Role of the Cell Wall Integrity and Filamentous Growth Mitogen-Activated Protein Kinase Pathways in Cell Wall Remodeling during Filamentous Growth[∇][†]

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Many fungal species including pathogens exhibit filamentous growth (FG) as a means of foraging for nutrients. Genetic screens were performed to identify genes required for FG in the budding yeast *Saccharomyces cerevisiae*. Genes encoding proteins with established functions in transcriptional activation (*MCM1*, *MATα2*, *PHD1*, *MSN2*, *SIR4*, and *HMS2*), cell wall integrity (*MPT5*, *WSC2*, and *MID2*), and cell polarity (*BUD5*) were identified as potential regulators of FG. The transcription factors *MCM1* and *MATα2* induced invasive growth by promoting diploid-specific bipolar budding in haploid cells. Components of the cell wall integrity pathway including the cell surface proteins Slg1p/Wsc1p, Wsc2p, Mid2p, and the mitogen-activated protein kinase (MAPK) Slt2p/Mpk1p contributed to multiple aspects of the FG response including cell elongation, cell-cell adherence, and agar invasion. Mid2p and Wsc2p stimulated the FG MAPK pathway through the signaling mucin Msb2p and components of the MAPK cascade. The FG pathway contributed to cell wall integrity in parallel with the cell wall integrity pathway and in opposition with the high osmolarity glycerol response pathway. Mass spectrometry approaches identified components of the filamentous cell wall including the mucin-like proteins Msb2p, Flo11p, and subtelomeric (silenced) mucin Flo10p. Secretion of Msb2p, which occurs as part of the maturation of the protein, was inhibited by the ß-1,3-glucan layer of the cell wall, which highlights a new regulatory aspect to cell wall remodeling in this organism. Disruption of ß-1,3-glucan linkages induced mucin shedding and resulted in defects in cell-cell adhesion and invasion of cells into the agar matrix.

Many fungal species undergo filamentous growth (FG) as a means of expanding into new environments (163, 165). The FG pattern is typically regulated by signal transduction pathways, which sense and respond to extracellular stimuli such as nutrient availability (172). FG is required for the virulence of fungal pathogens like *Candida albicans* (85, 165). In the FG mode, *C. albicans* and other fungal species express a highly varied collection of cell surface proteins (22) to modulate their adherence properties and create cell surface variation (53, 105). Changes in the adhesive properties of the fungal cell surface also contribute to the expansion of cells in connected mats of cells called biofilms, which in pathogens also promote virulence (108). Therefore, understanding the genetic pathways that underlie fungal foraging behaviors is an area of interest.

In the budding yeast *Saccharomyces cerevisiae*, nutrient limitation induces FG (invasive/pseudohyphal), which results in the formation of chains of elongated cells connected in branched filaments (31, 49, 84, 133). Both haploid (*MAT**a*** and *MATα*) and diploid (*MAT**a***/*MATα*) cell types undergo FG (84, 133). Among the phenotypes associated with FG is a delay in the G_2 phase of the cell cycle, reorganization of cell polarity, and changes in cell-cell and cell-substrate adherence (72, 139, 160). These changes are regulated by signaling pathways that include the RAS-cyclic AMP-protein kinase A pathway (100, 101), the target of rapamycin (TOR) pathway (134, 161), and a mitogen-activated protein kinase (MAPK) pathway commonly referred to as the FG pathway (10, 133). The FG pathway is regulated by the polarity establishment GTPase Cdc42p (78, 119), which is a member of the Rho family of small GTPases (117) and a global regulator of cell polarity and signal transduction (63). Cdc42p associates with the p21-activated kinase Ste20p (78, 119) to activate the Ser/Thr kinases Ste11p-Ste7p-Kss1p (93). Phosphorylation of the MAPK Kss1p activates the protein, which itself phosphorylates and activates transcription factors Ste12p and Tec1p (92) that induce the expression of target genes. Among the transcriptional targets of the FG pathway is the gene encoding the cell surface flocculin Flo11p (132, 140).

The cell surface glycoprotein Msb2p is a member of the signaling mucin family of proteins (21, 148), which connects to Cdc42p and is required for FG pathway activity (29). The adaptor protein Sho1p also functions in the FG pathway and associates with Msb2p and the guanine nucleotide exchange factor for Cdc42p, Cdc24p (145, 157). Most of the proteins that function in the FG pathway are required for the activation of other MAPK pathways in the cell (125, 129, 154), and it remains unclear how pathway-specific identity is maintained between MAPK pathways that share components (6). Under nutrient-limiting conditions, Msb2p is processed in its extracellular domain by the aspartyl protease Yps1p, which is required for the activation of the FG pathway (157).

In this study, genetic screens were performed to identify proteins that regulate FG in yeast. Analysis of the genetic data uncovered new insights into the filamentation response. We

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TABLE 1. Yeast strains

Name[a]	Description	Reference or source
PC312	*MATα ura3-52*	84
PC313	*MATa ura3-52*	84
PC538	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52*	29
PC622	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-SHO1::KanMX6*	29
PC344	*MATa/MATα ura3-52/ura3-52*	31
PC999	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA*	29
PC209[b]	*MATα ste4::LEU2 FUS1-lacZ FUS1-HIS3 ura3-52 far1 GAL-STE4 rad51 ade1 lys2*	29
PC2043	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA*	Karunanithi et al., submitted
PC367	*MATa ura3-52 slt2::URA3*	30
PC1029	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 flo11::KanMX6*	Karunanithi et al., submitted
PC1531	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sho1::HYG*	29
PC986[c]	*MATa ura3-52 leu2 trp1 his3*	Open Biosystems
PC948	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 msb2::KanMX6*	29
PC673	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste20::KanMX6*	This study
PC2382	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste12::KanMX6*	This study
PC693	*MATa ura3-52 leu2 ste12::LEU2*	This study
PC507	*MATa ura3-52 snf1::LEU2*	This study
PC670	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-SHO1::KanMX6 pbs2::URA3*	This study
PC624	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-SHO1::KanMX6 ste12::URA3*	This study
PC2963	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO10-HA*	This study
PC3008	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 SLG1-HA*	This study
PC3006	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FUS1-HA*	This study
PC3007	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 RAX2-HA*	This study
PC3009	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 CTR1-HA*	This study
PC2942	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 slg1::HAURA3HA*	This study
PC2912	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 flo10::HAURA3HA*	This study
PC1312	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-WSC2*	This study
PC3023	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-FLO10*	This study
PC549	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste20::URA3*	This study
PC368	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pbs2::URA3*	This study
PC3394	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 slt2::ura3*	This study
PC1310	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-MID2*	This study

[a] All strains are in the Σ1278b background unless otherwise indicated.
[b] Derived from the 252JHa background.
[c] PC986 represents the wild-type strain from the S288c background. Ordered deletions from the S288c background were also used (46).

found that haploid and diploid cells exhibit different agar invasion patterns on different substrates, and genes that regulate cell type specification contribute to the invasive growth pattern. We also found that cell wall remodeling, which is controlled by the cell wall integrity pathway (82) and the FG pathway, occurs under nutrient-limiting conditions. We further show that the cell wall integrity pathway contributes to FG pathway regulation at the level of the cell surface proteins that comprise the two pathways. Mass spectrometry and directed genomics approaches were used to identify components of the filamentous cell wall, which led to the finding that shedding of Msb2p is regulated by the ß-glucan layer. Our findings provide new insights into the combinatorial effects of signaling networks in mediating differentiation to the FG pattern. The critical role the cell wall plays in the filamentation response strengthens the prevailing idea that the cell wall is a logical structure to target in the control of fungal pathogenesis.

MATERIALS AND METHODS

Strains, plasmids, and microbiological techniques. Yeast strains are described in Table 1. Most strains are isogenic to the Σ1278b parental strain HYL333 (PC313) (84). Plasmids are listed in Table 2. pRS316 and related plasmids have been described previously (147). Overexpression constructs were obtained from an ordered collection obtained from Open Biosystems (45). Gene disruptions and *GAL1* promoter fusions were made by PCR-based methods (7, 88) using plasmids provided by John Pringle (Stanford University, Palo Alto, CA). Some disruptions were created by the use of antibiotic resistance markers on cassettes HYG and NAT (50). Internal epitope fusions were created as described previously (143) using plasmids containing the three copies of the Myc and hemagglutinin (HA) epitopes. Integrations were confirmed by PCR analysis. Plasmids pMCM1[1–97] (plasmid expressing residues 1 to 97 of MCM1), pMCM1[1–276], pMCM1[1–286], and pGAL-α2 were provided by G. Sprague (14). Deletion mutants were obtained from an ordered collection (166).

Yeast and bacterial strains were manipulated by standard methods (138, 142). All experiments were carried out at 30°C unless otherwise indicated. The ß-galactosidase assays were performed as described previously (30) and represent the average of at least two independent trials. YEPD (yeast extract, peptone, and dextrose), SCD (synthetic complete medium plus dextrose), and BBMB (YEPD plus 0.1 M citrate, ph 4.5, and 0.3% [wt/vol] methylene blue) plates were prepared by standard methods (2). For some experiments, the mating-specific reporter *FUS1* (97) was examined in cells lacking an intact mating pathway (*ste4Δ*). In this context, *FUS1* exhibits Msb2- and FG pathway-dependent expression (29). *FUS1-HIS3* expression was used to confirm *FUS1-lacZ* reporter data and was measured by spotting equal amounts of cells onto synthetic medium lacking histidine and containing 4-amino-1,2,4-triazole. The single-cell invasive growth assay (31) and a plate-washing assay (133) were performed to evaluate FG. Budding pattern was based on established methodology (27), using calcofluor white (CFW) and was confirmed for some experiments by visual inspection of connected cells. The assignment of process and function to genes was facilitated by public databases, particularly the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) (28, 58).

