

Hog1 Mitogen-Activated Protein Kinase (MAPK) Interrupts Signal Transduction between the Kss1 MAPK and the Tec1 Transcription Factor To Maintain Pathway Specificity^{∇†}

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In *Saccharomyces cerevisiae*, the mating, filamentous growth (FG), and high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) signaling pathways share components and yet mediate distinct responses to different extracellular signals. Cross talk is suppressed between the mating and FG pathways because mating signaling induces the destruction of the FG transcription factor Tec1. We show here that HOG pathway activation results in phosphorylation of the FG MAPK, Kss1, and the MAPKK, Ste7. However, FG transcription is not activated because HOG signaling prevents the activation of Tec1. In contrast to the mating pathway, we find that the mechanism involves the inhibition of DNA binding by Tec1 rather than its destruction. We also find that nuclear accumulation of Tec1 is not affected by HOG signaling. Inhibition by Hog1 is apparently indirect since it does not require any of the consensus S/TP MAPK phosphorylation sites on Tec1, its DNA-binding partner Ste12, or the associated regulators Dig1 or Dig2. It also does not require the consensus MAPK sites of the Ste11 activator Ste50, in contrast to a recent proposal for a role for negative feedback in specificity. Our results demonstrate that HOG signaling interrupts the FG pathway signal transduction between the phosphorylation of Kss1 and the activation of DNA binding by Tec1.

A fundamental question in cell biology is how cells respond correctly to extracellular signals. Cells must accurately translate extracellular cues such as hormones, nutrient levels, and environmental stresses into intracellular changes such as protein activation or changes in gene expression. Surprisingly, many signaling pathways share components, which poses a problem for the maintenance of signaling specificity. Thus, if two pathways that share components have evolved to transduce distinct signals into distinct outputs, then specific mechanisms presumably exist so that activation of either signal does not lead to the inappropriate induction of both outputs.

In *Saccharomyces cerevisiae* three mitogen-activated protein kinase (MAPK) pathways share multiple components, providing an excellent model system for studying the molecular mechanisms responsible for the maintenance of signaling specificity. As their names suggest, the mating, filamentous growth (FG), and high-osmolarity glycerol (HOG) pathways are functionally distinct signaling systems. In response to a peptide pheromone produced by cells of the opposite mating type, the mating pathway is activated in haploid cells, where it performs many functions, including arrest of the cell cycle, induction of genes necessary for the mating process, control of mating projection formation, and promotion of cellular and nuclear fusion (3, 9, 18, 19). In contrast, during nutrient limitation FG

pathway activation induces the transcription of a distinct set of genes which controls a set of morphological changes that occur during FG, including increased cell elongation, increased cell-cell adherence, and a shift to the bipolar budding pattern (9, 22, 28, 38). Finally, after a shift of the external environment to higher osmolarity, HOG pathway activity regulates ion channels, glycerol export, cell cycle progression, and the protein biosynthetic machinery, while inducing the transcription of genes whose products are involved in the synthesis of glycerol. Together, these responses restore the osmotic balance to regain homeostasis (13, 24, 36). We primarily focus here on the mechanism by which cross talk is avoided between the HOG and FG pathways.

Canonical MAPK cascades are composed of three sequential kinases: these are generically termed the MAPK kinase kinase (MAPKKK), the MAPKK, and the MAPK. In the FG pathway the three kinases of the cascade are named Ste11, Ste7, and Kss1, respectively (Fig. 1). Ste11 also acts as the MAPKKK in one branch of the HOG pathway. Its downstream pathway-specific target is the MAPKK, Pbs2, which in turn activates the MAPK Hog1. Several components upstream of Ste11 are also shared between the FG and HOG pathways, including two transmembrane proteins, Sho1 and Msb2, the membrane-associated GTPase Cdc42, the PAK family kinase Ste20, and an adapter called Ste50. Ste20 is activated by GTP-bound Cdc42 and is a MAPKKKK that phosphorylates and activates Ste11.

As mentioned above, Ste11 only acts in one branch of the HOG pathway. A second branch, called the Sln1 branch, also converges on activation of the MAPKK, Pbs2, but utilizes a two-component histidine kinase phosphorelay system similar to two-component systems in bacteria. In addition to Sln1, the histidine kinase found at the membrane, the branch includes

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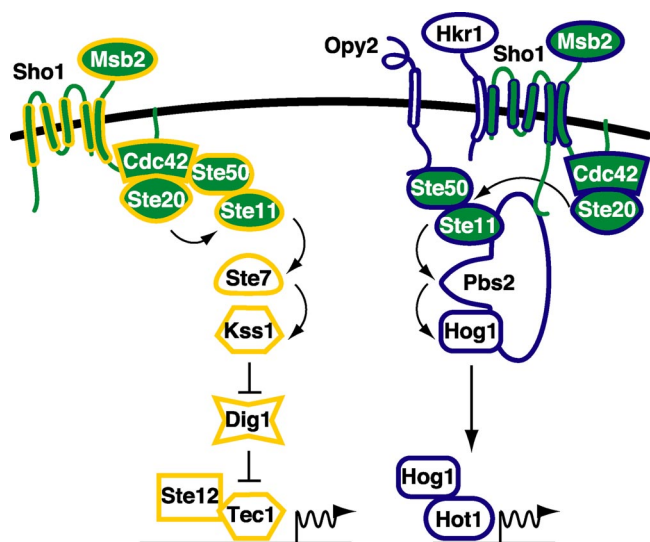


FIG. 1. FG and HOG MAPK signaling pathways. Components that are shared between the FG and HOG pathways are depicted in solid green. Components of the FG pathway are outlined in yellow, and components of the HOG pathway are outlined in blue. For simplicity, some interactions for which there exist evidence are not depicted such as those between Sho1 and Ste50 and Sho1 and Ste11. Also, the interaction between Ste50 and Cdc42 is not depicted in the HOG pathway, and only one of several possible transcription factors is depicted in the HOG pathway.

the phosphotransferase, Ypd1, the response regulator, Ssk1, and the redundant MAPKKs, Ssk2/Ssk22. This branch is not conserved in humans, but in many fungi, including pathogenic fungi, it appears that this branch plays a dominant role in sensing many extracellular stresses, including osmotic stress (1).

Although the receptors and the mechanisms by which they are connected to the downstream signaling pathway are well understood for many signaling pathways, including the mating pathway, considerably less is known regarding the early steps of signaling in the FG and HOG pathways. Recent work suggests that the mucin-like proteins, Msb2 and Hkr1, act in the HOG pathway as redundant osmosensors (50). However, Msb2 is also required for full activation of the FG pathway (16). A third transmembrane protein, Opy2, appears to function specifically in the HOG pathway (59) and, finally, a fourth transmembrane protein, Sho1, is shared between both the FG and the HOG pathways (31, 34). Since no FG pathway-specific membrane protein has been identified, how FG-specific nutritional signals are sensed remains unknown.

A remarkably complex network of protein-protein interactions connects the Msb2, Hkr1, Opy2, and Sho1 membrane proteins to the downstream kinases (Fig. 1). Sho1 functions as an adaptor that helps transmit the external signal to Ste11. Specifically, Sho1 binds to the putative osmosensors, Msb2 and Hkr1 (50). Sho1 also associates with Ste50 (51), which itself serves as an adaptor by binding to the N-terminal noncatalytic domain of Ste11, as well as to Opy2 and Cdc42 (43, 54, 59). An interaction between the SH3 domain of Sho1 and a polyproline motif in Pbs2 is essential for HOG signaling (31, 32, 41), and there is also evidence that Sho1 binds directly to Ste11 (51, 60).

The net functional result of these protein-protein interactions is thought to be the signal-induced recruitment of Ste11 to the plasma membrane where Cdc42-bound Ste20 can then phosphorylate and activate Ste11, ultimately leading to the activation of the pathway-specific MAPKs. In addition to transmitting the extracellular stimulus to the MAPK cascade, it is widely thought that this network of protein-protein interactions could prevent shared components from transmitting the signal to other pathways, thereby providing a mechanism for maintaining signaling specificity between pathways.

