* sets ( $P < 10^{-12}$ ) (Figure S8), 20 targets were found to be in common and only 50% (10/20) were reciprocally regulated (up in *dig1Δ* and down in *ste12Δ*) (Figure S8). As expected, *Ste12p* and *Dig1p*

showed reciprocal regulation of a subset of target genes (Figure S8; *FLO11/MUC1*, *PGU1*, *YLR042C*, and *BARI*). However, more genes were independently regulated only by *Dig1p* or only by *Ste12p* (Figure S8).

For example, the target with the largest change in expression identified in the *dig1Δ* volcano plot was *YOR387C* (> 111-fold increase) (Figure 2A and Table S1). Although *YOR387C* lacks associated GO terms, a literature search indicated that *YOR387C* is induced in zinc-limiting conditions (Higgins *et al.* 2003; Wu *et al.* 2008). Although *YOR387C* and its paralog *VEL1* (Higgins *et al.* 2003) were significantly regulated in the *dig1Δ* mutant, neither gene was found to be regulated in the *ste12Δ* mutant (Figure 4B and Table S1). Additionally, only the *dig1Δ* mutant showed a change in expression of *ZAP1* (Table S1), a major transcription factor induced by limiting zinc (Zhao and Eide 1997). Interestingly, the ability of cells to take up trace zinc was determined to be critical for flocculation (Yuan 2000), which is a closely related response to filamentous growth and mat formation. A



**Figure 5** Different ruffling patterns in colonies correspond to different levels of fMAPK pathway activity. (A) Bright-field microscopy of a wild-type colony grown in YEPD for 48 and 72 hr. Red arrow indicates same location on a mat at 48 hr when the region is smooth and 72 hr when the region has become ruffled. Different focal planes of the same colony are shown. (B) Immunoblot analysis using p42/p44 antibodies to detect P-Kss1p levels from extracts prepared from cells before and after ruffling. Immunoblots were also performed using antibodies to Kss1p and Pgk1p as loading controls. (C) Immunoblot analysis using p42/p44 antibodies to detect phosphorylated Kss1p at different parts of a colony. Immunoblots were also performed using antibodies to Kss1p and Pgk1p as loading controls. fMAPK, fungal MAPK; YEPD, yeast extract, peptone, and dextrose.

subset of other upregulated targets in the *dig1Δ* mutant were genes induced under low-zinc conditions [Figure 4B, *ZPS1* (Lyons *et al.* 2000)] as well as a zinc transporter [*ZRT1* (Zhao and Eide 1996)]. These genes were not strongly downregulated in the *ste12Δ* mutant. The expression profiles of *ZPS1* and *VEL1* were confirmed by qPCR analysis (Figure 4, C and D).

Likewise, the *ste12Δ* mutant showed downregulation of some target genes that were not altered in the *dig1Δ* mutant. One of the targets identified in the *ste12Δ* volcano plot was the retrotransposon *YDR365W-B* (Kim *et al.* 1998). Other retrotransposons—*YDR365W-B*, *YOL103W-B*, *YAR009C*, and *YDR210C-D* (Kim *et al.* 1998)—were also regulated by *Ste12p* but less so by *Dig1p* (Figure 4B). The expression profiles of *YDR365W-B* and *YAR009C* were confirmed by qPCR analysis (Figure 4, E and F). Thus, key transcriptional regulators of the fMAPK pathway operate through mechanisms that do not only involve reciprocal regulation between the two proteins.