Genetic screens. Wild-type cells (PC538) were transformed with a high-copy-number YEp24 library (18), and ~9,600 colonies were screened on synthetic dextrose medium with Ura (SD-Ura) at a density of ~600 colonies/plate for those that exhibited enhanced agar invasion. Plates were replica plated and washed thoroughly to identify hyperinvasive colonies. Twenty-five colonies were identified, two of which showed plasmid-dependent phenotypes by patching colonies on 5-fluoroorotic acid. Restriction digest mapping and sequence anal-

TABLE 2. Plasmids used in this study

Name in the present study	Alternate name	Function or description	Reference or source
PC2207	pRS316	CEN/URA3 control	147
PC188	YEp24	Control plasmid	Sprague laboratory
PC207	YCp50	CEN/URA3 control	Sprague laboratory
PC369	YEp24 library pool	High-copy-number library	18
PC3105	λ YES library pool	High-copy-number library	127
PC20a and -b	YCp50 library pool	CEN-based library	137
PC187	pSTE4	YCp50 plasmid	Sprague laboratory
PC1417	pFLO8	YEplac181-FLO8 URA3	84
PC3085	pGAL-MATa1	*MATa1* overexpression construct	Sprague laboratory
PC3133	pMSB2-lacZ	*CEN/URA3 MSB2-lacZ* translational fusion	121
PC1820	pMSB2-HA	CEN/URA3 expressing Msb2p-HA from its endogenous promoter	157
PC1043	pSVS1-lacZ	FG pathway reporter	132
PC1044	pYLR042C-lacZ	FG pathway reporter	132
PC1042	pPGU1-lacZ	FG pathway reporter	132
PC3080	pMCM11-91	MCM1 derivative	14
PC3081	pMCM11-156	MCM1 derivative	14
PC3079	pMCM11-187	MCM1 derivative	14
PC370	YEp24-MCM1	YEp24 library isolate	This study
PC371	Yep24-MATα1α2-BUD5	YEp24 library isolate	This study
PC206	pMPT5	YCp50 library isolate	This study
PC3083	YEp24 MATα1α2	YEp24 plasmid	This study
PC3082	pλ YES	Vector control	This study
PC527	pPHD1	λYES library isolate	This study
PC528	pSIR4	λYES library isolate	This study
PC529	pMSN1	λYES library isolate	This study
PC530	pHMS2	λYES library isolate	This study
PC1236	pλ YES-MID2	λYES library isolate	This study
PC1271	pλ YES-WSC2	λYES library isolate	This study
PC3084	YCp50MATα1α2-BUD5	λYES library isolate containing only the *MATα1α2 BUD5* genes	This study
PC3086	YEp24-MATα1α2-BUD5EagI deletion	λYES library isolate containing only the MATα1α2 genes	This study

ysis were used to confirm plasmid inserts. For the λ YES screen, ~50,000 colonies transformed with the λ YES library (127) were examined by plate washing on synthetic medium supplemented with 2% galactose (Gal). Plasmid-dependent isolates were similarly identified. In a third screen, plasmids that induce *FUS1-HIS3* expression in an *ste4Δ* strain (PC538) were identified by screening λ YES overexpression library transformants for growth on SD-His medium supplemented with 3-amino-1,2,4-triazole. In a separate screen, the *MPT5* gene was identified as a suppressor of a mutant (designated aa9) that exhibited elevated *FUS1-HIS3* expression. YCp50 library transformants (137) were plated at a density of ~200 colonies/plate on SD-Ura plates. Colonies were replica plated onto SD-Ura-His medium, and colonies that failed to grow were identified. *MPT5* was a plasmid-dependent isolate identified in the screen that suppressed *FUS1-HIS3* signaling in the aa9 mutant.

Analysis of Msb2p Secretion in cell wall mutants. To determine the effect of cell wall genes on Msb2p-HA secretion, haploid (*MAT***a**) ordered deletion collection isolates (166) were transformed with a plasmid carrying a functional epitope-tagged *MSB2* gene (pMSB2-HA). Transformants were pinned to SD-Ura medium, and once grown, were transferred to a 96-well plate containing 100 μl of water and pinned to SD-Ura medium overlaid with a nitrocellulose disc filter (0.4-μm pore size; HAHY08550; Millipore) and incubated for 48 h at 30°C. Filters were rinsed in distilled water to remove cells and probed with antibodies to the HA epitope (12CA5.16.4). To identify cell wall genes that when overexpressed influence the secretion of Msb2p-HA, a strain containing an integrated *MSB2-HA* gene at the *MSB2* locus and driven by its endogenous promoter (PC999) was transformed with a collection of plasmids containing defined yeast open reading frames under the control of the *GAL1* promoter (45). Transformants were selected on SD-Ura medium and screened by pinning to S+Gal-Ura medium on nitrocellulose filters. Pinned colonies were incubated for 2 days at 30°C, and filters were washed in a stream of water. Filters were probed by immunoblot analysis as above.

Microscopy. Differential interference contrast and fluorescence microscopy using rhodamine, fluorescein isothiocyanate, yellow fluorescent protein, and cyan fluorescent protein filter sets were performed using an Axioplan 2 fluorescent microscope (Zeiss) with a Plan-Apochromat 100×/1.4 (oil) objective (numerical

aperture, 0.17). Digital images were obtained with an Axiocam MRm camera (Zeiss). Axiovision, version 4.4, software (Zeiss) was used for image acquisition and analysis.

Immunological techniques. Immunoblot analysis was performed as described by Vadaie et al. (157). Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 10% precast gels (Bio-Rad, Hercules, CA) and electrophoretically transferred to Protran BA85 nitrocellulose membranes (VWR International Inc., Bridgeport, NJ). The membranes were incubated in blocking buffer (5% nonfat dry milk, 10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% Tween 20) for 1 h. The membranes were then incubated for 18 h at 4°C in blocking buffer containing either a mouse monoclonal antibody directed against HA (12C5; Roche Diagnostics, Indianapolis, IN) or a mouse monoclonal immunoglobulin G antibody directed against green fluorescent protein (Roche Diagnostics, Indianapolis, IN). After the membranes were washed, incubated with alkaline phosphatase-conjugated secondary antibody, and washed free of unbound secondary antibody, ECL Plus immunoblotting reagents were used to detect HA- and green fluorescent protein-tagged proteins (Amersham Biosciences, Piscataway, NJ). Monoclonal antibodies specific for actin were obtained from Chemicon (MAB1501; Chemicon; Billerica, MA).

Cell wall purification and isolation of secreted proteins. To generate yeast and filamentous cell walls, wild-type cells (PC538) were grown for 8 h in YEPD medium, YEP-Gal medium (2% Gal), or YEPD medium supplemented with 10 μg/ml alpha factor. Cells were harvested by centrifugation, washed in phosphate-buffered saline (PBS) buffer (140 mM NaCl, 3 mM KCl, 4 mM NaH$_2$PO$_4$, 1 mM KH$_2$PO$_4$), and resuspended in PBS at a titer of 10^8 cells/ml. The cells were transferred to ice-cold Fast-Prep tubes and subjected to 36 cycles of shaking on a Fast-Prep machine (MP Biomedical, Solon, OH). Each cycle was for 20 s at speed setting 6 on the machine, and the samples were cooled on ice for 60 s between each cycle. Broken cell walls were collected by centrifugation (5,000 × g for 10 min) and washed twice with PBS. SDS was added to the resuspended cell wall preparation to a concentration of 1%, and the samples were then subjected to a 10-min boiling step to remove any proteins that might be nonspecifically associated with the wall. The SDS-boiled cell wall preparations were then washed twice in PBS and treated for 30 min at 37°C with 1% ß-mercaptoethanol. The cell walls

were then collected and washed twice by centrifugation with ice-cold water to give purified cell wall preparations, which were lyophilized.

Secreted proteins were isolated by growing wild-type (PC313) cells for 48 h in SD medium (2% glucose). The cells were removed by centrifugation, and the proteins were precipitated by polyethylene glycol (PEG 8000) or trichloroacetic acid (TCA). The PEG precipitation was based on established methodology (4). A 50% PEG solution was added in a dropwise manner to collected medium to give a final PEG concentration of 8%. MgCl$_2$ was added to a final concentration of 10 mM. After incubation at 4°C for 30 min with stirring, the sample was centrifuged at 14,000 × g for 15 min, and the resultant pellet was lyophilized. For the TCA precipitation, acetone and TCA were added to the medium to give a final concentration of 50% acetone and 12.5% TCA, and the proteins were allowed to precipitate in a 24-h incubation at −20°C. The precipitated proteins were collected by centrifugation, washed twice with −20°C acetone, and lyophilized.

TFMS digestion. Under controlled anhydrous conditions, trifluoromethanesulfonic acid (TFMS) digests glycosidic linkages without cleavage of peptide bonds and can be used to digest cell wall glucans and release intact deglycosylated cell wall proteins (11). Cell walls and secreted protein samples were lyophilized to dryness. A 20-mg sample of the secreted protein or a 20-mg cell wall sample from each type of cell wall was placed in a 15-ml Corex tube and relyophilized, and 1.25 ml of a solution of 16% anisole in TFMS was added. The samples were subjected to digestion by TFMS at 4°C for 6 h (Sigma Aldrich, St. Louis, MO). The TFMS solution was purged with N$_2$ prior to adding it to the sample, and the tube was covered with parafilm and placed in a large chamber purged with N$_2$ gas. The samples were periodically purged with N$_2$ to maintain a water-free digestion. The digestions were stopped by the dropwise addition of 3.75 ml of a solution of pyridine-methanol-H$_2$O (3:1:1). The samples were continually swirled in a dry ice-ethanol bath during the addition of the pyridine-methanol-H$_2$0 and incubated on dry ice for an additional 20 min, followed by a 20-min incubation at −20°C. The samples were then allowed to thaw, and 1 ml of a 5% ammonium bicarbonate solution was added. The released cell wall proteins were collected by adding acetone and TCA (final concentrations of 50% acetone and 12.5% TCA) and allowing the proteins to precipitate during a 24-h incubation at −20°C. The proteins were collected by centrifugation at 5,000 × g for 10 min, washed three times with −20°C acetone, dried briefly, and resuspended in 1% SDS, and protein concentrations were determined using a Bio-Rad detergent-compatible protein assay kit (Bio-Rad, Hercules, CA). The deglycosylated proteins were then subjected to electrophoresis in SDS polyacrylamide gels. Following Coomassie blue staining, gel slices containing proteins were cut from the gels and sent for mass spectrometry analysis.