One consequence of MAPK signaling is the activation of specific genes through the control of DNA-binding transcriptional activators. The FG pathway controls a heteromeric activator composed of Ste12 and the FG pathway-specific transcription factor, Tec1 (27). The inactive and unphosphorylated form of the FG pathway MAPK, Kss1, binds to and inhibits Ste12-Tec1; however, when phosphorylated by Ste7, Kss1 acts to activate Ste12-Tec1, allowing transcription of FG pathway-specific genes (15, 30). In addition to loss of the inhibitory activity of unphosphorylated Kss1, activation of Ste12-Tec1 involves inactivation of an inhibitor called Dig1 (14, 37, 52). Ste12-Tec1 appears to have two modes by which it associates with DNA. In one mode, each DNA-binding subunit recognizes its cognate sequence: Ste12 recognizes a motif called a pheromone response element (PRE), while Tec1 associates with a motif called a TEA/ATTS consensus sequence (TCS). This composite element has been termed a filamentation response element (FRE) (27). In most contexts, however, no Ste12 binding site is apparent, suggesting that recognition is mediated primarily by Tec1-TCS binding (12, 61). During osmotic stress, active Hog1 is imported into the nucleus (21, 44), where it directly interacts with several different factors at promoters to induce transcription (35, 40, 45). These factors include Hot1, the repressor Sko1, and the redundant stress responsive transcription factors Msn2/4 (24).

Recent work has established a mechanism for signaling specificity maintenance between the mating and FG pathways during signaling through the mating pathway (2, 8, 11). During exposure to pheromone the mating pathway is activated and the MAPK Fus3 becomes phosphorylated. However, it has been appreciated for some time that Kss1 is also phosphorylated and activated under these conditions (7, 10, 46). Activation of Kss1 should result in erroneous transcription of FG genes, but this does not occur. Fus3 maintains specificity by phosphorylating threonine 273 of Tec1, the FG pathway-specific transcriptional activator, causing Tec1 to be recognized and ubiquitinated by the SCF ubiquitin ligase, resulting in its rapid destruction. Through this cross-pathway inhibition, Fus3 actively prevents filamentation genes from being improperly activated upon exposure of cells to mating pheromone.

In contrast to the mating-FG pathway axis, it is not fully understood how inappropriate cross talk with the mating and FG pathways is avoided upon activation of the HOG pathway. Previous work has shown that both Hog1 and Pbs2, the MAPK and MAPKK of the HOG pathway, are required to maintain signaling specificity during osmotic stress (34). Deletion of either gene results in erroneous expression of both mating and FG pathway genes during HOG pathway activation. Furthermore, it seems that the primary role that Pbs2 plays in cross talk suppression is to activate Hog1, whose kinase activity is re-

quired for signaling specificity. Again, this suggests Hog1 actively inhibits cross-pathway activation. In the present study we further investigate the mechanism that prevents cross talk between the HOG and FG pathways. Surprisingly, we found that in wild-type cells both Ste7 and Kss1 are phosphorylated in response to osmotic stress, but signaling specificity must be maintained since transcription of FG pathway genes does not occur under such conditions (17, 35, 58). We show that signaling specificity is preserved because HOG signaling interrupts signaling to Tec1. In contrast to the mechanism of cross talk suppression between the mating and FG pathways, we find that HOG signaling prevents DNA binding of Tec1 rather than inducing its destruction. We also find that nuclear accumulation of Tec1 is not affected by HOG signaling. Inhibition by Hog1 is apparently indirect since it does not require any of the consensus S/TP MAPK phosphorylation sites on Tec1, its DNA-binding partner Ste12, or the associated regulators, Dig1 or Dig2. It also does not require the consensus MAPK sites of the Ste11 activator, Ste50, in contrast to a recent proposal for a role for negative feedback in specificity.

MATERIALS AND METHODS

Yeast strains, plasmids, and genetic techniques. Strains used in the present study have the Sigma 1278b background, whose full genotype is *trp1 leu2 ura3 his3*, and are listed in a strain table (see Table S2 in the supplemental material). All alleles on plasmids were expressed from their natural promoters and are listed in a plasmid table (see Table S3 in the supplemental material).

After the yeast knockout collection strains (Open Biosystems) were grown in deep-well 96-well plates in yeast extract-peptone-dextrose for 2 to 3 days, the medium was removed, and 200 μ l of transformation mix was added (43% polyethylene glycol, 85 mM lithium acetate, 7.5 mM Tris-HCl [pH 8.0], 0.75 mM EDTA, 0.25 mg of single-stranded DNA/ml, 0.5 μ g of *FRE-lacZ* plasmid DNA/ml). Plates were vortex mixed by using a Multitube Vortexer (VWR) and incubated at 42°C for 3 h. Then, 1 ml of SC-Ura was added to each well, and 150 μ l of this dilution was transferred to a fresh 96-well plate containing 1.2 ml of medium. These plates were grown for 2 to 3 days.

Extract preparation and immunoblotting for Western analyses. Cells were grown to mid-log phase (optical density at 600 nm [OD₆₀₀] of 0.6 to 0.8). The culture was split, and appropriate samples were treated with 1 M KCl or α -factor for 15 min. Cells were collected by centrifugation and snap-frozen. Pellets were resuspended in 100 μ l of lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 100 mM NaF, 5 mM EDTA, 1% NP-40, 15 mM 4-nitrophenylphosphate, 30 mM Na₂S₂O₅, 0.1 mM Na₃VO₄, 80 mM β -glycerophosphate, 1 mM dithiothreitol [DTT], 1:100 Sigma phosphatase inhibitor cocktails 1 and 2, and 1:260 Sigma protease inhibitor cocktail). Zirconia/silica beads (Biospec Products) were added. Cells were disrupted in a Mini-Beadbeater 8 (Biospec Products) by four cycles of disruption at full power for 1 min, with cooling on ice for 2 min between each cycle. The extract was removed by pipetting, and the beads were washed in 100 μ l of lysis buffer; this material was then added to the extract. Cell debris was removed by centrifugation at 14,000 \times g for 10 min. The protein concentration was determined by a BCA protein assay (Pierce). Portions (100 μ g) of whole-cell extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5 or 10% gels and transferred to nitrocellulose. Immunoblotting was performed with the following antibodies at the indicated dilutions: anti-p42/44 (1:1,000; NEB), anti-myc (1:1,000; 9E10; Covance), anti-Ste7 (1:2,000; a gift from Brad Cairns), anti-phospho-Ste7 (1:1,000; custom generated at Phosphosolutions, Inc.), anti-tubulin (1:2,000; Abcam), anti-mouse immunoglobulin G-horse radish peroxidase (IgG-HRP; 1:3,000; Bio-Rad), anti-rabbit IgG-HRP (1:3,000; Bio-Rad), and anti-rat IgG-HRP (1:3,000; Jackson Laboratories). Membranes were stripped by incubation in 0.2 M NaOH for 5 min and reprobed as indicated.

Pheromone and salt time course experiments were performed as described by Bao et al. (2), except that for the salt time course, the cultures were treated with 1 M KCl for the indicated times. Polyclonal Ste12 antibodies were used at 1:1,000 (a gift from the Herskowitz lab).

Coimmunoprecipitation experiments. Cultures (100 ml) were grown to an OD₆₀₀ of 0.8 to 1.0, collected by centrifugation, and snap-frozen. Pellets were resuspended in 400 μ l of lysis buffer (50 mM HEPES-KOH [pH 7.6], 250 mM

potassium acetate, 10 mM EDTA, 20% glycerol, 1 mM DTT, 5 mM 4-nitrophenylphosphate, 10 mM Na₂S₂O₅, 80 mM β -glycerophosphate, Sigma phosphatase inhibitor cocktails 1 and 2 [1:100], and Sigma protease inhibitor cocktail [1:260]), and zirconia/silica beads were added. Cells were disrupted, and the lysate was collected as described for Western extract preparation. Potassium acetate was added to a final concentration of 400 mM, and then polyethylenimine was slowly added to a concentration of 0.1%. Cell debris was removed by centrifugation, and the protein concentration was measured by using a BCA protein assay (Pierce). After the protein concentration was normalized, M2 FLAG agarose (Sigma) washed three times in lysis buffer was added to the extracts, and the samples were rocked for 15 min. Beads were washed three times with lysis buffer and then boiled in sample buffer (30 mM Tris-HCl [pH 6.8], 5 mM DTT, 6% SDS, 20% glycerol, bromophenol blue). The samples were subjected to SDS-PAGE on a 7.5% gel, transferred to polyvinylidene difluoride, and immunoblotted with anti-FLAG (1:2,000; Sigma) and anti-Dig1 (1:1,000; a gift from Mike Tyers).