#### Changes in colony patterning correspond to changes in MAPK pathway activity

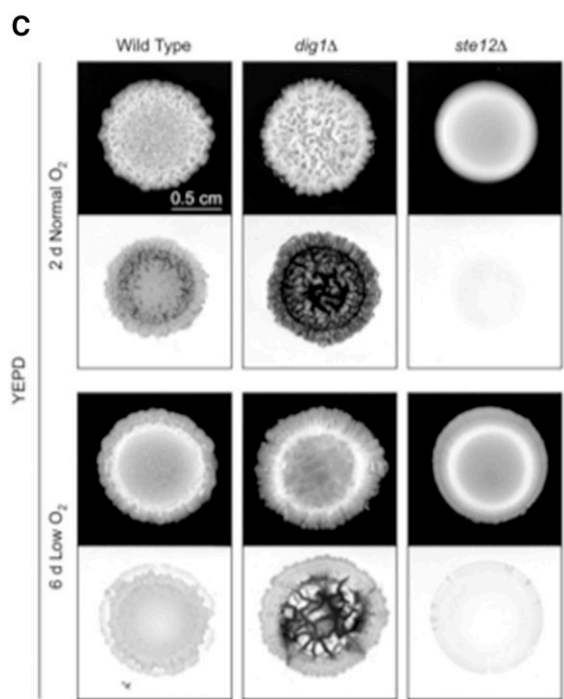
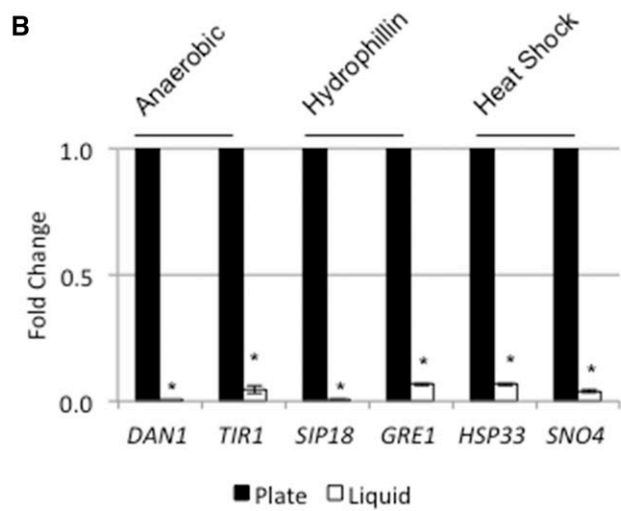
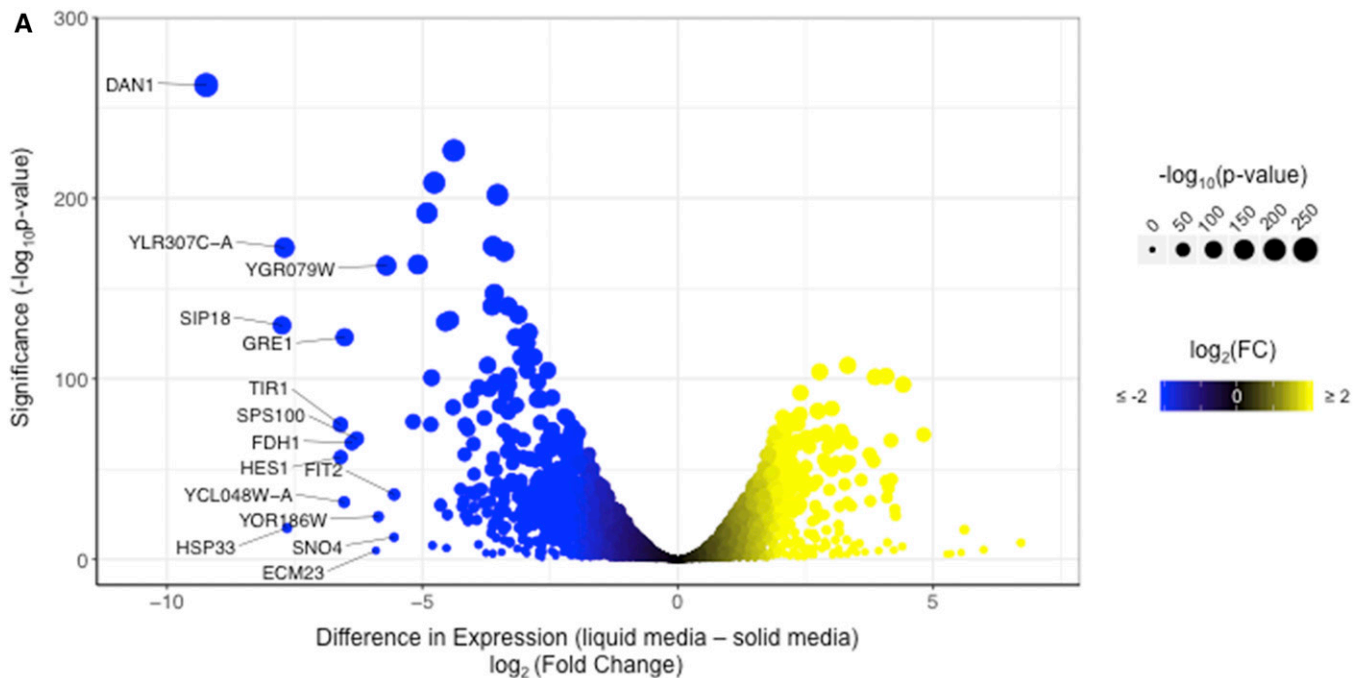
We also examined the development of colony pattern formation under different conditions and over time. Colonies showed different ruffling patterns under different conditions. For example, growth in preferred carbon sources such as glucose media (YEPD) resulted in colony perimeters that were smooth compared to a ruffled colony interior (Figure 5A, adjacent photographs of a colony are shown at different focal planes on the *z*-axis). Such differences were not observed in more uniformly ruffled colonies grown on a nonpreferred carbon source (YEP-Gal) (Video S1). Over time, the smooth regions of the colony became ruffled (Figure 5A, red arrow marks the same location in the colony).

One explanation for differences in colony pattern formation might be that different parts of the colony are exposed to

different environments. The fMAPK pathway, which is sensitive to nutrient levels, may induce different responses in different parts of the colony. To test this possibility, samples from regions of the colony were collected and examined for fMAPK activity by phosphorylation of the MAP kinase *Kss1p* (Cullen 2015a). Samples collected from smooth colony perimeters showed low P~*Kss1p* levels (day 5, smooth). Samples collected from the same physical location that had become ruffled (day 6, ruffled) showed high P~*Kss1p* levels (Figure 5B). This result indicates that as cells in colonies consume nutrients and subsequently starve, the fMAPK pathway is activated to promote ruffle formation. In line with this possibility, colony interiors showed higher levels of P~*Kss1p* compared with colony perimeters (Figure 5C). We note that in this experiment, the levels of total *Kss1p* protein were also lower at colony perimeters. *KSS1* is a target of the fMAPK pathway, and the regulation of total *Kss1p* may be a way that fMAPK regulates its activity through positive feedback (Roberts *et al.* 2000). Therefore, signaling pathways may exhibit different activities, and induce target genes to different levels, in a manner that is influenced by nutrient levels (and time) to control adhesion-dependent surface growth.

#### Genes that respond to oxygen, desiccation, and temperature stress were induced during colony surface growth

Growth on surfaces presents unique challenges compared to the uniform growth in liquid cultures. To further explore the response to colonial growth on surfaces, expression profiling data sets were compared between cells grown in liquid (Adhikari and Cullen 2014) and cells grown on an agar surface (this study). Growth on surfaces caused the differential expression of 3267 genes when compared to growth in liquid (Table S1, wild-type liquid–solid,  $|\log_2FC| > 0.585$  and  $FDR < 0.05$ ). This represents ~54% of the yeast genome



**11** **Figure 6** Target genes induced during surface growth correspond to the response to specific stresses. (A) Volcano plot showing the difference in expression and significance of all targets between growth in liquid media vs. solid media. x-axis is  $\log_2(\text{Fold Change})$ ; y-axis is  $-\log_{10}(P\text{-value})$ . Labeled targets are the top 15 induced targets during surface growth. (B) Relative fold change of the indicated gene was determined by qPCR analysis. Expression normalized to *ACT1* in the indicated mutants, as determined by qPCR analysis. The experiment was performed in triplicate and error bars represent the standard difference between experiments. \*  $P < 0.05$  for differences compared to wild-type. (C) Wild-type cells and the indicated mutants were spotted on YEPD 2% agar media in normal and low-oxygen conditions. Colonies were grown for the indicated times to compare colony patterns and invasive growth. Inverted images of the invasive scars are shown.  $\log_2(\text{FC})$ ,  $\log_2(\text{Fold Change})$ ; qPCR, quantitative PCR; YEPD, yeast extract, peptone, and dextrose.