Protein identification by mass spectrometry analysis. Protein identification by liquid chromatography-tandem mass spectrometry (MS/MS) was carried out by Midwest Bio Services LLC (Overland Park, KS) from SDS-polyacrylamide gel electrophoresis slices. The slices were subjected to a trypsin digestion, and the released peptides were concentrated on a peptide trap column. The peptides were separated by microcapillary C$_{18}$ reverse-phase column chromatography. Individual peptides were then subjected to MS/MS analysis. Proteins were identified using the TURBOSEQUEST software, and the sequences of the parent peptides were inferred by matching the MS/MS spectra to the *S. cerevisiae* protein databases. Peptides identified as having a correlation coefficient (XC) value of >2.5 were considered to be significant matches. As an additional analysis, the data were screened for asparagines containing an attached GlcNac.

mRNA level determination using quantitative PCR. Total RNA was isolated from 25-ml cultures grown in YEP-Gal medium for 8 h using hot acid-phenol extraction. cDNA synthesis was carried out using 1 μg of RNA and an iScript cDNA Synthesis Kit (Bio-Rad; Hercules, CA) according to the manufacturer's instructions. One-tenth of the synthesized cDNA was used as the template for real-time PCR. Real-time PCRs were performed using 25-μl reaction mixtures on a Bio-Rad MyiQ Cycler with iQ SYBR green Supermix (170-888; Bio-Rad). Reverse transcription-quantitative PCR was performed using the following amplification program: initial denaturation for 8 min at 95°C, followed by 35 cycles of denaturation for 15 s at 95°C and annealing for 1 min at 60°C. Melt curve data collection was enabled by decreasing the set point temperature after the final annealing step by 0.5°C. The specificity of amplicons was confirmed by generating the melt curve profile of all amplified products. *ACT1* was used as the reference gene for normalization of mRNA levels. Gene expression was quantified as described previously (120). Primers were based on Voynov et al. (162) and were as follows: *FLO11*, 5′-GTTCAACCAGTCCAAGCGAAA (forward) and 5′-GTAGTTACAGGTGTGGTAGGTGAAGTG (reverse); *FLO10*, 5′-CTTCTTGTGAGTCTGGTGTCTGTTCCGAA (forward) and 5′-GGCACTATTTTCGATGTCAGAACTAACCGC (reverse); and *ACT1*, 5′-GGCTTCTTTGA

CTACCTTCCAACA (forward) and 5′-GATGGACCACTTTCGTCGTATTC (reverse).

RESULTS

Genetic screens identify new regulators of FG. Genetic approaches were undertaken to identify genes that regulate FG. Agar invasion is a hallmark for FG, and mutants that perturb FG typically exhibit agar invasion phenotypes (133). In one approach, an overexpression library (λ YES [127]) and a high-copy-number library (YEp24 [18]) were screened for genes that induce invasive growth in a wild-type strain (PC538) of the filamentous (Σ1278b) background. Because haploid strains of the Σ1278b background exhibit poor agar invasion on SD medium, library transformants were screened for enhanced invasion on SD medium by the plate-washing assay (133). Seven genes were identified (Table 3). In most cases, multiple library isolates were not identified, which indicates that the screens were not saturating. Three of the genes have previously been established as regulators of FG, *BUD5* (87), *PHD1* (47, 115), and *HMS2* (91) (Table 3), and two came out of a recent large-scale screen, *SIR4* and *MSN2* (62) (Table 3), which validates the identification of filamentation regulatory genes by this approach. Two genes not previously connected to FG were also identified (*MCM1* and *MATα2*) (Table 3).

In a related approach, genes were identified in a screen for genes that when overexpressed influence the activity of a transcriptional reporter (*FUS1-HIS3*), which in Σ1278b strains lacking an intact mating pathway (*ste4Δ*) is dependent on FG pathway components for expression (29). Two genes were identified that induced reporter expression (*WSC2* and *MID2*) (Table 3), and in a separate screen one gene was identified that dampened it, *MPT5* (65) (Table 3). In total, 10 genes were identified, half of which represent potentially new ties to FG regulation (Table 3). A subset of the genes required the FG pathway to exert their invasive effects; others did not (Table 3). Likewise, a subset of genes required the glucose-regulatory protein kinase Snf1p to exert their effects (18, 56, 74, 75) (Table 3). Therefore, the screens identified genes that influence invasive growth through at least two different filamentation control pathways. Functional classification of the genes showed connections between FG and cell type specification, transcriptional regulation, cell polarity, and cell wall integrity (Table 3).

MCM1 and MATα2 induce constitutive invasion by promoting diploid-specific polarity in haploid cells. We focused on genes that represent potentially new connections to FG regulation. *MCM1* encodes an essential transcription factor of the MADS box family (23, 39, 98) that is required for the expression of mating and stress-responsive genes (60). YEp24-*MCM1* induced agar invasion (Fig. 1A). The N terminus of Mcm1p is sufficient for DNA binding and transcriptional activation (14). A transcription-defective allele, *MCM1$^{1-91}$*, did not induce agar invasion, whereas full-length *MCM1* (*MCM1$^{1-286}$*) and transcription-competent alleles *MCM1$^{1-156}$* and *MCM1$^{1-187}$* induced invasive growth (data not shown). This result indicates that the effect of Mcm1p on FG requires its transcriptional activation function. Mcm1p functions at the same promoters as Ste12p for some genes although the proteins are thought to bind to different sites (110). Introduction of YEp24-MCM1 in the *ste12Δ* mutant

TABLE 3. Genes identified in genetic screens that influence FG

Gene	Library[a]	Screen[b]	MAPK[c]	Snf1[d]	Reference or source for connection to FG	Process	Function
BUD5	YEp24	HIG	No	ND	87	Cell polarity	GTP/GDP exchange factor for Rsr1p (Bud1p) required for axial and bipolar budding patterns
MPT5	YCp50	SFR	Yes	ND	This study	Cell wall integrity	Member of the Puf family of RNA-binding proteins
WSC2	λYES	HFR	Yes	ND	This study	Cell wall integrity	Sensor-transducer of the stress-activated PKC1-MPK1 pathway maintenance of cell wall integrity
MID2	λYES	HFR	Yes	ND	This study	Cell wall integrity	Sensor-transducer of the stress-activated PKC1-MPK1 pathway maintenance of cell wall integrity
HMS2	λYES	HIG	Partial	No	91	Transcription	Protein with similarity to heat shock transcription factors
PHD1	λYES	HIG	Partial	No	48, 115	Transcription	Transcriptional activator that enhances pseudohyphal growth
MSN2	λYES	HIG	No	No	62	Transcription	Transcriptional activator related to Msn4p; activated under stress conditions
MCM1	YEp24	HIG	No	ND	This study	Transcription; cell type	Cell-type- and pheromone-specific transcription factor MADS box family
MATα2	YEp24	HIG	No	ND	This study	Transcription; cell type	Cell-type- and pheromone-specific transcription factor homeobox family
SIR4	λYES	HIG	Partial	Yes	62	Transcription; cell type	Silencing regulator involved in assembly of silent chromatin domains at silent mating-type loci

[a] The sources for the libraries are as follows: YCp50, Rose et al. (137); YEp24, Carlson and Botstein (18); and λYES, Ramer et al. (127).
[b] HIG, library isolates that induce hyperinvasive growth; HFR, elevated expression of the *FUS1-HIS3* reporter; SFR, suppression of the *FUS1-HIS3* reporter.
[c] Determined by expression of FG pathway reporters and the *FUS1* reporter and suppression of the invasion defect of the *ste12* mutant (Fig. 1 and 2C).
[d] Determined by the ability to suppress the agar invasion defect of the *snf1* mutant. ND, not determined.

caused hyperinvasive growth (Table 3), which indicates that Mcm1p induces invasive growth independent of the FG pathway.