Chromatin immunoprecipitation (ChIP). Cells were grown to mid-log phase (OD₆₀₀ of 0.4 to 0.5). Then, 50 ml of culture was fixed with 1% formaldehyde at room temperature for 15 min. Glycine was added to a final concentration of 125 mM, and the samples were incubated at room temperature for 5 min. Cells were washed twice with Tris-buffered saline, collected by centrifugation, and snap-frozen. Pellets were resuspended in 500 μ l of lysis buffer (50 mM HEPES [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, Sigma protease inhibitor cocktail [1:260]), and zirconia/silica beads were added. Cells were disrupted in a Mini-Beadbeater 8 by five cycles of disruption at full power for 1 min, with cooling on ice for 2 min between each cycle. The lysate was collected. The chromatin-containing pellet was separated by centrifugation and resuspended in 300 μ l of lysis buffer. Sonication was performed by using a water bath sonicator (Bioruptor) set to cycle at 30 s on and 1 min off for 30 min. Cell debris was removed by centrifugation at 14,000 for 10 min.

Next, 3 μ l of 9E10 antibody was added to each sample, and the mixtures were rocked at 4°C for 2 h. Protein G-Sepharose was washed three times in phosphate-buffered saline (PBS) and twice in lysis buffer. Portions (20 μ l) of washed beads were added to each sample. After 1.5 h at 4°C the beads were washed twice with lysis buffer, twice with high salt lysis buffer (500 mM NaCl), twice with wash buffer (10 mM Tris-Cl [pH 8.0], 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA), and once with Tris-EDTA (pH 8.0). Next, 100 μ l of elution buffer (50 mM Tris-Cl [pH 8.0], 10 mM EDTA, 1% SDS) was added, and the samples were incubated at 65°C for 15 min. The eluate was collected by centrifugation. The beads were washed with Tris-EDTA plus 0.67% SDS plus proteinase K, and the supernatant was added to the eluate from the previous step, followed by incubation at 65°C overnight.

DNA was purified by using Qiagen PCR purification columns. RNase was added to the purified DNA, and the samples were incubated at 37°C for 1 h. Samples were analyzed by using quantitative PCR.

β -Galactosidase assays. Single-tube β -galactosidase assays were done as described by Bao et al. (2), except that after the cultures were split, one set was treated with 0.4 M NaCl for 5 h before collection. For the 96-well β -galactosidase assays, cells were grown to log phase in 96-well plates, and their OD₆₀₀ was measured by using an optical plate reader (Molecular Devices). Then, 10 μ l of culture was added to 10 μ l of permeabilization buffer (0.227 M Na₂HPO₄, 0.142 M NaH₂PO₄, 20 mM KCl, 4 mM MgSO₄, 5% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 40 mM 2-mercaptoethanol), followed by incubation at room temperature for 10 min. A 180- μ l substrate solution (0.113 M Na₂HPO₄, 71 mM NaH₂PO₄, 10 mM KCl, 2 mM MgSO₄, 20 mM 2-mercaptoethanol, 20 mM chlorophenolred- β -D-galactopyranoside) warmed to 37°C was added. Readings were taken every 2 min at OD₅₅₀ using an optical plate reader. The β -galactosidase value was calculated as follows: [(rate - average blank rate)/rate sample volume]/[(OD₆₅₀ - average blank OD₆₅₀)/OD₆₅₀ volume].

Immunofluorescence. Samples collected before or after treatment with 0.4 M NaCl for 15 min were resuspended in 1 ml of buffer KP plus formaldehyde (90 mM potassium phosphate [pH 6.4], 3.7% formaldehyde) and fixed by incubation at 30°C for 10 min. Samples were washed three times with 1 ml of buffer KP (100 mM potassium phosphate [pH 6.4]) and once with 1 ml of buffer KPS (100 mM K₂HPO₄, 1.2 M sorbitol, 36 mM citric acid [pH 5.9]). The samples were resuspended in 250 μ l of buffer KPS and stored overnight at -20°C. The samples were defrosted and digested by the addition of 2 μ l of 10 mg of Zymolyase 100T/ml at 30°C with nutation for 20 min. Digested cells were washed once with buffer KPS (after digestion spin at 2,000 rpm) and resuspended in ca. 20 to 40 μ l of buffer KPS depending on the size of the pellet. Then, 10- μ l portions of cells were applied for 2 min to glass slides (ER-267W; Erie Scientific) with wells precoated with 0.1% poly-L-lysine for 5 min. The cells were aspirated, and the slides were plunged sequentially into cold methanol for 3 min and 10 s into cold acetone

(methanol and acetone were stored at -20°C). The wells were washed twice with bovine serum albumin (BSA)-PBS ($1\times$ PBS with 10 mg of BSA/ml), followed by incubation with 20 μl of 9E10 antibody diluted 1:2,000 in BSA-PBS for 1.5 h in a humid chamber. The wells were washed five times with BSA-PBS and then incubated with 20 μl of anti-mouse TRITC (tetramethyl rhodamine isothiocyanate)-conjugated secondary diluted 1:1,000 in BSA-PBS for 2 h in a humid chamber. The wells were washed five times with BSA-PBS and 1.5 μl mounting medium (1 mg of phenylenediamine/ml, 40 mM K_2HPO_4 , 10 mM KH_2PO_4 , 150 mM NaCl, 0.1% NaN_3 , 50 μg of DAPI/ml, 5% Vectashield, 90% glycerol [pH 8]) was added. Slides are analyzed with the Zeiss Axiovert 200M inverted microscope, and the images were analyzed with AxioVision 4.6 software.

Mass spectrometry (MS). (i) **Extract preparation.** One-liter cultures were grown to an OD_{600} of ~ 1 and collected by centrifugation. The cell pellet was resuspended in 5 ml of buffer H0.3 (25 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 0.5 mM EGTA, 2 mM MgCl_2 , 20% glycerol, 0.02% NP-40, 300 mM KCl, 1 mM DTT, 5 mM 4-nitrophenylphosphate, 10 mM $\text{Na}_2\text{S}_2\text{O}_5$, 80 mM β -glycerophosphate, Sigma phosphatase inhibitor cocktails 1 and 2 [1:100], Sigma protease inhibitor cocktail [1:260]) and dropped slowly in liquid nitrogen. Frozen cell pellets were lysed by using a mortar and pestle. The extract was slowly thawed and brought to a volume of 16 ml with buffer H0.3. Cell debris was removed by centrifugation. M2 FLAG agarose (Sigma) was washed three times in buffer H0.3 and then added to the extract and rocked for 2 h at 4°C . The beads were washed three times with buffer H0.3. 3XFLAG peptide was added, followed by rocking for 30 min at 4°C . The eluate was collected and precipitated overnight at 20°C by the addition of 9 volumes of 100% ethanol.

(ii) **Sample preparation.** To each of the three samples, 8 M urea and 100 mM Tris (pH 8.5) were added to solubilize the protein. The subsequent mixture was then reduced by adding 1 M tris (2-carboxyethyl) phosphine (TCEP) (for a final concentration of 5 mM TCEP), followed by incubation at room temperature. To alkylate, iodoacetamide (10 mM final concentration) was added, and the samples were subsequently incubated at room temperature while in the dark for 15 min. Endoproteinase Lys-C (0.1 $\mu\text{g}/\mu\text{l}$) was then added in the amount of 2.0 μl and shaken for 4 h, while incubated in the dark at 37°C . The addition of 100 mM Tris (pH 8.5) diluted the solutions to 2 M urea. Calcium chloride (100 mM) was then added for a final concentration of 1 mM CaCl_2 . A 10.0- μl portion of trypsin (0.5 $\mu\text{g}/\mu\text{l}$) was added. The resulting mixtures were then shaken for 18 h and incubated in the dark at 37°C . To neutralize the mixtures, 15.0 μl of formic acid (90%) was added for a final concentration of 5% formic acid. The tubes were centrifuged for 30 min at 2°C on a tabletop centrifuge.