[6091 genes (Lin *et al.* 2013)]. Functional classification of the top 15 induced targets (Figure 6A, >25-fold increase in expression) during surface growth included genes induced during anaerobic stress [*DAN1* (Sertil *et al.* 1997) and *TIR1* (Cohen *et al.* 2001), Figure 6A], desiccation/dehydration [*SIP18* (Miralles and Serrano 1995) and *GRE1* (Garay-Arroyo and Covarrubias 1999), Figure 6A], and elevated temperature [*HSP33* (Wilson *et al.* 2004) and *SNO4* (Samanta and Liang 2003), Figure 6A]. An expanded list of the top 50 (>15-fold increase in expression) induced targets included genes that regulate metal homeostasis, RNA processing, and the metabolism of lipids and nonfermentable carbon sources, as well as genes of unknown function (Figure S9).

The most differentially induced target during surface growth was the gene that encodes the anaerobic-responsive cell wall mannoprotein *Dan1p* (Mrsa *et al.* 1999) (Figure 6A). qPCR analysis showed that *DAN1* expression was induced during surface growth (Figure 6B). Examination of the *dan1Δ* mutant from an ordered gene deletion collection (Ryan *et al.* 2012) showed a defect in colony ruffling (Figure S10, *dan1Δ*). Thus, *Dan1p* may impact colony patterning in response to stresses associated with surface growth. In addition to *DAN1*, the four members of the Tir family of anaerobic-responsive cell wall mannoproteins (Cohen *et al.* 2001) also showed transcriptional induction during surface growth (Figure 6A and Table S1, wild-type liquid–solid), which was verified in a study on the regulation of the cell wall (Chow *et al.* 2018). Therefore, anaerobic-specific changes to the cell wall also occur during surface growth in yeast.

Because targets that encode anaerobic-responsive proteins were among the top induced targets during colony surface growth [*DAN1* and the *TIR* genes (Figure 6A)], the impact of oxygen in colony ruffling and invasive growth was examined. *S. cerevisiae* undergoes aerobic alcohol fermentation by what is known as the Crabtree effect (Crabtree 1929). In yeast, this includes the repression of genes involved in aerobic respiration in the presence of glucose during exponential growth (De Deken 1966). Studies that explore this phenomenon in yeast have done so in liquid culture (De Deken 1966; Hagman *et al.* 2014), but the role of oxygen in regulating aspects of colonial growth has not been explored. To determine the role of oxygen on colony pattern formation, wild-type and *dig1Δ* colonies grown in normal atmospheric oxygen (20% oxygen) were compared to colonies grown in low oxygen (5–15% oxygen). Comparing the colonies at similar sizes (2-day normal oxygen and 6-day low oxygen) showed that both were less ruffled in low oxygen (Figure 6C). Wild-type and *dig1Δ* colonies showed reduced invasive growth in low oxygen (Figure 6C). Additionally, wild-type, *dig1Δ*, and *ste12Δ* colonies grown in limiting oxygen (5–15% oxygen or 0% oxygen) grew more slowly than in normal oxygen (20% oxygen) (Figure S11). Slower growth was not bypassed with excess glucose (Figure S11, 8% Glu) as might be predicted by the Crabtree effect in yeast. Therefore, oxygen levels impact colony patterning and invasive growth.