The other YEp24 plasmid identified in the screen contained the *BUD5* and *MATα2* genes. Restriction digest analysis showed that *BUD5* and *MATα2* independently induced invasive growth (Table 3). *BUD5* encodes the GTPase activating protein for Rsr1p and is required for bud site selection (25). Altering bud site selection has a potent effect on agar invasion
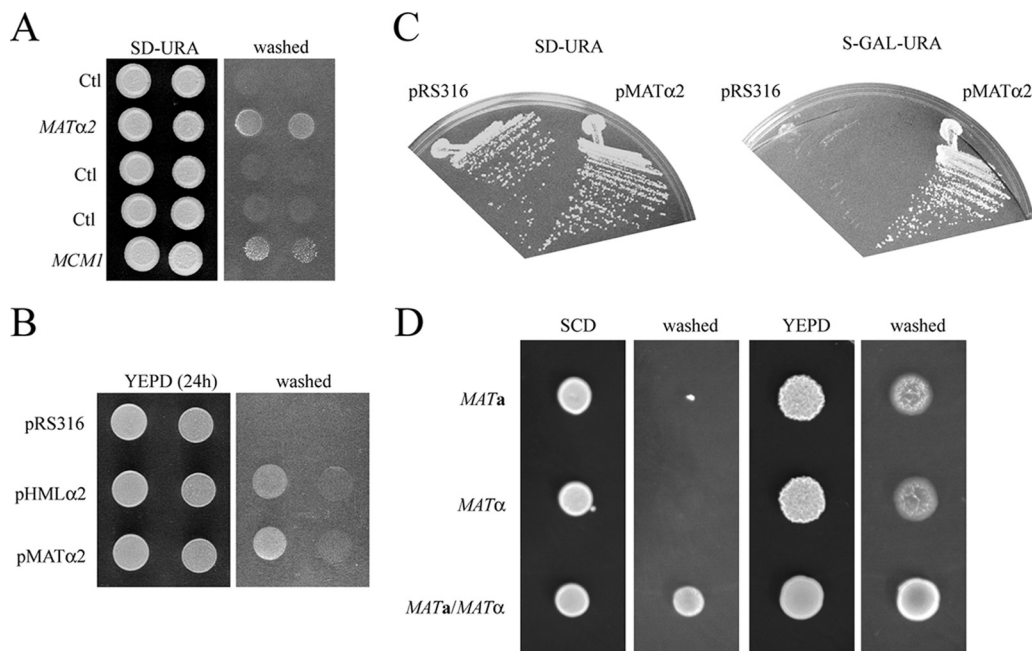

FIG. 1. High-copy-number *MCM1* and *MATα2* induce constitutive invasion by promoting diploid-like behaviors. (A) Constitutive agar invasion of cells harboring YEp24-Mcm1 (Mcm1) and YEp24-MATα2 (*MATα2*) compared to strains harboring control (Ctl) YEp24 plasmids. Equal concentrations of cells were spotted onto SD-Ura medium for 4 days. The plate was photographed (left panel), washed in a stream of water, and photographed again (right panel). (B) *MATα2* and *HMLα2* containing plasmids confer similar constitutive agar invasion phenotypes in wild-type cells (PC313) after a 24-h incubation on rich YEPD medium. (C) *MATα2* suppresses the growth defect of cells overexpressing *STE4* (PC2431), compared to a control plasmid (pRS316). Cells were patched onto SD-Ura and S-Gal-Ura medium for 2 days at 30°C and photographed. (D) Comparison of the invasive properties of haploid and diploid cells. *MATα* (PC312), MAT**a** (PC313), and *MAT**a**/MATα* (PC344) cells were spotted onto YEPD and SCD media. The plates were incubated for 2 days at 30°C, and the plates were photographed, washed, and photographed again.

(32), and the fact that *BUD5* was identified in the screen is not surprising. Like *MCM1*, *MATα2* conferred invasive growth in the *ste12Δ* mutant, indicating that its effect on invasive growth was independent of the FG pathway (Table 3).

MCM1 and *MATα2* function as determinants of cell type in yeast and when inappropriately expressed induce diploid-like behaviors in haploid cells (41, 59, 153). Consistent with this view, introduction of the silent copy of *MATα2*, *HMLα2*, induced invasive growth to a similar degree as *MATα2* (Fig. 1B). Moreover, overexpression of *MATα2* suppressed the mating pathway-induced growth arrest caused by overexpression of the G_β subunit *STE4* (Fig. 1C). Under high-nutrient conditions, haploid cells bud in an axial pattern, whereas diploid cells bud in a bipolar pattern (27) that is similar to the distal-unipolar pattern of haploid cells undergoing invasive growth (32). Haploid cells containing p*MATα2* exhibited bipolar budding (80% bipolar, 19% axial, and 1% random; $n = 200$ cells) compared to the axial pattern of cells containing a control plasmid (pRS316; 5% bipolar, 95% axial, and <1% random; $n = 200$ cells). Cells carrying p*MCM1* showed a similar effect (data not shown). Consistent with the idea that diploid-type budding leads to an invasive growth phenotype, diploid cells (*MATa*/*MATα*) exhibited constitutive agar invasion on synthetic medium and invaded better than haploid (*MATa* or *MATα*) cells (Fig. 1D). This phenotype explains the isolation of genes that promote diploid-type budding in the genetic screen. Both haploid and diploid cells exhibited similar invasive growth on rich medium (Fig. 1D) although diploid colonies were smooth in appearance, likely due to lower levels of *FLO11* expression in diploid compared to haploid cells (86). Mutants that confer bipolar budding in haploid cells (e.g., *bud3Δ*, *bud4Δ*, and *bud10Δ*) also induce constitutive invasion (32). The invasive pattern induced by *MATα2* was constitutive (rather than hyperinvasive) as it was observed in mid-log-phase cells after a 24-h incubation on YEPD medium (Fig. 1B) when wild-type haploid cells typically exhibit an axial budding pattern and are not invasive (31). The *SIR4* gene was also identified in the screen (Table 3) and may similarly induce invasive growth by derepression of silent mating type loci (131). Mcm1p and MATα2p did show slight differences in invasive growth in that Mcm1p induced a "speckled" pattern (Fig. 1A). Mcm1p may also contribute to FG regulation through transcriptional induction of other filamentation target genes (see below).

The cell wall integrity pathway contributes to FG. The genetic screens identified a potential connection between the cell wall integrity pathway and FG (Table 3). The cell wall integrity pathway is a MAPK pathway that senses and responds to cell wall stress during vegetative growth and in response to a variety of challenges including pheromone-induced morphogenesis and heat shock (68, 82). Cell surface components of the pathway, Wsc2p and Mid2p (83, 111, 126, 159), which came out of the genetic screen (Table 3), and the MAPK Slt2p/Mpk1p (79) were tested for a role in invasive growth. The *slt2Δ* and *wsc2Δ* mutants and to a lesser degree the *mid2Δ* mutant showed defects in invasive growth by the plate-washing assay (Fig. 2A). A third cell surface component, Slg1p/Wsc1p (159), also showed an invasive growth defect when absent from cells (see below). The *slt2Δ* mutant showed defects in cell-cell adhesion and cell elongation based on microscopic examination of invaded cells (Fig. 2B) and a modest reduction in *FLO11*

expression (Fig. 2C) but not to the same degree as FG pathway mutants (Fig. 2C). Cells overexpressing *MID2* and *WSC2* were somewhat hyperinvasive (Fig. 2A) and were hyperfilamentous (Fig. 2B).

MID2 and *WSC2* induced *FUS1* expression when overexpressed (Table 3). Because in this genetic context (*ste4Δ*) the *FUS1* reporter shows dependency on Msb2p, Sho1p, and other FG pathway components (29), the cell wall integrity pathway may feed into FG pathway regulation. A connection between the FG (Ste11p-Ste7p-Kss1p) and cell wall integrity (Bck1p-Mkk1p/Mkk2p-Slt2p) pathways has not previously been established. However, activated MEK kinases Ste7p and Mkk1p exhibit cross talk between the cell wall integrity and mating pathways (167). In protein glycosylation mutants, the cell wall integrity pathway and a pathway that resembles the FG pathway (Msb2p-Sho1p-Ste20p-Ste11p-Ste7p-Kss1p-Ste12p) are both activated and are required for viability (29, 30). In this context, an activated allele of the cell wall integrity MAPK kinase kinase *BCK1* stimulates cross talk to the Ste12p pathway (30). We found that overexpression of *MID2* and *WSC2* induced expression of FG pathway reporters *SVS1*, *PGU1*, and *YLR042C* (132) by approximately twofold (Fig. 2D) and the *FUS1* reporter by a factor greater than threefold (Fig. 2E). The boost in *FUS1* expression required Msb2p, Ste20p, and Ste12p (Fig. 2E). Sho1p was only partially required to mediate Wsc2p- and Mid2p-dependent *FUS1* expression (Fig. 2E). Slt2p was not required to mediate the elevated *FUS1* expression (Fig. 2E), which indicates that the cross talk between the pathways occurs upstream of Slt2p. These results connect cell surface components of the cell wall integrity and FG pathways and uncover a difference between Msb2p and Sho1p in signal transmission in the FG pathway.