(iii) **Multidimensional protein identification technology.** Upon completion of the digestion, the proteins were pressure loaded onto a fused silica capillary desalting column containing 3 linear cm of strong-cation-exchange material (5- μm particle diameter) followed by 3 cm of C_{18} (5- μm particle diameter reverse-phase resin) packed into a deactivated capillary (250- μm inner diameter). Using 1.5 ml of buffer A (95% water, 5% acetonitrile, 0.1% formic acid), the desalting columns were washed overnight. After the desalting process, a capillary (100- μm inner diameter) consisting of a 10- μm laser pulled tip packed with 10 cm of 3- μm Aqua C_{18} material (Phenomenex, Ventura, CA) was attached to the filter union (desalting column-filter union-analytical column). The resulting split columns were placed inline with a Surveyor MS pump (version 2.3; ThermoFinnigan, Palo Alto, CA) and analyzed by using a customized five-step separation method (for 90, 120, 120, 120, and 150 min, respectively).

Step 1 utilized only buffer A (95% water, 5% acetonitrile, 0.1% formic acid) and buffer B (80% acetonitrile, 20% water, 0.1% formic acid). It began with 5 min of 100% buffer A, followed by several buffer B gradients: 5 min of 0 to 10%, 40 min of 10 to 45%, and 10 min of 45 to 100%. A 20-min period of 100% buffer B treatment ensued, and the gradient program ended with 10 min of 100% buffer A.

Steps 2 to 5 utilized buffers A, B, and C (500 mM ammonium acetate, 5% acetonitrile, and 0.1% formic acid). Steps 2, 3, and 4 each began with 3 min of 100% buffer A and continued with 7 min of 20% buffer C, a 5-min gradient from 0 to 10% buffer B, a 75-min gradient from 10 to 45% buffer B, and then 5 min of a 45 to 100% buffer B gradient. Next, 10 min of 100% buffer B followed, and then the sequence ended with 10 min of 100% buffer A. The buffer C portions consisted of 20% for step 2, 50% for step 3, and 80% for step 4.

Step 5 began in a similar fashion (3 min of 100% buffer A, 7 min of 100% buffer C, and a 5-min gradient from 0 to 10% buffer B), and yet its 10 to 45% buffer B gradient lasted for 85 min, and the 45 to 100% buffer B gradient was for 10 min. A period of 10 min of 100% buffer B and then a gradient of 0 to 100% buffer B ensued, with the run ending with 10 min of 100% buffer A.

By increasing the salt concentration (buffer C) peptides "bump" off of the strong cation-exchange material and then, with a gradient of increasing hydrophobicity (buffer B), the peptides can elute from the reverse phase into the ion source. To elute the peptides from the micro capillary column, a distal 2.5-kV

spray voltage was applied. The applied voltage caused the peptides to directly electrospray into an LTQ two-dimensional ion trap mass spectrometer (ThermoFinnigan). First, a cycle of one full-scan mass spectrum (300 to 2,000 m/z) and then five data-dependent tandem MS (MS/MS) spectra at a 35% normalized collision energy was performed throughout each step of the multidimensional separation. The aforementioned high-pressure liquid chromatography solvent gradients and MS functions were all controlled by the Xcalibur data system (version 1.4).

(iv) **Analysis of MS/MS spectra.** As each step was executed, its spectra were recorded to a RAW file. These data was then converted into ".ms2" format through the use of RawXtract (version 1.8). From the .ms2 files, poor-quality MS/MS spectra were removed by using an automated spectral quality assessment algorithm known as a PARC filter (4). The remaining MS/MS spectra were searched with the SEQUEST algorithm (20) against the SGD_S-cerevisiae.fasta database (created on 16 December 2005). Protein sequences from the database were reversed (decoy database) to assess the false-positive rate (39). A computer cluster consisting of 100 1.2 GHz Athlon CPUs was used to perform the search (47). The SEQUEST search did not employ any enzyme specificity, and the final data set was filtered by using the DTASelect (version 2.0) program (unpublished data). The type of digestion method used was specified ($-\text{trypstat}$ for tryptic digests) so as to specifically filter for peptides with trypsin specificity. A user-specified false-positive rate was used to dynamically set XCorr and DeltaCN thresholds through quadratic discriminant analysis. These data sets were then further filtered to remove contaminants (i.e., keratin) through the use of Contrast (version 2.0). A minimum of two peptides and half-tryptic status ($-p\ 2\ -y\ 1$) were set in the Contrast.params file.

RESULTS

Screen of the *S. cerevisiae* gene deletion collection for mutants that display a signaling specificity defect. Although both the HOG and the FG pathways have been extensively studied, it remains unclear how signaling specificity between the pathways is maintained. In an attempt to gain insight into how cross talk might be prevented, we carried out a genetic screen of the *S. cerevisiae* gene deletion collection for mutants that cause erroneous activation of the FG MAPK output in response to HOG signaling.

To accomplish this goal, we transformed the Yeast Knock-Out collection (Open Biosystems) with a plasmid harboring an *FRE-lacZ* reporter construct, which requires Tec1 for its expression (27). We then screened the transformants using a high-throughput 96-well plate β -galactosidase assay. This initial screen identified candidates for mutants that displayed increased expression of the reporter gene compared to a wild-type control strain (step 1 in Fig. 2A). As expected, both the *hog1* Δ and the *pbs2* Δ strains showed increased expression of the reporter construct, indicating that our assay could identify mutants known to affect an output of the FG pathway. Also, three of four nonessential components of the middle module of the mediator complex and several other mediator components, including *cdk8* Δ (*srb10* Δ), showed increased *FRE-lacZ* expression, a finding consistent with previous reports showing that mediator can act as a negative regulator (33, 55). We anticipated that our initial screen would yield false-positive identification of mutants due to assay variability, reporter gene-specific effects, and mutations unlinked to the marked gene knockout. To control for false-positive results due to assay variability, mutants with β -galactosidase activities that were at least 1.35 standard deviations above the median value of the plate on which it resided were selected for retesting in triplicate (step 2 in Fig. 2A). This set corresponded to 365 mutants. Of these, we identified 32 mutants that displayed an increase in *FRE-lacZ* expression that was at least one standard