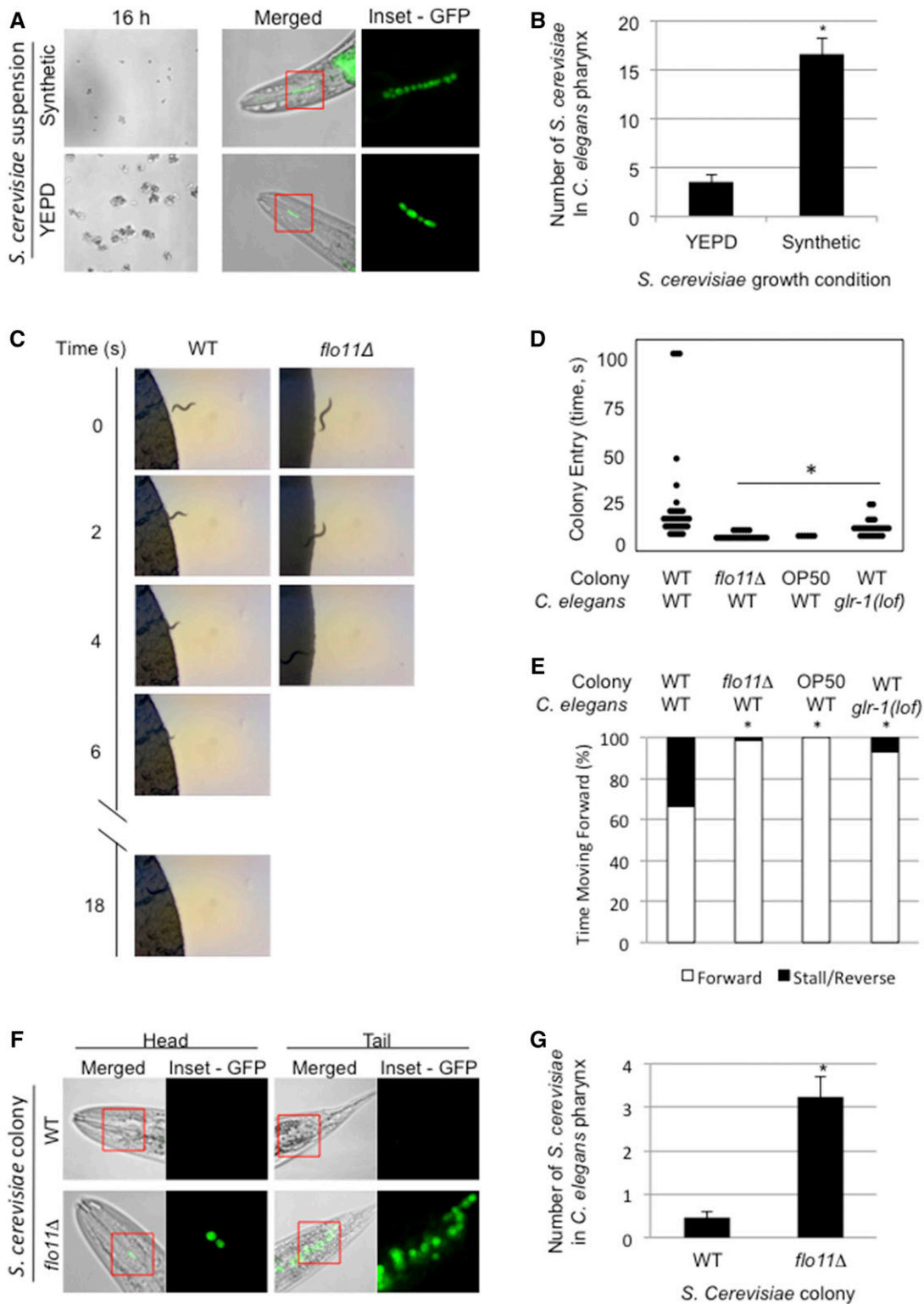
Another class of genes induced during surface growth has been described as regulated during desiccation. These included the gene that encodes the hydrophilin *Sip18p* (Miralles and Serrano 1995; Dang and Hinch 2011) (Figure 6A). A second hydrophilin, *Gre1p*, (Garay-Arroyo and Covarrubias 1999) was also among the top 10 induced targets (Figure 6A). Four of six hydrophilins and putative hydrophilins were induced during surface growth (Table S1, wild-type liquid–solid). Hydrophilins such as *Sip18p* allow for survival under desiccation stress and during the dehydration/rehydration process (Rodríguez-Porrata *et al.* 2012). The induction of hydrophilin-encoding genes *SIP18* and *GRE1* during surface growth was confirmed by qPCR analysis (Figure 6B). By testing mutants from an ordered deletion collection (Ryan *et al.* 2012), we found that the loss of either hydrophilin, *SIP18* or *GRE1* (Figure S10A; *gre1Δ* and *sip18Δ*), increased mat ruffling and hyper-invasive growth, indicating that intracellular hydration may be a trigger for both invasive growth and colony ruffling (Figure S10B; *gre1Δ* and *sip18Δ*). Therefore, cells in mats may experience desiccation stress, and genes induced under this condition can impact colony patterning.

Genes encoding the heat-shock proteins *Hsp33p* (fourth most-induced target), *Sno4p*, *Hsp26p*, and *Hsp12p* were also induced during mat growth. Additionally, as shown below, a ruffled colony showed a temperature differential of 0.8° between its warmest and coolest regions (see Figure 7D, below). Modest differences in temperature have previously been shown to effect metabolism in *S. cerevisiae* (Jones and Hough 1970). *Spg4p*, described as being essential for growth at high temperature (Martinez *et al.* 2004), was also among the top targets. Using an ordered deletion collection, we explored the roles of *SNO4* and *HSP26* (Bentley *et al.* 1992) (Figure S10A; *sno4Δ* and *hsp26Δ*), as the *hsp33Δ* mutant did not exist in the deletion collection. Deletion of either of these genes resulted in smoother colonies.

None of the mutants tested were necessary for growth in low-oxygen (5–15% O<sub>2</sub>), high-temperature (37°), or desiccated conditions (Figure S11). This may be a result of functional redundancy among the major induced targets. Taken together, oxygen, temperature, and desiccation stresses impact colonial growth in yeast. Genes induced during surface growth impact colony patterning that may aid in the response to these stresses.

#### ***Flo11p-dependent adhesion protects cells in colonies from a nematode predator***

*Flo11p* is an established regulator of filamentous growth, and its role in maintaining contacts between cells to form filaments has obvious benefits during surface penetration (e.g., invasive growth) into new environments. However, the broader functional implications of *Flo11p*-dependent adhesion between cells on the colony surface are not clear. The interactions among yeast cells mediated by a related flocculin, *Flo1p*, can provide protection from toxins and antibiotics (Smukalla *et al.* 2008), and cell adhesion has more generally



**Figure 7** Impact of Flo11p on predator avoidance in yeast colonies. (A) Suspensions of GFP-labeled WT cells (PC6733) grown for 16 hr in synthetic or YEPD media (16 hr). Merged fluorescent and brightfield images of heads of mounted *C. elegans* showing fluorescent *S. cerevisiae* inside the animal pharynx after 45 min feeding (merged). Fluorescent images of individual *S. cerevisiae* inside the animal pharynx (inset = GFP). (B) Bar graph showing difference in average number of *S. cerevisiae* cells in *C. elegans* pharynx between YEPD- and synthetic-grown suspensions. Error bars are SE. \*  $P < 0.05$ . (C) Selected images from videos showing WT *C. elegans* entering WT or *flo11Δ* yeast colonies. A set of representative videos are available in the supplement (Videos S8–S15). (D) Dot plot showing the time required for worms to enter the indicated colonies. WT (N2) or *glr-1(lof)* *C. elegans* worms were placed on plates containing WT or *flo11Δ* yeast, or the *E. coli* food strain OP50. (E) Stacked bar graphs comparing *C. elegans* forward movement to stalls and/or reversals in the indicated yeast or *E. coli* colonies. (F) Three-day-old colonies of WT (PC538) and *flo11Δ* (PC1029) cells expressing-plasmid

1643 been postulated to provide protection to individual cells of a  
1644 colony (Granek and Magwene 2010). In bacterial communi-  
1645 ties, the growth of cells in mats can deter predation by other  
1646 microbes and multicellular predators, like nematodes (Darby  
1647 *et al.* 2002). To explore whether cell adhesion contacts might  
1648 similarly protect fungal cells from predation, we developed a  
1649 mock predator–prey assay between *S. cerevisiae* and the nem-  
1650 atode *C. elegans*.