The HOG and FG pathways have opposing effects on cell wall integrity. The FG and high-osmolarity glycerol (HOG) MAPK pathways require overlapping components and are thought to function in mutually exclusive activation states (34, 109, 121, 164). The HOG MAPK pathway functions to promote osmotolerance (12) in part through cell wall remodeling (43, 61). Cells lacking an intact HOG pathway were resistant to CFW(>0.24 mg/ml CFW on YEPD medium), whereas cells lacking an intact FG pathway were sensitive to CFW (<0.03 mg/ml CFW on YEPD medium) (data not shown), which indicates that the two pathways may have opposing effects on cell wall integrity. In support of this possibility, overexpression of *SHO1*, which is required for the activation of both pathways (29, 94), induced CFW sensitivity that was dependent on Pbs2p and that was exacerbated in the *ste12Δ* mutant (Fig. 3A). The addition of sorbitol, which stimulates the HOG pathway (122, 123), suppressed CFW sensitivity of cells overexpressing *SHO1* (Fig. 3A). The cell morphological phenotypes bore out this result. Overexpression of *SHO1* induced cell polarization that was dependent on the FG pathway and was inhibited by the HOG pathway (Fig. 3B). In the presence of CFW, overexpression of Sho1p induced cell lysis that was suppressed by the addition of sorbitol (Fig. 3B). The HOG and FG pathways may have opposing effects on cell wall integrity through induction of different transcriptional targets. Indeed, expression profiling identifies nonoverlapping cell wall targets of the two pathways (42, 121).
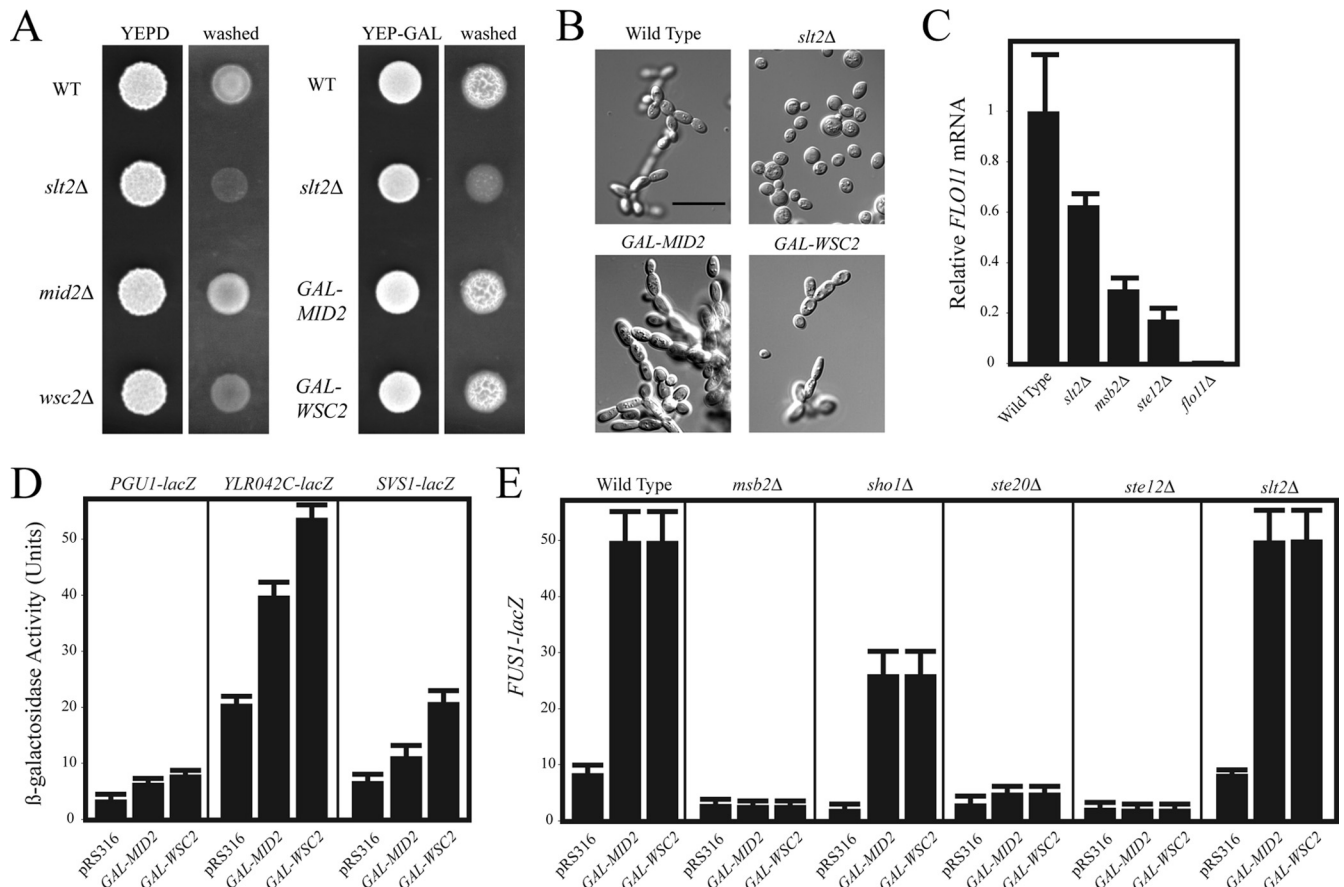
FIG. 2. The role of the cell wall integrity pathway in FG and FG pathway regulation. (A) Wild-type (WT; PC538), *slt2Δ* (PC367), *GAL-MID2* (PC1310), and *GAL-WSC2* (PC1312) strains were spotted onto YEPD or YEP-Gal medium for 4 days at 30°C. The plates were photographed, washed in a stream of water to reveal invaded cells, and photographed again. (B) Examples of wild-type (PC538), *slt2Δ* mutant (PC367), *GAL-MID2* (PC1310), and *GAL-WSC2* (PC1312) cells grown in YEP-Gal medium. Bar, 20 μm. (C) The level of *FLO11* mRNA in wild-type (PC538), *slt2Δ* (PC367), *msb2Δ* (PC948), *ste12Δ* (PC539), and *flo11Δ* (PC1029) mutants. *FLO11* mRNA levels were normalized to *ACT1* mRNA levels, and normalized values are shown. RNA was prepared from cells grown for 8 h in YEP-Gal medium and examined by quantitative PCR analysis. (D) Expression of FG pathway reporters *SVS1-lacZ*, *PGU1-lacZ*, and *YLRO42C-lacZ* in cells carrying plasmids overexpressing *WSC2* and *MID2* alongside a wild-type control (pRS316). (E) *FUS1-lacZ* expression in cells containing overexpression plasmids for *WSC2* and *MID2* alongside a wild-type control (pRS316) in wild-type (PC538), *msb2Δ* (PC948), *sho1Δ* (PC1531), *ste20Δ* (PC673), *ste12Δ* (PC2382), and *slt2Δ* (PC3394) strains. For experiments shown in panels D and E, cells were grown for 8 h in YEP-Gal medium. Assays were performed in duplicate, with error bars representing standard deviation between experiments.

Mass Spectrometry approaches identify components of the filamentous cell wall. To better understand how the FG pathway contributes to cell wall remodeling, we identified targets of the FG pathway by a proteomics approach. The yeast cell wall is composed of glucans, mannoproteins, and a small amount of chitin (68, 112, 150). Cell wall mannoproteins are incorporated into the wall by noncovalent attachment (17, 67), disulfide bridge formation (102, 113), and glycosylphosphatidylinositol (GPI) anchor modification (20, 54, 169), which leads to a covalent linkage to ß-1,3-glucan through ß-1,6-glucan (66, 69). Genomic approaches show that a complex regulatory network underlies the biosynthesis, maintenance, and reorganization of the cell wall (80, 81). For example, the expression of more than half of the cell wall proteins is cell cycle regulated (19, 152), and this regulation contributes to the localized incorporation of proteins into the cell wall (149).

Recent advances in mass spectrometry technology have enabled rapid and sensitive identification of cell wall glycoproteins (168). However, most cell wall proteins are modified by N- and O-linked glycosylation, which complicates their identification by their molecular masses. We used a novel approach to identify cell wall proteins, by treatment of purified cell wall proteins with TFMS to remove glycosidic linkages (11). As a result, we identified a number of components of the filamentous cell wall (Table 4), including Flo11p (86), Ccw12p (103, 124), Pir1p (155), Hsp150p (141), and Sed1p (52). Cell wall proteins were also identified in vegetative cells and cells treated with mating pheromone as a control (Table 4). In addition to cell wall proteins, glycolytic enzymes, secreted proteases, and other proteins were identified, in line with other proteomic analyses and directed approaches to characterize proteins in the yeast cell wall (40, 89, 116).

Several proteins were identified that were not entirely expected. Pry2p is a pathogen-related protein of unknown function that has not previously been connected to the cell wall. Flo10p is a presumptive cell wall protein that is not thought to
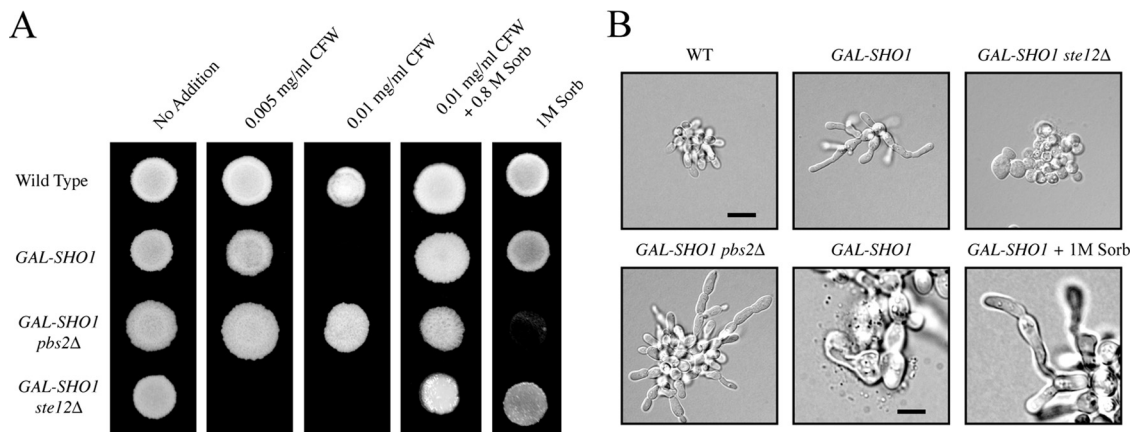
FIG. 3. Overexpression of *SHO1* exerts opposing effects on cell wall integrity through the FG and HOG pathways. (A) Wild-type (PC538), *GAL-SHO1* (PC622), *GAL-SHO1 pbs2Δ* (PC670), and *GAL-SHO1 ste12Δ* (PC624) strains were spotted onto YEPD medium containing CFW and/or sorbitol (Sorb) at the indicated concentrations. (B) The cell morphologies of cells in panel A. More than 200 cells were examined for this experiment, and representative cells are shown. Bar, 5 μm. WT, wild type.

be expressed in Σ1278b strains due to transcriptional silencing (51, 130). *FLO10* is expressed to some degree as a result of rapid "switching" at the transcriptional level, presumably to introduce variation in the cell surface (53, 105). We confirmed that the *FLO10* gene is expressed at low levels in comparison to the *FLO11* (Fig. 4B). *FLO10* expression, like *FLO11* expression (Fig. 2C) (140), was regulated to some degree by the FG pathway (Fig. 4C). Indeed, *FLO10* was identified as an FG pathway target by expression profiling (C. Chavel, H. M. Dionne, B. Birkaya, J. Joshi, and P. J. Cullen, unpublished data). An Flo10p-HA fusion protein was constructed and identified by immunoblot analysis (Fig. 4A) among a collection of epitope-tagged cell wall proteins. Our ability to detect the Flo10p protein by immunoblot analysis and mass spectrometry may indicate that the protein is relatively stable. As for other mucin-like proteins Msb2p (157), Hkr1p (121), and Flo11p (Karunanithi, S. R., N. Vadaie, B. Birkaya, H. M. Dionne, J. Joshi, L. Grell, and P. J. Cullen, submitted for publication), Flo10p-HA was found to be secreted from cells by colony immunoblot analysis (Fig. 4D).