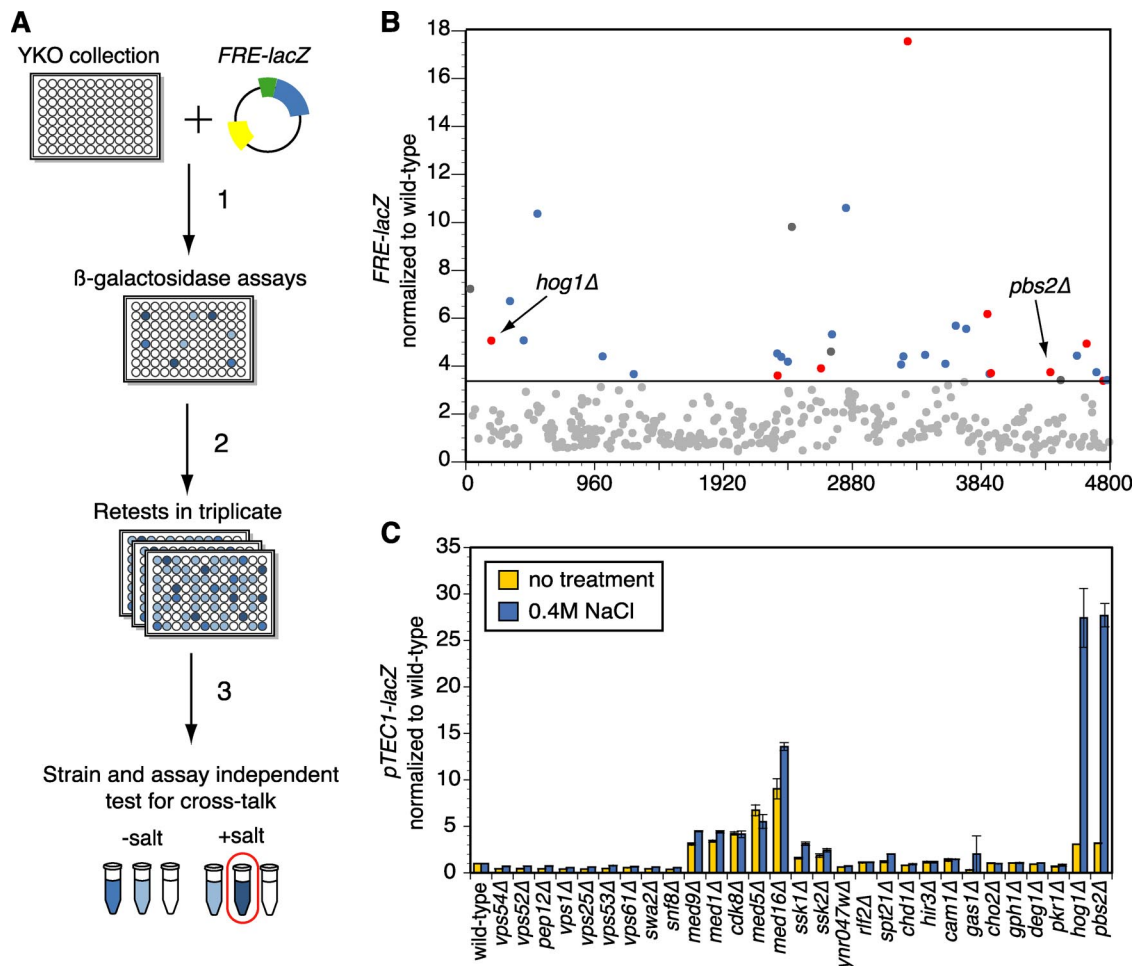


FIG. 2. Screen of the yeast gene knockout collection. (A) Scheme. (Step 1) The Yeast Knockout Collection (YKO) was transformed with a plasmid encoding an *FRE-lacZ* reporter gene. Transformants were screened for increased expression of *lacZ* using a 96-well β -galactosidase assay. (Step 2) The 365 mutants with the highest levels of *lacZ* expression normalized to the median of each plate were rearranged and retested in triplicate. (Step 3) The genes from the mutants that were one standard deviation above wild type in the retests were knocked out in the Sigma strain background. An integrated pTEC1-*lacZ* reporter was used with single-tube β -galactosidase assays to test for increased expression of *lacZ* in response to osmstress. (B) Data obtained from retesting experiments. The strains were taken from the deletion collection and are in the S288C strain background. The black line represents one standard deviation above the mean value for wild-type strains assayed. Mutants represented by red dots were found to display increased *FRE-lacZ* output when reconstructed and reassayed in the Sigma 1278b strain background, while mutants represented by blue dots were not. *hog1* Δ and *pbs2* Δ serve as positive controls and are indicated. Mutants represented by gray dots were not assayed in the Sigma strain background. (C) pTEC1-*lacZ* expression levels for the knockout strains reconstructed in the Sigma 1278b strain background. The knockout strains were made from YM2932. Strains of the indicated genotypes were assayed after being cultured for 5 h in the presence or absence of 0.4 M NaCl.

deviation above the average expression of the set of 365 mutants normalized to a wild-type control strain on each plate (Fig. 2B).

To control for false-positives due to unlinked mutations and for reporter-specific effects, we reconstructed the gene knockouts for the mutants that passed the first two tests in the filamentation-competent Σ 1278 strain background. These were made in a strain that contained a chromosomally integrated pTEC1-*lacZ* reporter gene construct. The resulting strains were assayed by using a single tube β -galactosidase assay. To distinguish between mutants that were simply defective in negative regulation of the FG pathway from bona fide cross talk mutants, we also assayed each reconstructed mutant in the presence or absence of osmstress (step 3 in Fig. 2A, data are shown in Fig. 2C). Cross talk mutants would be expected to display a salt-dependent increase in the FG output,

whereas mutants defective in negative regulation would display similar outputs regardless of the presence of osmstress.

Upon analysis, we found that many of the initial candidates appeared to be false-positive results (Fig. 2C). For unknown reasons, these included a large group of vesicle trafficking mutants. Of the remaining candidates, only the *hog1* Δ and *pbs2* Δ mutants displayed salt-induced activation of the pTEC1-*lacZ* reporter gene (Fig. 2C). These data suggested that if unidentified components are required to prevent cross talk, they may be redundant or essential. Also, known components of either pathway could be involved in cross talk suppression, as exemplified by the phosphorylation of Tec1 on T273 by Fus3 which prevents cross talk between the mating and FG pathways.

Activation of the HOG pathway in wild-type cells results in the phosphorylation of Ste7 and Kss1. Since we were unable to

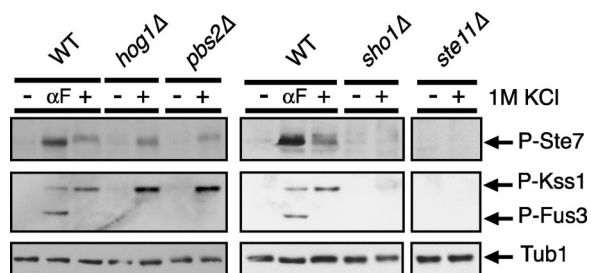


FIG. 3. Ste7 and Kss1 are phosphorylated upon osmostress. Shown are immunoblots of wild-type, *hog1Δ*, *pbs2Δ*, *sho1Δ*, and *ste11Δ* strains (F1950, YM2144, YM2145, YM2174, and YM1144, respectively). Cells were either untreated (–), treated with 1 M KCl for 15 min (+), or treated with α mating pheromone (α F) for 15 min. Phosphoproteins were detected with phospho-specific antibodies. Bands corresponding to the phosphorylated form of the indicated proteins are shown. Wild-type treated with mating pheromone serves as a control for the antibodies used. Ste7 is also the MAPKK in the mating pathway and is robustly activated in response to α F. This results in the phosphorylation of the mating MAPK, Fus3. Tubulin is used as a loading control.

identify new components involved in maintaining signaling specificity between the HOG and FG pathways, we focused on gaining a better understanding of the role that Hog1 plays in this process. The MAPKKK Ste11 is shared between the FG and HOG pathways. Genetic data suggest that activation of the Sho1/Msb2/Hkr1/Opy2 branch of the HOG pathway results in activation of Ste11 and its target in the HOG pathway, the Pbs2 MAPKK. It has been suggested that in *hog1Δ* and *pbs2Δ* strains, HOG pathway signaling results in the erroneous phosphorylation of the FG and mating pathway MAPKK Ste7 by Ste11 (34). We tested this hypothesis by using an antibody we developed previously that recognizes a peptide corresponding to the dually phosphorylated activation loop of Ste7 (49). Immunoblotting of extracts prepared from strains treated with 1 M KCl salt for 15 min revealed that Ste7 was phosphorylated in both *hog1Δ* and *pbs2Δ* strains (Fig. 3). Furthermore, Kss1, the MAPK in the FG signaling cascade, is also phosphorylated in response to salt treatment of cells (Fig. 3). These data correlate with the erroneous transcriptional induction of FG pathway targets in *hog1Δ* cells in response to osmostress that has been reported previously (17, 35, 58).

Strikingly, we found that Ste7 was also phosphorylated and activated in wild-type cells treated with salt. We also found that Ste11 was required for Ste7 phosphorylation under these conditions (Fig. 3). Ste7 phosphorylation was partially dependent on Sho1, which is consistent with its position upstream of the MAPK cascade in both pathways (Fig. 3). As in *hog1Δ* and *pbs2Δ* cells, the osmostress-induced signal was propagated to Kss1 (Fig. 3). However, previous work has indicated that, in contrast to *hog1Δ* and *pbs2Δ* strains, this activation of Kss1 in wild-type cells does not lead to transcription of FG pathway target genes. Given this observation, our data suggest that an inhibitory mechanism for maintaining signaling specificity exists downstream of phosphorylation of the Kss1 MAPK. Presumably, such a mechanism would interrupt the ability of the Ste12-Tec1 transcription factor to activate its target genes.