1651 *C. elegans* is a free-living nematode that is commonly used  
1652 in research laboratories (Brenner 1974), and has been used  
1653 to study the effects of *C. albicans* and *S. cerevisiae* accumula-  
1654 tion in the gut (Jain *et al.* 2009; Bois *et al.* 2013). *C. elegans*  
1655 feed on bacteria and other microbes including yeasts (Félix  
1656 and Braendle 2010). The pharynx, a neuromuscular pump,  
1657 contracts and relaxes rhythmically to draw in liquid and sus-  
1658 pended particles to grind them up before transporting them  
1659 to the intestine. Although the pharynx pumps continuously,  
1660 previous studies have shown that microbe size is a determin-  
1661 ing factor in *C. elegans* feeding; large food is often excluded  
1662 during this grazing behavior (Avery and Shtonda 2003;  
1663 Shtonda and Avery 2006; Fang-Yen *et al.* 2009). One function  
1664 of cell adhesion molecules is to allow cells to adhere to each  
1665 other, leading to large colonies. To observe the impact of such  
1666 adhesion, wild-type *S. cerevisiae* cells were grown in condi-  
1667 tions that either permit cell–cell adherence (Figure 7A, YPD)  
1668 or suppress adherence (Figure 7A, synthetic). *C. elegans* were  
1669 transferred into suspensions of *S. cerevisiae* as per established  
1670 methods (Bois *et al.* 2013). We found that less-adherent *S.*  
1671 *cerevisiae* (Figure 7A, synthetic) were taken into the pharynx  
1672 of *C. elegans* in greater numbers than *S. cerevisiae* that ad-  
1673 hered to each other (Figure 7A, YPD). This finding is consis-  
1674 tent with the idea that cell–cell adhesion protects cells from  
1675 ingestion by *C. elegans*.

1676 We next assessed how yeast cells growing in adhesive  
1677 colonies might impact the feeding behavior of *C. elegans*.  
1678 To test this possibility, the time required for worms to pene-  
1679 trate yeast colonies from nose to tail was measured for  $\leq$   
1680 100 sec (Figure 7, C and D and Videos S8–S15). Worm stall-  
1681 ing and reversing out of the colony was also examined (Fig-  
1682 ure 7E). After 3 days of colony growth, a wild-type yeast  
1683 strain that formed normal, ruffled colonies slowed *C. elegans*  
1684 as it attempted to penetrate the colony (Figure 7, C–E, and  
1685 Videos S8 and S9). In some cases, worms abandoned enter-  
1686 ing the colony entirely. In contrast, a *flo11Δ* colony, which  
1687 fails to form ruffled colonies, did not deter worms from enter-  
1688 ing (Figure 7, C–E, and Videos S10 and S11). *flo11Δ* col-  
1689 onies were equally vulnerable to nematode entry as a colony  
1690 of OP50, an *E. coli* laboratory food strain for *C. elegans*  
1691 (Brenner 1974) (Figure 7, D and E, and Videos S12 and  
1692 S13). Additionally, *C. elegans* transferred directly into  
1693 *flo11Δ S. cerevisiae* colonies ingested more cells than *C. ele-*

1699 *gans* transferred into wild-type *S. cerevisiae* colonies (Figure  
1700 7, F and G). It is important to note here that our results out-  
1701 line an association between a lack of physical barrier and a  
1702 higher rate of being consumed by *C. elegans*. As such, we  
1703 expect any mutant that lacks the physical barrier conferred  
1704 by adhesive colonies would be similarly vulnerable.

1705 *C. elegans* might be excluded from wild-type colonies due  
1706 to the physical barrier encountered by wild-type colonies  
1707 expressing *FLO11*. Additionally, exclusion might also result  
1708 from a change in a worm’s sensory response to the colony  
1709 surface. Wild-type *C. elegans* halt their forward locomotion  
1710 and initiate backward movement in response to a light touch  
1711 to their anterior-most tip (nose). This avoidance of touch to  
1712 the worm’s nose has been termed the nose-touch response  
1713 (Kaplan and Horvitz 1993), a behavior involving the ASH <sup>10</sup>  
1714 polymodal nociceptive sensory neurons (with a small contri-  
1715 bution from the FLP and OLQ sensory neurons) (Kaplan and  
1716 Horvitz 1993). The glutamate-gated ion channel GLR-1 func-  
1717 tions in the downstream command interneurons and is re-  
1718 quired for the nose-touch response (Hart *et al.* 1995; Maricq  
1719 *et al.* 1995). To test the possibility that *C. elegans* do not  
1720 effectively enter yeast colonies due to the activation of this  
1721 mechanosensory avoidance response, experiments were per-  
1722 formed with *C. elegans glr-1(n2461)* loss-of-function (*lof*)  
1723 mutant animals, which are defective for nose touch, exposed  
1724 to a wild-type yeast colony. The *glr-1(lof)* mutant animals  
1725 penetrated colonies faster than wild-type worms, though this  
1726 was still slower than wild-type worms penetrating *flo11Δ*  
1727 yeast colonies (Figure 7D, and Videos S14 and S15). In ad-  
1728 dition, the *glr-1(lof)* mutant animals had fewer stalls and  
1729 reversals upon initial colony entry than wild-type *C. elegans*  
1730 (Figure 7E). Taken together, our data reveal a clear associa-  
1731 tion between the formation of adhesive contacts between  
1732 yeast cells in surface-growing colonies and predation in a  
1733 laboratory setting, both by becoming too large to eat and also  
1734 by forming a physical barrier. Further experiments are  
1735 needed to establish if protection against macroscopic preda-  
1736 tors is actually an evolutionary driving force for this  
1737 phenotype.