Many of the proteins identified by mass spectrometry were targets of signaling pathways that control FG. Targets of the FG pathway (*FLO11*, *FLO10*, *MSB2*, and *SCW10*; Ste12p/Tec1p targets) and the cell wall integrity pathway (*SED1*, *CIS3*, *HSP150*, *PGK1*, *PST1*, and *PIR3*; Rlm1p targets) (64) were identified. Targets of the Swi4p/Swi6p transcription factors (3, 8, 104), which regulate progression through the G₁ phase of the cell cycle and which themselves are induced by the cell wall integrity pathway (5), were identified (*MSB2*, *SCW10*, *SCW4*, *TOS1*, and *ECM33*). Targets of the transcription factor Skn7p, which is regulated by the cell wall regulatory Rho GTPase Rho1p (1), were identified (*SCW4* and *ECM33*) (Table 4). Targets of other transcription factors that regulate FG were also identified including Sok2p (for *SED1*) (115) and Mcm1p (for *PCK1* and *HSP150*) (Table 4). Therefore, the cell wall integrity pathway, the FG pathway, and other transcriptional regulatory pathways function to populate the filamentous cell wall with a specific collection of cell wall mannoproteins.

The ß-glucan layer influences mucin secretion, cell-cell adhesion, and FG. We previously showed that Msb2p, like other members of the mucin family (21), is processed and that the extracellular domain of the protein is secreted from cells (157). Indeed, Msb2p was identified as a secreted protein by mass spectrometry analysis (Table 4). To determine whether Msb2p secretion is regulated by the cell wall, a collection of cell wall mutants was screened for altered shedding of Msb2p-HA by colony immunoblot analysis. Of ~100 mutants examined, 11 showed altered secretion of Msb2p-HA (Fig. 5B; see also Fig. S1 in the supplemental material). Several of the genes identified (*SLG1*, *EXG1*, *KRE11*, *ECM15*, and *SMI1*) function in the biosynthesis and assembly of the ß-glucan layer (13, 81, 158). *SLG1* encodes the cell wall integrity sensor Slg1p/Wsc1p. Defects in most of the genes resulted in enhanced secretion of Msb2p (Fig. 5B), which suggests that the ß-glucan layer contributes to mucin retention. Defects in the ß-glucan layer would be expected to increase the porosity of the cell wall (118), which may explain the elevated levels of Msb2p secretion. *KRE11* also functions in protein transport and may contribute to Msb2p secretion through delivery of the protein to the cell surface (144). In one mutant, altered shedding of Msb2p-HA correlated with altered expression of the *MSB2* gene as determined by examining *MSB2-lacZ* expression in cell wall mutants (Fig. 5C, *gas1Δ*). In most mutants, however, *MSB2-lacZ* expression did not correlate with secretion of the protein (Fig. 5C). For example, the *slg1Δ* mutant showed reduced expression of *MSB2-lacZ*, which might result from an auto-feedback mechanism, given that the FG pathway controls *MSB2* expression (29) and elevated shedding of the Msb2p-HA protein. We interpret this result to indicate that although there is less Msb2p in the cell, its ability to be retained in this mutant is compromised, resulting in elevated secretion from cells. Several cell wall genes including *SLG1* were also found to be required for invasive growth (Fig. 5D and E).

The fact that relatively few cell wall genes were identified might reflect buffering of cell wall functions (81). This possibility is supported by the fact that combinations of cell wall

TABLE 4. Components of the filamentous cell wall and related proteins identified by MS analysis

Protein	Culture preparation(s)[a]	No. of peptides[b]	Regulatory element(s)[c]	Process[d]	Function
Msb2	TCA	2	**Ste12**, Dig1, Tec1, **Swi4/Swi6**	Cell wall organization/signal transduction	Mucin family member involved in the filamentous growth pathway
Flo11	Y, Gal, α	1	**Ste12**, Tec1, Flo8, Dig1	Cell wall organization	GPI-anchored cell surface flocculin; pseudohyphal formation
Flo10	Gal	1		Cell wall organization	Lectin-like protein with similarity to Flo1p; involved in flocculation
Hsp150	Y, Gal, α, PEG, TCA	1	**Mcm1, Rlm1**	Cell wall organization	O-mannosylated heat shock protein attached to the cell wall
Scw10	TCA	3	**Ste12, Swi4/Swi6**, Dig1, Mbp1	Cell wall organization	Cell wall protein with similarity to glucanases
Sed1	Y, Gal, α, PEG	3	**Sok2**, Hap2, Nrg1, Mot3, Adr1, **Rlm1**	Cell wall organization	Major stress-induced structural GPI cell wall glycoprotein
Scw4	PEG, TCA	1	**Swi6, Skn7**	Cell wall organization	Cell wall protein with similarity to glucanases
Tos1	TCA	2	Gcn4, **Swi4**, Fkh1	Cell wall organization	Covalently-bound cell wall protein of unknown function
Ecm33[f]	TCA	2	Abf1, Mbp1, **Swi6, Skn7**, Fkh1, Fkh2	Cell wall organization/cell polarity	GPI-anchored protein; possible role in apical bud growth
Exg2	α	1		Cell wall organization	Exo-1,3-beta-glucanase, involved in cell wall beta-glucan assembly
BglI	TCA	3		Cell wall organization	Endo-beta-1,3-glucanase; major protein of the cell wall
Exg1	TCA	2	Fkh2, Mbp1	Cell wall organization	Major exo-1,3-beta-glucanase of the cell wall; beta-glucan assembly
Pir3	PEG	2	**Rlm1**	Cell wall organization	O-glycosylated cell wall protein; cell wall stability
Pry2	TCA	1		Cell wall organization	Unknown
PstI[f]	TCA	1	Cbf1, Ume6, **Rlm1**	Cell wall organization	Cell wall protein that contains a putative GPI attachment site
Ccw12[f]	Y, Gal, α	3	**a1-α2**[e]	Cell wall organization	Cell wall mannoprotein
Pir1	Y,Gal	1		Cell wall organization	O-glycosylated protein required for cell wall stability
Cis3	α, TCA	1	**Rlm1**	Cell wall organization	Mannose-containing glycoprotein of the cell wall; PIR family
Cdc19	PEG	2	**Ste12**	Glycolysis	Pyruvate kinase
Tpi1	TCA	1	Ino4	Glycolysis	Triose phosphate isomerase, abundant glycolytic enzyme
Eno1	PEG, TCA	3	Ino2	Glycolysis/gluconeogenesis	Enolase I, a phosphopyruvate hydratase
Pck1	PEG	1	**Mcm1**, Sip4, Adr1, Cat8	Glycolysis/gluconeogenesis	Phosphoenolpyruvate carboxykinase, a key enzyme in gluconeogenesis
Pgk1	PEG, TCA	1	Tye7, **Rlm1**	Glycolysis/gluconeogenesis	3-Phosphoglycerate kinase
Tdh3	PEG, TCA	1	Hsf1, Msn4, Gcr1, Cin5	Glycolysis/gluconeogenesis	Glyceraldehyde-3-phosphate dehydrogenase
Hxt6	PEG	1	Adr1	Hexose transport	High-affinity glucose transporter of the major facilitator superfamily
Spa2	PEG	1		Cell polarity	Component of the polarisome
Bar1	TCA	2	**Ste12**, Dig1	Protein processing	Aspartyl protease
Ahp1	PEG, TCA	1		Thioredoxin	Thiol-specific peroxiredoxin
Pma1	Gal, α	1		Ion transport	Plasma membrane H+-ATPase
Ste6	α	1	**Ste12, Mcm1**	Protein export	ATP-binding cassette transporter export of a-factor
Tef2	Y, Gal, α	1	Flh1, Hsf1	Translation	Translational elongation factor EF-1 alpha; also encoded by TEF1
Tsa1	PEG	1	HapI, Yap1	Thioredoxin	Thioredoxin peroxidase
Mpp6	TCA	1		Translation	Nuclear RNA binding protein that associates with the exosome