Hog1 prevents Tec1 from binding to filamentation gene promoters in a substantially kinase-dependent manner. As described in the introduction, the available evidence strongly

suggests that activation of the FG pathway leads to the binding of Tec1, the FG pathway-specific subunit of the Ste12-Tec1 transcription factor, to the promoters of target genes (61). Since Hog1 is required to prevent cross talk, we reasoned that it might prevent Tec1 from associating with promoters. We tested this hypothesis by performing ChIP, followed by quantitative PCR on *tec1Δ* mutant strains complemented by a *myc6-TEC1* allele. Three promoters were tested that had been shown in previous studies to be direct targets of Tec1: *PGU1*, *FLO11*, and *CLN1* (29). Primer pairs were chosen that were centered on TCS sequences found in the promoters of these genes. Because salt treatment of cells results in dissociation of transcription factors from DNA (42), we chose to examine myc6-Tec1 association in the absence of salt. Under these conditions, in a wild-type strain, we found little or no signal in ChIP assays indicative of an association of myc6-Tec1 with the promoter regions of *PGU1*, *FLO11*, or *CLN1* relative to an analysis of an isogenic strain harboring an untagged *TEC1* allele (Fig. 4A). This may not be surprising since we did not subject cells to conditions known to activate the FG pathway. However, even in the absence of added osmostress, we found a dramatic increase in Tec1 occupancy at all three targets in cells lacking *HOG1* (Fig. 4A). This increase was partially dependent on *SHO1* (Fig. 4A). To test whether this increase in Tec1 association could be trivially explained by an increase in Tec1 levels, we performed quantitative immunoblotting studies of these strains. As shown in Fig. 4B, quantification of the immunoblots revealed essentially identical levels of Tec1 present in *hog1Δ* versus wild-type cells.

We tested the role of the enzymatic activity of Hog1 in inhibiting the association of myc6-Tec1 with target promoters. Impairing the kinase activity of Hog1 by mutating the active-site lysine (K52) required for ATP binding or by mutating either of the activating phosphorylation sites (T274 or Y176) also produced increased association of Tec1 with its target promoters (Fig. 4A). The effects of these *hog1* mutants on myc6-Tec1 binding to endogenous promoters roughly paralleled their effects on *FRE-lacZ* expression (Fig. 4C). That is, impairing kinase activity resulted in increased *FRE-lacZ* output. However, it is important to note that none of the mutants produced the same phenotype as the *HOG1* deletion, suggesting either that they display sufficient residual kinase activity to inhibit Tec1 or that there is a kinase-independent role for Hog1 in specificity in addition to its kinase-dependent role.

HOG pathway signaling does not induce degradation of Tec1. During activation of the mating pathway Kss1 is activated, but activation of Kss1 does not result in transcription of FG pathway genes. As discussed above, Tec1 is degraded when the mating pathway is active, preventing erroneous cross talk between the mating and FG pathways. We tested whether signaling specificity between the FG and HOG pathways is mediated by a similar mechanism. As expected, myc6-Tec1 was rapidly degraded when haploid *MATa* strains were treated with α -factor. However, myc6-Tec1 steady-state levels remained unchanged when cells were treated with a 1 M KCl osmostress (Fig. 5A). We also tested whether Ste12 is degraded in response to salt but found that, like Tec1, its steady-state levels were not affected by salt treatment. These data, as well as the results shown in Fig. 4B, indicate that the Hog1 MAPK inhibits

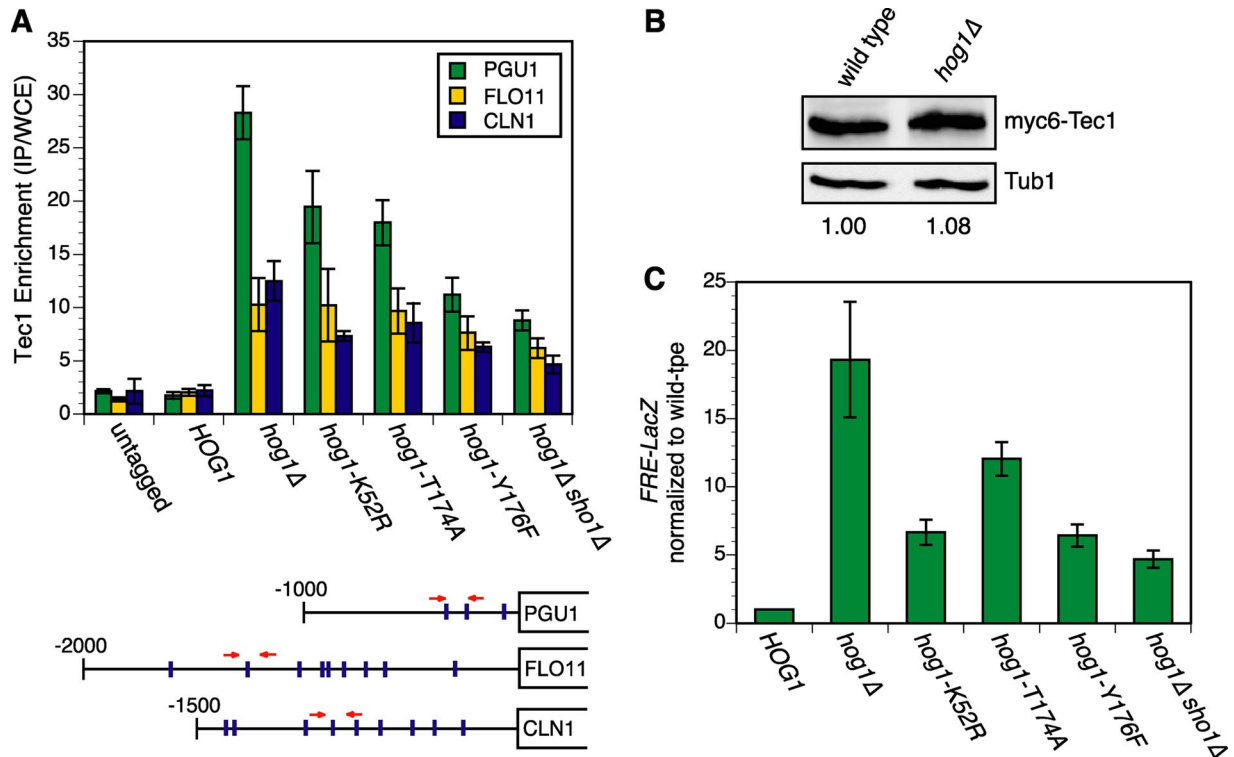


FIG. 4. Hog1 limits Tec1 binding to FG pathway promoters. (A) ChIP assays. Shown is the ChIP signal corresponding to Tec1 association with the indicated promoters in a *hog1Δ tec1Δ* strain (YM1993) transformed with *TEC1* (untagged) or *myc6-TEC1* and a plasmid harboring the indicated *HOG1* allele (*hog1Δ* and *hog1Δ sho1Δ* [YM2173] were transformed with insert-less vector). *PGU1*, *FLO11*, and *CLN1* promoters are diagrammed. The blue rectangles represent putative Tec1 binding sites, and the arrows represent the primers used for ChIP. (B) Tec1 protein levels. Shown are anti-myc and anti-tubulin immunoblots of a *tec1Δ* and *hog1Δ tec1Δ* strain (YM1934 and YM1993) transformed with *myc6-TEC1*. Protein levels were measured using fluorescent secondary antibodies. (C) *FRE-lacZ* expression. Shown are β -galactosidase activities of a *hog1Δ* strain (YM2144) transformed with plasmids harboring the indicated *HOG1* alleles (*hog1Δ* and *hog1Δ sho1Δ* [YM2188] were transformed with the corresponding insert-less vector).

Tec1 not by inducing its destruction but rather by directly or indirectly affecting its activity.

Tec1 maintains its nuclear localization during activation of the HOG pathway. Changes in the localization of proteins in response to signaling pathway activation are a common mode of regulation. For example, inactive Hog1 is localized to the cytoplasm, but upon phosphorylation the active kinase rapidly enters the nucleus. As discussed above, we discovered that HOG signaling inhibits the ability of Tec1 to bind to DNA.