### 1738 ***Flo11p*-dependent ruffles impact colonial** 1739 **heat dissipation**

1740 Colony ruffling increases the surface-to-volume ratio and may  
1741 provide benefits such as efficient thermoregulation, or other  
1742 such adaptations to the environment (Palková and Váchová  
1743 2006). We also observed that heat-shock response proteins  
1744 were among the most induced surface-growth targets (Figure  
1745 6A, *HSP33* and *SNO4*). To test whether ruffles made by  
1746 *Flo11p*-dependent adhesion impact the temperatures of col-  
1747 onies, ruffled wild-type colonies and smooth *flo11Δ* colonies  
1748 were examined by infrared imaging (Figure 8A). Thermal  
1749

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1694 borne GFP (PC2560). *C. elegans* transferred into the colony for 45 min before mounting. Merged fluorescent images of head and tail regions of *C.*  
1695 *elegans* showing fluorescent *S. cerevisiae* inside the animal (merged). Fluorescent images of individual *S. cerevisiae* inside the animal (inset = GFP). (G)  
1696 Bar graph showing difference in average number of *S. cerevisiae* cells in the *C. elegans* pharynx between WT and *flo11Δ* colonies. Error bars are SE.  
1697 \*  $P < 0.05$ . WT, wild-type; YEPD, yeast extract, peptone, and dextrose.



1755 imaging showed that the average temperature of a ruffled  
1756 colony was 0.31° Cooler than the average temperature of a  
1757 smooth colony (Figure 8B, black bars,  $P < 0.05$ ,  $n = 6$ ). The  
1758 average coolest region of a ruffled colony was 0.33° Cooler  
1759 than the average coolest region of a smooth colony (Figure  
1760 8B, white bars,  $P < 0.05$ ,  $n = 6$ ). Additionally, the coolest  
1761 regions of the ruffled colonies (Figure 8C,  $<30^\circ$ ) were the  
1762 ruffles themselves (Figure 8C, merged). In a study of cell wall  
1763 stresses, *flo11Δ* cells were also shown to have modest tem-  
1764 perature sensitivity at 37° (Chow *et al.* 2018). In summary,  
1765 Flo11p-dependent ruffling might aid thermoregulation in  
1766 yeast colonies.

## 1767 Discussion

1770 Here, we explored the role of signaling pathways that regulate  
1771 filamentous growth in mediating adhesion-based surface re-  
1772 sponses, including mat formation and colony patterning.  
1773 Fungal cells commonly grow on surfaces, which poses unique  
1774 challenges due to the heterogeneity of the nutrients within  
1775 different parts of the colony and direct exposure to environ-  
1776 mental conditions. Understanding the responses to growth on  
1777 surfaces is important because fungal pathogens exhibit mat  
1778 growth and form invasive filaments (e.g., hyphae) on the  
1779 surface of the host, and on inert surfaces; during early steps  
1780 in host colonization.

### 1781 Analysis of signaling pathways that coregulate 1782 adhesion-dependent surface responses

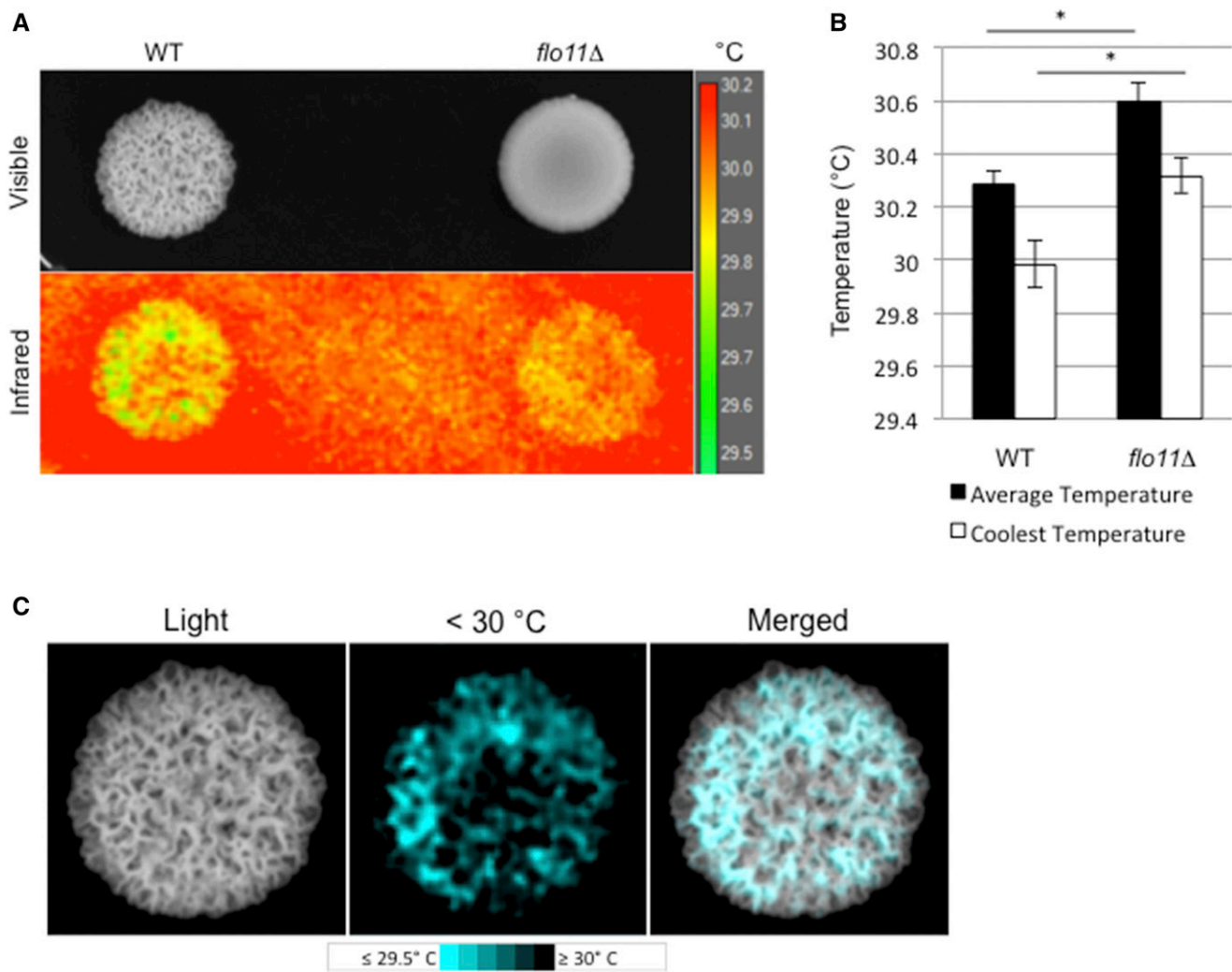
1783 Intracellular signaling pathways can operate in functionally  
1784 interconnected networks (Levchenko 2003). How multiple  
1785 pathways operate in a coordinated manner to achieve mor-  
1786 phogenetic responses with high fidelity remains a mystery. By  
1787 examining the major signaling pathways that regulate fila-  
1788 mentous growth in yeast, we have identified new roles for the  
1789 filamentation network in regulating colony patterning and  
1790 mat growth. Thus, the filamentation regulatory pathways  
1791 may have a general function in regulating the growth of cells  
1792 in surface communities.