[a] Proteins were identified from cells grown in YEPD (Y), YEP-Gal (Gal), or YEPD medium supplemented with 1 μg/ml α-factor (α) or from cells grown in conditioned medium precipitated with TCA or PEG.
[b] As identified by mass spectrometry.
[c] Transcription factors that bind to consensus sequences found in the promoters of genes encoding proteins identified in the analysis. Proteins shown in boldface are established regulators of the FG or cell wall integrity pathways.
[d] Process and function were determined by information gathered at the *Saccharomyces* Genome Database (http://www.yeastgenome.org/).
[e] Reported in reference 41.
[f] Data analysis of the mass spectrometry data for asparagine residues with an attached GlcNac. N-linked GlcNac is not removed by TFMS digestion. The following N-linked modified peptides were identified: for Ecm33, K.VQTVGGAIEVTG**N**FSTLDLSSLK.S; for PstI, K.SPVETVSDSLQFSF**N**GNQTK.I and K.SPVETVSDS LQFSF**N**GNQTK.I; and for Ccw12, K.**N**GTSTAAPVTSTEAPK.N (the modified asparagines are underlined and in boldface; trypsin cleavage sites are indicated by the periods).
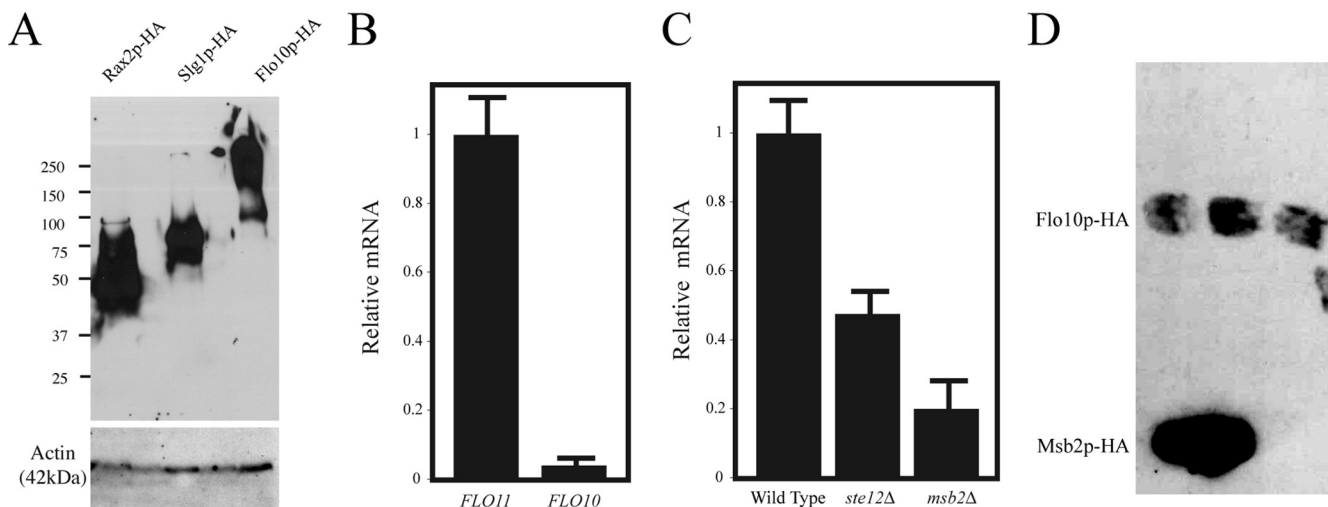
FIG. 4. The expression and shedding of Flo10p-HA. (A) Cells expressing the Flo10p-HA (PC2963), Rax2p-HA (PC3007), and Slg1p-HA (PC3008) fusion proteins were examined by immunoblot analysis with anti-HA antibodies (top panel) and anti-actin antibodies (bottom panel). (B) mRNA levels of *FLO11* and *FLO10* normalized to *ACT1* mRNA levels determined by quantitative PCR. RNA was prepared from cells grown for 8 h in YEP-Gal medium. (C) mRNA levels of *FLO10* normalized to *ACT1* levels in wild-type (PC538), *ste12Δ* (PC539), and *msb2Δ* (PC948) strains. RNA was prepared from cells grown for 8 h in YEP-Gal medium. (D) Cells expressing Flo10p-HA and Msb2p-HA were patched onto YEPD medium overlaid with a nitrocellulose filter for 24 h and incubated at 30°C. Cells were washed off the filter, which was probed with antibodies against the HA epitope. The results were confirmed by spotting cell-free (conditioned) medium onto nitrocellulose, followed by immunoblot analysis (data not shown).

mutants exacerbate Msb2p shedding (N. Vadaie and P. J. Cullen, unpublished observations). To address this possibility, the effect of overexpression of cell wall genes on Msb2p-HA secretion was examined. Of ~80 genes examined, 24 perturbed Msb2p-HA secretion when overexpressed (Fig. 5F; see also Fig. S1 in the supplemental material). Overexpression of cell wall genes typically caused elevated Msb2p-HA retention (Fig. 6A). *EXG1*, which encodes the major exo-1,3-ß-glucanase of the cell wall (158), showed phenotypes with respect to Msb2p-HA secretion when it was disrupted or overexpressed (Fig. 5). Overexpression of several of the cell wall proteins also induced hyperpolarized growth (Fig. 5F).

The above results suggest that loss of ß-1,3-linkages results in shedding of Msb2p and may therefore also influence FG. To directly test this possibility, cells were treated with sublethal concentrations of ß-glucanase and assessed for agar invasion. ß-Glucanase treatment resulted in a complete loss of agar invasion (Fig. 6A). ß-Glucanase treatment inhibited the ruffled colony morphology (Fig. 6A), indicative of cell-cell adhesion (133). Flo11p-dependent cell-cell adhesion was also reduced by ß-glucanase treatment (Fig. 6B). Treatment of cells with ß-glucanase caused greater than 90% of cell-associated Msb2p-HA and Flo11p-HA to be released from cells (data not shown). The defect in cell-cell adhesion was more severe than observed in the *flo11Δ* mutant (Fig. 6B), in line with the idea that cell adhesion is mediated by Flo11p-dependent and Flo11p-independent mechanisms (96). A subpopulation of cells exposed to ß-glucanase showed morphological abnormalities (Fig. 6A, right panels), which might be expected based on the fact that the cell wall is required to maintain cell shape, cellular asymmetry, and the polarized localization of cytoskeletal proteins (128). Part of the invasive growth defect might also result from failure of filamentous cells to polarize correctly. Therefore, the ß-1,3-glucan layer is essential for FG.

DISCUSSION

We report the results of independent genetic screens to identify new regulators of FG in yeast. The 10 genes identified in this study reflect connections between FG and cell polarity, cell type specification, and cell integrity. This collection adds to a growing body of knowledge in which several hundred genes have been identified that contribute to FG (62, 90, 99, 114). One feature of the high-copy-number plasmid library screening approach is that it has the potential to identify essential genes (*MCM1*), genes buffered by genetic redundancy, and genes that when tagged perturb their function (e.g., *WSC2* and *MPT5*). Because FG is a complex response, multiple independent approaches continue to improve on our understanding of the response.