Tec1 has been reported to be localized to the nucleus, suggesting that the effects we observe in the absence of osmotic stress could not be explained by its sequestration in the cytoplasm. However, in principle its export during osmotic stress could prevent Tec1 from binding DNA under acute stress conditions. Using indirect immunofluorescence, we tested this hypothesis by assaying the localization of myc6-Tec1 using anti-myc epitope antibodies. We confirmed that Tec1 is a nuclear protein (Fig. 5B, top panel). In response to osmotic stress, we found

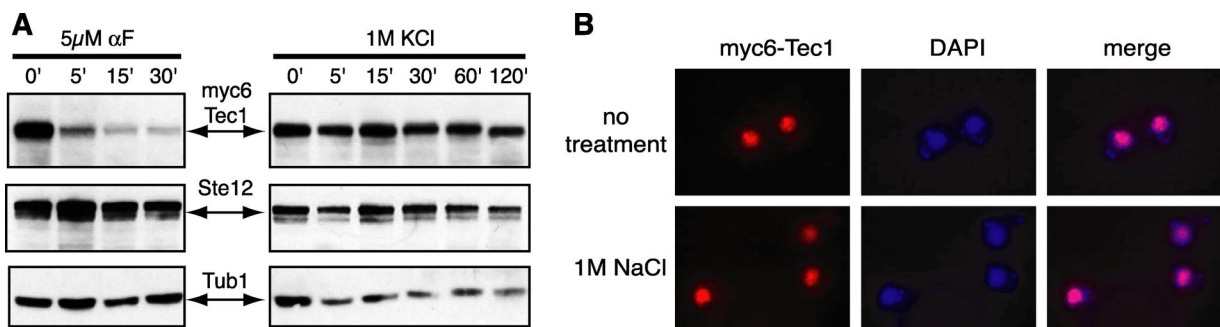


FIG. 5. HOG signaling does not affect stability or localization of Tec1. (A) Immunoblots showing myc6-Tec1 and Ste12 protein levels in response to treatment of wild-type cells with 5 μ M α -factor or 1 M KCl for the indicated time periods. (B) Indirect immunofluorescence images of myc6-Tec1 localization in a *tec1Δ* strain (YM1934) transformed with *myc6-TEC1* after treatment of cells with 1 M NaCl for 10 min.

that Tec1 remained localized to the nucleus (Fig. 5B). Thus, control of Tec1 localization does not seem to explain how Hog1 prevents Tec1 from associating with target promoters.

Consensus MAPK phosphorylation sites on Tec1, Dig1, Dig2, and Ste12 are not required to prevent cross talk. Our data indicate that osmostress results in the phosphorylation of Kss1. However, this event apparently does not lead to association of Tec1 with its target promoters. Therefore, we reasoned that a component of the FG pathway that functions downstream of Kss1 might be negatively regulated by Hog1 via direct phosphorylation (see Fig. 1A). For example, phosphorylation of Tec1 by Hog1 could alter its affinity for DNA. MAPKs almost always phosphorylate substrates on ST/P dipeptides. Therefore, we constructed serine-to-alanine or threonine-to-alanine mutations in every consensus MAPK phosphorylation site in Tec1, Dig1, and a related protein, Dig2. Although Dig2 appears to play a less important role in the FG pathway than Dig1, in the interest of completeness we included it in our analysis. The mutants were examined for their effects on *FRE-lacZ* expression by expressing the alleles from centromeric plasmids in strains harboring knockouts of the corresponding genes. As shown in Fig. 6A, none of the mutants in Tec1, Dig1, or Dig2 caused increased activation of the FG pathway comparable to either the *dig1Δ* or *tec1-T273M* mutants. This was also true of multiple mutants (indicated in Fig. 4A as *dig1-6A*, *dig1-5A*, and *tec1-7A*) that harbor mutations at all of the consensus sites (note that for Tec1, T273, the target of Fus3 was left intact in the *tec1-7A* allele to assess the role of the other sites). In addition, previous work has shown that mutation of all of the consensus MAPK phosphorylation sites on Ste12, either singly or together, does not result in increased signaling to the FG pathway (48).

Hog1 does not regulate the association of Tec1 and Dig1. Since Hog1 does not appear to directly regulate any of the known components in the FG pathway downstream of Kss1 activation, we sought to identify proteins that associate with Hog1 or Tec1. For these experiments, we constructed strains in which the protein was fused to a triple-FLAG epitope tag. The corresponding allele was either integrated into the chromosome in the case of Hog1 or expressed from a centromeric plasmid in the case of Tec1. The proteins were immunopurified, and the protein compositions of the eluates were analyzed by MS using multidimensional protein identification technology (25, 26, 56). For Hog1-FLAG3X, we performed purifications in the absence or presence of salt so that we could differentiate between proteins that bind the inactive versus active forms of Hog1. However, we did not identify any proteins that associated with Hog1 consistently in replicate purifications (data not shown). MS of immunopurified material from a 3XFLAG-Tec1 strain yielded several proteins for which some coverage was obtained in an otherwise wild-type strain but for which no coverage was obtained in a *hog1Δ* strain (see Table S1 in the supplemental material). These proteins are candidates for factors that mediate the regulation of Tec1 by Hog1. One of the proteins identified through this analysis was Dig1. Dig1 is a known inhibitor of Tec1, and it is possible that during osmostress Hog1 either directly or indirectly prevents Dig1 from releasing Tec1. As described above, we found that Hog1 does not regulate Dig1 by phosphorylation of its consensus MAPK sites; however, it is possible that Hog1 regulates the

interaction between Dig1 and Tec1 by some other means, such as phosphorylation on a nonconsensus MAPK site. Since low-coverage identifications in MS-based analysis display variability, we tested whether the presence of Hog1 affects the Tec1-Dig1 interaction by using coimmunoprecipitation assays using 3XFLAG-Tec1. As shown in Fig. 6B, immunoblotting of the immunoprecipitates from *HOG1* cells with polyclonal antibodies against Dig1 revealed two bands. Based on previous data, the slower-migrating form is likely to be a phosphorylated form of Dig1 (52). However, identical results were obtained in *hog1Δ* cells (Fig. 6B), suggesting that the absence of Dig1 in the immunopurified material from the *hog1Δ* strain reflected variability in protein identification rather than a bona fide difference. Thus, Hog1 does not appear to regulate association of Tec1 with Dig1.

Rck1 and Rck2 are not required for signaling specificity. Rck2 is a protein kinase, homologous to mammalian MAPK-activated protein kinases, that both interacts with Hog1 and is phosphorylated by Hog1 in response to osmotic and oxidative stress (5, 6, 53). When activated, Rck2 subsequently phosphorylates EF-2, resulting in a reduction in translation (53). We tested whether in addition to its role in regulating translation, Rck2 or a closely related homologue, Rck1, play an undiscovered role in maintaining signaling specificity between the HOG and FG pathways. Although deletion strains of Rck1 and Rck2 were present in the gene deletion collection and were therefore assayed in the screen described in Fig. 2, Rck2 is one of the few known substrates of Hog1 and merited a more careful analysis. β -Galactosidase assays using *FRE-lacZ* were performed on *rck1Δ*, *rck2Δ*, and *rck1Δ rck2Δ* strains (Fig. 6C). However, the data demonstrated that neither protein is required to maintain signaling specificity to the FG pathway.

MAPK phosphorylation sites on Ste50 are not required for signaling specificity. During the preparation of this manuscript it was reported that feedback phosphorylation of Ste50 by Hog1 downregulates both the FG and HOG pathways (23). It was also reported that mutation of five consensus MAPK phosphorylation sites on Ste50 resulted in cross talk between the two pathways. We attempted to confirm the latter result by constructing and assaying the identical mutant. However, we found that the *ste50-5A* mutant, in contrast to the *hog1Δ* mutant, did not display increased expression of the *FUS1-lacZ* mating pathway reporter gene used by Hao et al., nor did it affect the expression of pTEC1-*lacZ*, a reporter gene that is more commonly used to read out the activity of the FG MAPK pathway (Fig. 6D). This was true either in the absence or presence of osmostress. Finally, we found no effect of this *ste50* allele on the phenotype of *hog1Δ* mutants in either assay (Fig. 6C).