1794 We also have identified the chromatin remodeling com-  
1795 plex, SAGA, to be a regulator of filamentous growth. SAGA  
1796 and the previously characterized Rpd3p pathway (Bernstein  
1797 *et al.* 2000; Chavel *et al.* 2010) are involved in the epigenetic  
1798 modification of chromatin. SAGA is an evolutionarily con-  
1799 served chromatin remodeling complex and a member of the  
1800 histone acetyltransferase family of proteins (Wang and Dent  
1801 2014). SAGA's functions are diverse but include changes in  
1802 transcription that result in cell differentiation (Wang and  
1803 Dent 2014; Hirsch *et al.* 2015). In yeast, SAGA controls the  
1804 expression of a specific set of growth-promoting genes  
1805 (Bruzzone *et al.* 2018). In fission yeast, TORC1 and TORC2  
1806 converge to regulate SAGA in response to nutrient availabil-  
1807 ity (Laboucaré *et al.* 2017). Moreover, components of SAGA  
1808 have recently been shown to impact the virulence of  
1809 *Fusarium* (Gao *et al.* 2014).

1811 Expression profiling was performed to evaluate the roles  
1812 for a subset of pathways that regulate adhesion-dependent  
1813 surface growth. We identified an unexpectedly large number  
1814 of differentially expressed genes. Many of the genes were  
1815 regulated by a single pathway. However, as might be expected  
1816 of a dense network of functionally connected pathways, each  
1817 pathway impacted every other pathway by regulating over-  
1818 lapping targets as well as genes encoding pathway compo-  
1819 nents. A key example of this overlap, even in the expanded set  
1820 of pathways, was the gene encoding the adhesion molecule  
1821 Flo11p. This may be expected given the critical roles that  
1822 Flo11p plays in regulating filamentous growth (Rupp *et al.*  
1823 1999), mat formation (Reynolds and Fink 2001), and colo-  
1824 nial patterning (Granek and Magwene 2010). Moreover, the  
1825 *FLO11* gene is a hub where many signaling pathways and  
1826 transcription factors converge (Rupp *et al.* 1999). The other  
1827 gene was *PGU1*, which encodes a secreted plant cell wall-  
1828 degrading enzyme. Given that one surface that budding yeast  
1829 commonly encounter is the surface of plants, especially rot-  
1830 ting fruit, it may not be surprising that yeast induce this gene  
1831 when undergoing surface growth. Transcriptional induction  
1832 of the *PGU1* gene may also result from the growth of cells on  
1833 agar, which is a potential substrate, and may be independ-  
1834 ently regulated. Therefore, one function of the filamenta-  
1835 tion regulatory network may be to coordinately regulate  
1836 target genes that are needed to respond to the challenges  
1837 of growing on surfaces.

1838 We further uncovered new connections between signaling  
1839 pathways that regulate adhesion-dependent surface growth.  
1840 One connection was between the RAS and RTG pathways,  
1841 which coregulated a substantial number of target genes. The  
1842 coregulated targets support a function for the RAS pathway in  
1843 mitochondrial control. The RAS pathway is a global nutrient-  
1844 sensing pathway in yeast and other organisms (Zaman *et al.*  
1845 2008). The fact that RAS coregulates targets of the RTG  
1846 pathway supports the idea that RAS plays a critical role in  
1847 the response to mitochondrial stress. Our results are consis-  
1848 tent with previous observations that connect RAS to the over-  
1849 all regulation of the mitochondria. RAS is required for growth  
1850 on nonfermentable carbon sources, mitochondrial enzyme  
1851 content (Dejean *et al.* 2002), and citrate synthase activity  
1852 (Swiegers *et al.* 2006; Chavel *et al.* 2014). Interestingly, com-  
1853 ponents of the RAS pathway, including the GTPase activating  
1854 protein *Iralp* and adenylate cyclase *Cyr1p*, associate with  
1855 mitochondrial membranes, which might impact RAS path-  
1856 way function or activity at this site (Belotti *et al.* 2012). More-  
1857 over, RAS has been implicated in working with RTG to  
1858 promote longevity in yeast (Kirchman *et al.* 1999). Thus,  
1859 the functional interaction between the two pathways to reg-  
1860 ulate aspects of mitochondrial health might impact the cell's  
1861 overall life span. Further experiments will be required to de-  
1862 termine how the RAS and RTG pathways coordinate the re-  
1863 sponse to mitochondrial problems. Though the nature of this  
1864 relationship is unclear, subsequent studies may better char-  
1865 acterize the mechanism by which each pathway regulates the  
1866 other's hallmark targets.