Cell type and invasive growth. The genetic analysis uncovered distinct invasion patterns between haploid and diploid cells under different conditions. Diploid cells exhibit constitutive invasive growth on synthetic medium and invade the agar better that haploid cells. This property explains the identification of cell-type-regulatory transcription factors Mcm1p and MATα2p as invasion-promoting factors. Haploid cells bud in an axial pattern (26, 27), and starvation triggers the switch to distal-unipolar budding that induces invasive growth (32). The axial budding pattern of haploid cells has been postulated to promote rapid mating and diploid formation of adjoining cells in germinating asci (48). Haploid and diploid cells also show different sensitivities to osmotic stress (41) and in their cell wall composition (36). The different budding patterns between haploid and diploid cells may reflect fundamental behavioral differences between the two cell types. Our results confirm the central role that the reorganization of cell polarity (budding pattern) plays in contributing to invasive growth.
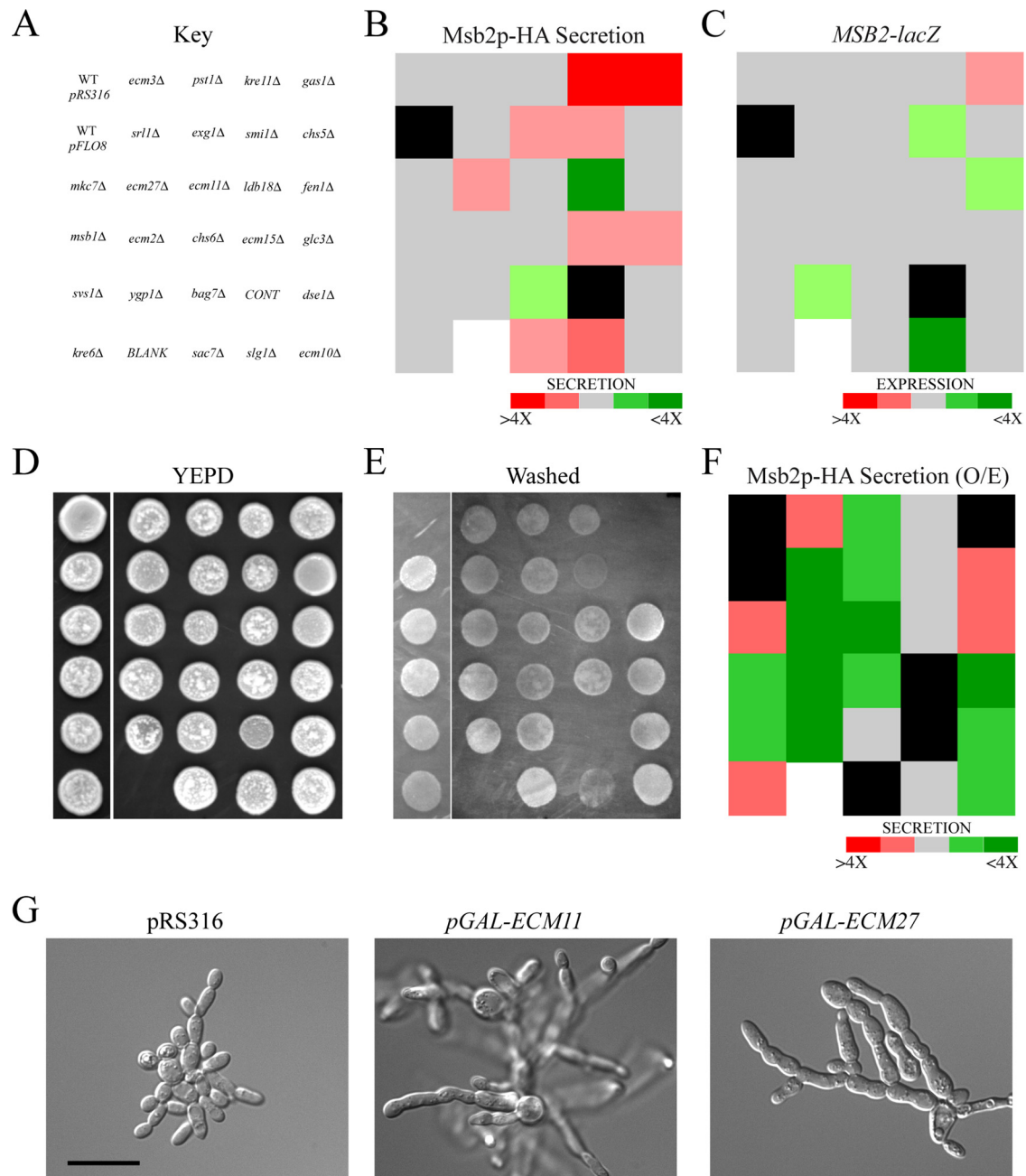
FIG. 5. The contribution of cell wall proteins in Msb2p-HA shedding, *MSB2-lacZ* expression, and invasive growth. (A) The key refers to open reading frame deletions in the *MAT*a collection (S288c background; [166]) that lack cell wall proteins. WT pRS316, wild-type strain PC986 transformed with pRS316; WT pFLO8, wild-type strain PC986 transformed with pFLO8; CONT, contaminant. (B) Msb2p-HA secretion in cell wall mutants. Deletion strains were transformed with pMSB2-HA (PC1820), and transformants were grown for 48 h on nitrocellulose filters atop SD-Ura medium. Cells were washed off the filters, which were probed by immunoblot analysis using anti-HA antibodies. Red, elevated secretion; green, reduced secretion; gray, equivalent secretion relative to neighboring colonies and wild type; black, not tested. (C) *MSB2-lacZ* expression in cell wall mutants. Cell wall mutants were transformed with p*MSB2-lacZ* (PC3133), and transformants were incubated for 8 h at 30°C in S-Gal-Ura medium to induce *MSB2* expression. Red, elevated expression; green, reduced expression; gray, no change; black, not determined. (D) Cell wall mutants were transformed with p*FLO8* (PC1417), and transformants were spotted onto YEPD medium and examined for agar invasion after 2 days by washing in a stream of water. (E) Washed YEPD plate. (F) The effect of overexpression (O/E) of cell wall genes on Msb2p-HA secretion. The color scheme is equivalent to that used in panel B. (G) Two examples where overexpression of cell wall genes induces hyperinvasive growth. Cells were grown for 16 h in S-Gal-Ura medium and examined by microscopy at a magnification of ×100. Bar, 20 μm. The complete list of secretion of Msb2p-HA in deletion mutants and overexpression plasmids as well as *lacZ* data is presented in Fig. S1 and Table S1 in the supplemental material.
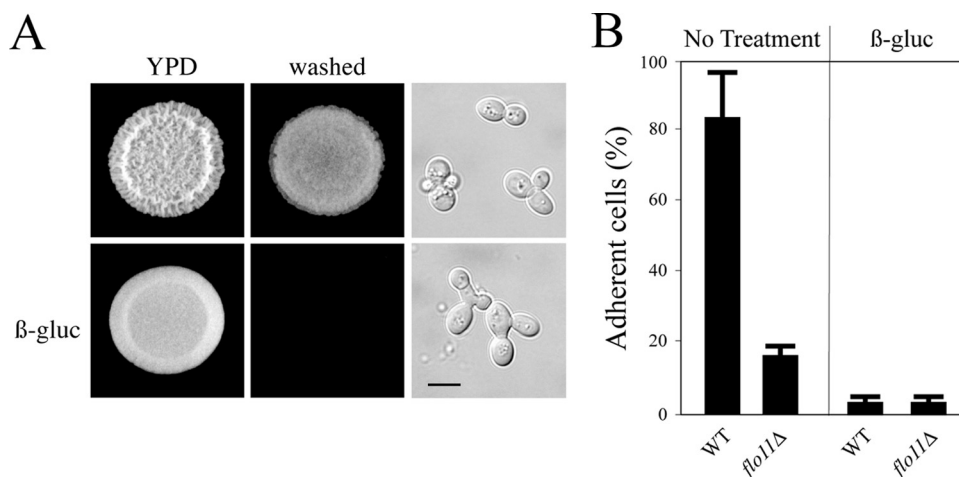
FIG. 6. Disruption of ß-1,3-glucan linkages abolishes invasive growth and cell-cell adhesion. (A) Wild-type cells (PC538) were spotted onto YEPD medium ± 10 mg/ml zymolyase (ß-1,3-glucan laminaripentaohydrolase [ß-gluc]), which hydrolyzes glucose polymers at ß-1,3-glucan linkages (37, 173). Colonies were incubated for 4 days. The plates were photographed, washed, and photographed again. At right are examples of morphologies of washed cells. Aberrant cells were observed at a frequency of 5%. Bar, 5 μm. (B) Cell-cell adhesion is mediated by ß-1,3-glucan linkages. Wild-type ([WT] PC538) and *flo11*Δ (PC1029) cells were grown under conditions that promote FG (YEP-Gal medium for 16 h) and assessed for cell-cell adhesion with or without a 1-h treatment with 10 mg/ml zymolyase. Cell-cell adherence was assessed by microscopic examination at a magnification of ×100. Cells were considered adherent if they were associated with more than four other cells. The experiment was repeated twice. Error bars represent standard deviation between experiments.

A role for cell wall remodeling in FG. The major finding from this study is that the cell wall integrity pathway contributes to FG. The inclusion of a new MAPK pathway in the repertoire of regulatory pathways that influence FG is an important contribution to the overall understanding of the FG response. By comparison, the pheromone response pathway does not play a major role in FG (84, 133), and the HOG pathway has an inhibitory role (34, 121, 164). It remains unclear whether the remaining MAPK pathway (sporulation/Smk1p pathway) (71) contributes to FG.

The cell wall integrity pathway functions in enumerable cellular contexts (82) and may contribute to FG by a variety of mechanisms. The cell wall integrity pathway is regulated by the TOR nutrient-sensing pathway (70) and is activated upon entry into stationary phase when nutrients become limiting (156). FG is also induced by nutrient limitation (31, 49), and although the cells do not enter G$_0$, they are subject to TOR pathway regulation (33, 134, 161). In *C. albicans*, the cell wall integrity pathway is required for morphogenesis (106) and is activated by cellular contact with the agar surface (76). In *Aspergillus nidulans*, the orthologous protein kinase C-dependent pathway is required for maintaining the hyphal growth rate (136).

One way the cell wall integrity pathway appears to regulate FG is by modulating the activity of the FG pathway. Wsc2p and Mid2p have different roles in FG in that Wsc2p loss/overexpression has more striking phenotypes with respect to agar invasion and the induction of FG pathway reporters. Both proteins promote FG pathway activity by a mechanism that requires the signaling mucin Msb2p. This connection might result from direct interactions between the cell surface proteins or indirectly, for example, by regulating genes that influence *MSB2* expression. In *Candida glabrata*, the Ste20p homolog has been shown to activate the cell wall integrity pathway (16) although in this species Ste20p does not appear to mediate nitrogen limitation-induced FG. Although Mid2p,

Wsc2p, Slg1p, and Slt2p/Mpk1p contribute to FG pathway activity, the phenotypes that result from their deletion/overexpression are relatively moderate, distinguishing them from core components of the FG pathway.

Communication between MAPK pathways in fungi occurs in complex environmental settings and when cells undergo differentiation. For example, the cell wall integrity pathway is activated during pheromone response to promote efficient mating (15, 24, 170). The HOG and cell wall integrity pathways act sequentially in response to global cell wall damage (9, 42, 44, 55, 146). In *C. albicans*, the adaptor protein Sho1p (135) is thought to regulate both the Cek1p and Hog1p MAP kinase pathways in cell wall biogenesis and chlamydospore formation (38). In *Neurospora crassa*, combinatorial effects of filamentation and cell integrity kinase pathways oppose the osmosensing OS2 pathway (95). In some situations, the cell wall integrity, FG, and HOG pathways act in parallel, such as in response to a protein glycosylation defect (30) and to produce the multicellular cell types associated with sexual development in *N. crassa* (95).

The FG pathway itself contributes to cell wall functions by inducing expression of cell wall proteins, namely the mucin-like glycoprotein Msb2p (29), the flocculins Flo11p (51, 140) and Flo10p (51), and other proteins. These mucin-like proteins might themselves play a role in cell wall integrity. For example, the adhesin glycoprotein Fig2p promotes cell wall integrity during mating (171). We show that retention/shedding of Msb2p is influenced by the ß-glucan layer of the cell wall. This finding has important implications with respect to cell wall regulation in that cell wall remodeling may be part of a cycle in which core remodeling influences the retention of cell wall mucins, which themselves regulate cell wall properties such as cell-cell adhesion and cellular signaling. By parallel activation of the FG and cell wall integrity pathways, mucin expression and retention at the cell surface are maximized.

Our results support the overall notion that targeting the fungal cell wall is an effective strategy in the design of drugs to inhibit the virulence of fungal pathogens (35, 107, 151). Damage to the ß-glucan layer of the cell wall potently inhibits invasive growth (Fig. 6). Although some effective fungicides exist, like capsofungin, which irreversibly inhibits 1,3-ß-glucan synthase (57), lead compounds are needed to attenuate invasive fungal infections of immunocompromised patients (73), and other potential cell wall targets remain largely unexplored (77). Further characterization of filamentation-dependent cell wall remodeling in budding yeast is likely to provide useful insights into the regulatory inputs of cell wall regulation that may pertain to fungal pathogenesis.

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