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**Figure 8** Effect of cell–cell adhesion on thermoregulation. (A) Visible and infrared images of WT and *flo11Δ* colonies. Colonies were incubated at 30° and removed from the incubator. The image was taken at < 30 sec after removal of lid. Scale temperature in degrees Celsius. (B) Difference in average and coolest recorded temperatures between WT and *flo11Δ*. \*  $P < 0.05$ ,  $n = 6$ . (C) Visible light (Light) and thermal images of cool colony regions (< 30°) of the WT colony from (A). “Merged” shows the overlapping of cool regions with colony ruffles. WT, wild-type.

We also show that *Dig1p* and *Ste12p*, which are commonly thought to reciprocally regulate the same set of target genes, actually regulated a partially nonoverlapping set of targets. One that stood out in the *Dig1p*-regulated set were genes that play a role in zinc uptake and metabolism. This observation is consistent with a previous set of findings that show unique expression profiles and binding sites for this activator–repressor pair (Breitkreutz *et al.* 2003; Zeitlinger *et al.* 2003).

**Yeast respond to surface growth by expressing a subset of stress-response genes**

We also explored the hypothesis that the activity of signaling pathways may change in different parts of a colony over time. We found this to be the case: the activity of the fMAPK pathway was different in different parts of the colony and in different aged colonies. This finding has broad implications in understanding how target genes might be induced during surface growth. For example, it might be expected that other time

points or harvesting from certain regions of the colony might result in different expression profiles. The comparative RNA-seq analysis performed here may be an oversimplification of the actual gene expression changes that occur within a colony, which may vary in different parts of a colony and over time.

In the study, we also examined genes that were differentially expressed during surface growth as compared to growth in liquid culture. The genes that were differentially expressed tell us about the challenges of growing in the unique environment of surface growth. Several of the differentially expressed genes identified fit with what one expects cells to encounter when growing on surfaces. One might expect that cells experience anaerobic stress within colonies compared to when growing in liquid conditions, where they are uniformly aerated by shaking. Likewise, cells in colony exteriors would be expected to be vulnerable to desiccation. Interestingly, we show that colony growth patterns change in response to these

stresses. Specifically, differentially regulated genes impact colony patterning in response to stress.

One prominent class of differentially expressed genes regulates the heat-shock response. This discovery was at first perplexing, given that cells were grown in liquid and on plates at the same temperature. However, thermal imaging verified that colonies do not have a uniform temperature. This may result from the generation of heat due to metabolic activities of cells within the colony, which may lead to temperature differences in different parts of the colony. We also show that ruffles dissipate heat more efficiently than other parts of the colony. The increased surface area of ruffles may promote heat dissipation. Therefore, another function of colony pattern formation might be to efficiently dissipate heat that results from cellular metabolic activities.

### **Adhesive contacts between yeast cells may offer protection from macroscopic predators**

Yeast and other microbes produce colonies that have a ruffled appearance due to contacts between cells. One established reason for cooperation among individuals in microbial populations is protection (West *et al.* 2007). For example, cells in the exterior of flocs protect cells in the interior from toxins and antibiotics (Smukalla *et al.* 2008). Microbial cells are also subject to predation by macroscopic predators. Macroscopic predators, like nematodes, graze on microbial communities. Predator-prey interactions have been studied in bacterial populations, which have developed diverse strategies to evade macroscopic predators. Depending on the species, bacteria have been shown to evade predators by the formation of mats (Darby *et al.* 2002; DePas *et al.* 2014; Nandi *et al.* 2016), by the secretion of proteases (Vaitkevicius *et al.* 2006) and exopolysaccharides that interfere with the predator's responses (Begun *et al.* 2007), and by phase variation, in which bacterial cells express different classes of cell surface proteins to evade detection (Dahl *et al.* 2011).

Although predator-prey studies are less-well characterized in fungal species, yeast and other fungal microorganisms have ecologically relevant interactions with animals in the wild, including grazing by wasps (Stefanini *et al.* 2016), flies (Fischer *et al.* 2017), and other invertebrates. Using two well-characterized laboratory genetic models, we provide evidence that adhesive contacts between yeast cells may deter efficient predation by nematodes. While individual cells in suspension were vulnerable to being ingested, cells that adhered to each other and formed clumps were too large to be eaten. Once cells settled out of suspension and grew into a colony, adhesive colonies formed a physical barrier to deter penetration by worms and also trigger the "nose-touch" mechanosensory behavioral avoidance response. Although the predator-prey model set up in the laboratory may or may not reflect a true ecological relationship between the two species, experiments using model organisms can provide insights into encounters that may occur in the wild. Our data provide an empirical framework to test the hypothesis that

the physical interaction among yeast cells in communities can protect individuals from macroscopic predation in the wild.

In conclusion, we have explored the roles of filamentous growth regulatory pathways in regulating multiple adhesion-dependent surface responses in yeast. Dissection of the regulatory pathways by comparative RNA-seq and mutant phenotype analysis revealed new components and targets, which emphasize the common and unique roles that pathways play in coordinately regulating colonial surface responses in this organism. Stress-responsive genes that respond to challenges associated with surface growth were also characterized. The signaling components, target genes, and stress-responsive genes identified here might be common among other fungi, including pathogens. Our results may extend specifically to early steps in fungal virulence, where cells must attach to and penetrate surfaces to colonize the host.

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Author contributions: J.C. designed and performed experiments and wrote the paper. I.S. analyzed the data. S.-J. performed experiments and analyzed the data. O.M. performed experiments. O.G. analyzed the data. D.M.F. designed experiments. A.K. analyzed the data. P.J.C. designed experiments and wrote the paper. The authors have no competing interests in the study.

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