DISCUSSION

Many signaling pathways that regulate distinct outcomes share components, leading to the proposal of a number of models to explain how erroneous cross talk between pathways is avoided. These include scaffold-mediated insulation, in which signaling occurs in a protein complex from which shared components either do not dissociate in their active states or, if they do, they are rapidly inactivated (e.g., by dephosphorylation) so that other pathways are not erroneously activated. A

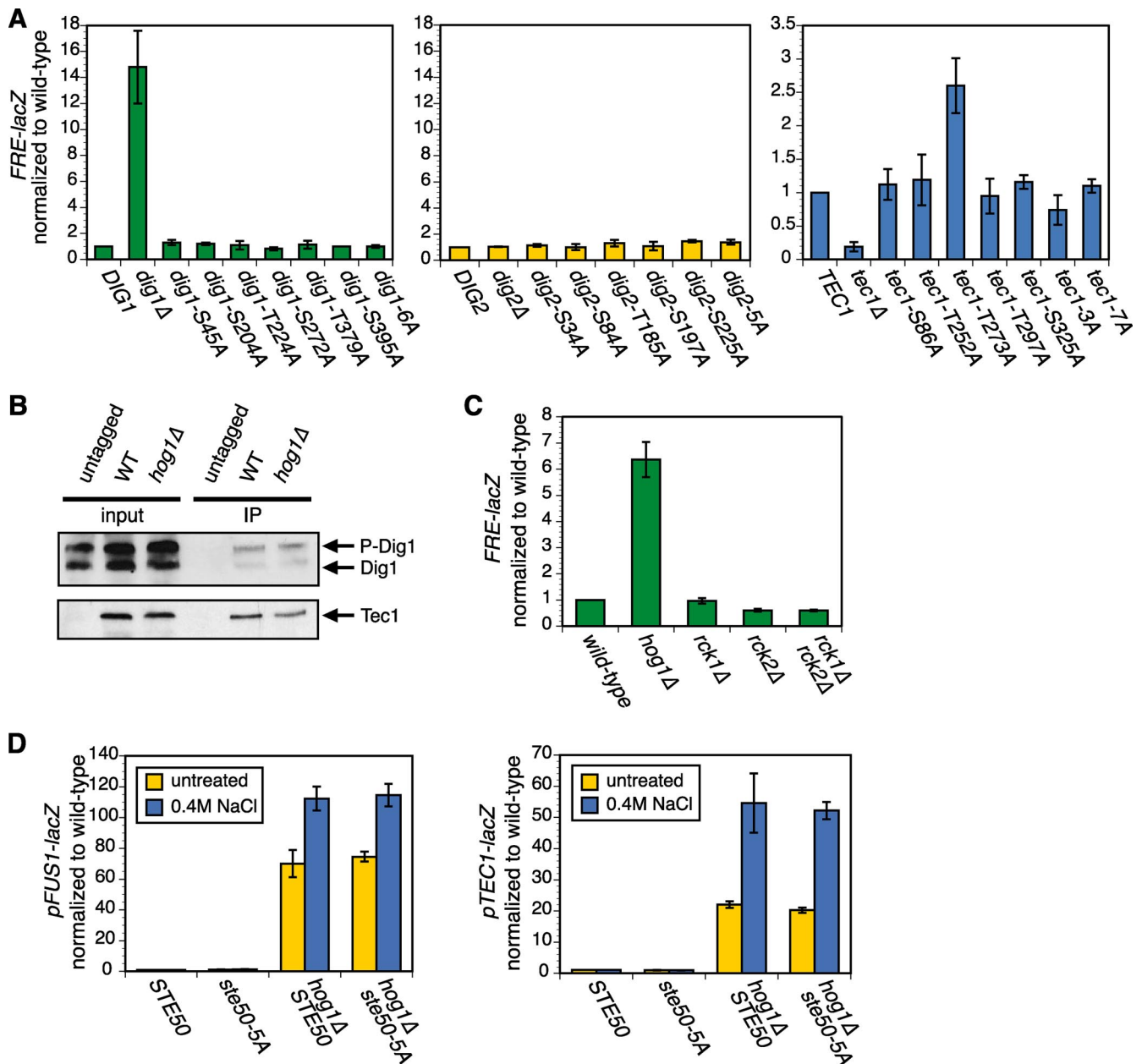


FIG. 6. Cross talk suppression between the FG and HOG pathways does not require the consensus MAPK sites on Dig1, Dig2, and Tec1, nor does the HOG signaling affect the Tec1-Dig1 interaction. (A) *FRE-lacZ* expression in a *dig1Δ*, *dig2Δ*, and *tec1Δ* strain (YM1598, YM2411, and YM1934, respectively) transformed with the indicated plasmids. *tec1-T273A* shows elevated expression of *FRE-lacZ* due to cross talk from the mating pathway. *tec1-3A* is *tec1-S462A S469A T476A*. (B) Coimmunoprecipitation of Dig1 with 3XFLAG-Tec1 in both wild-type and *hog1Δ* strains (YM2614 and YM2615). (C) *FRE-lacZ* expression in a wild-type, *hog1Δ*, *rck1Δ*, *rck2Δ*, and *rck1Δ rck2Δ* double strain (F1950, YM2144, YM2347, YM2348, and YM2352, respectively). (D) *FUS1-lacZ* and pTEC1-*lacZ* expression in a *ste50Δ* and *ste50Δ hog1Δ* double (YM1958, YM3526, YM3527, and YM3528) transformed with the indicated *STE50* allele. A difference in the growth and medium conditions is the likely explanation for why *hog1Δ* does not show as strong an induction of pTEC1-*lacZ* in response to salt as in Fig. 2.

second, more active, mechanism to maintain specificity occurs when signaling through one pathway inactivates pathway-specific components of the second pathway. This cross-pathway inhibition is exemplified in *S. cerevisiae* by the destruction of the FG pathway transcriptional activator, Tec1, in response to phosphorylation by the mating pathway MAPK, Fus3 (Fig. 7). In the present study, we sought to understand how specificity is maintained between the HOG and FG MAPK pathways. Our

results strongly suggest that cross-pathway inhibition also operates in this case but that the specific molecular mechanisms involved are distinct from those known to act to maintain specificity between the mating and FG pathways.

Our first indication that an inhibitory mechanism was at play between the HOG and FG MAPK pathways was that we found that both the Ste7 and the Kss1 FG pathway kinases were activated in response to osmotic stress, despite the fact that in

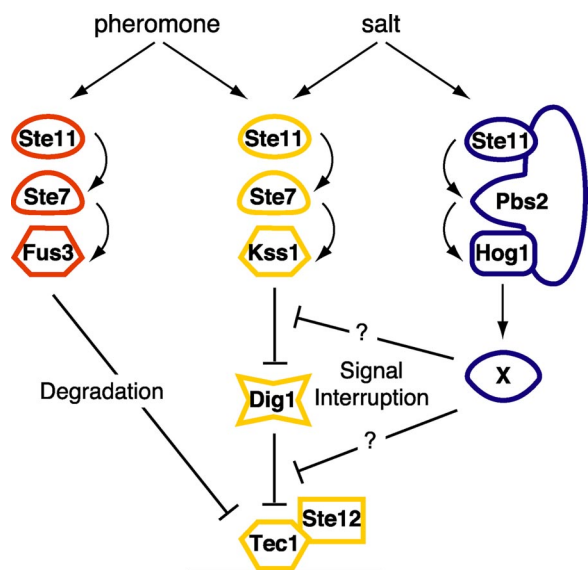


FIG. 7. Model of Tec1 regulation.

wild-type cells osmostress does not activate FG pathway target genes. Thus, even when all scaffolds and protein complexes are intact, the many protein-protein interactions in the HOG pathway apparently do not prevent erroneous activation of these kinases. We confirmed both that HOG signaling is required to suppress cross talk between the two pathways and that Hog1 kinase activity is important for specificity, suggesting an active inhibitory mechanism. Our data suggest that Hog1 either phosphorylates and inactivates an activator of Tec1 or activates an inhibitor of Tec1, thereby interrupting signaling. While this manuscript was in preparation, Hao et al. reported that Kss1 is phosphorylated in response to osmostress (23). They further provided evidence that the shared component Ste50 was phosphorylated under these conditions. Based on the phenotype of a mutant lacking five consensus MAPK phosphorylation sites on Ste50, these researchers suggested that specificity is maintained through Hog1-dependent inhibition of Ste50, which limits the duration of Kss1 activation during osmostress. It was further suggested that by limiting the time of Kss1 activation, specificity would in some way be maintained. However, in contrast to these findings, we observed that the identical mutation in Ste50 did not produce the cross talk phenotype of *hog1Δ* cells using either FG or mating pathway reporter genes. Thus, we conclude that Hog1 instead targets another protein to promote signaling specificity.

Our ChIP studies suggest that HOG signaling inhibits the association of the FG pathway activator Tec1 with its target genes. Specifically, in *hog1Δ* cells or in cells expressing catalytically deficient forms of Hog1, we observed a dramatic increase in the association of Tec1 with three different target promoters, even in the absence of osmostress. This increase in Tec1 occupancy was not associated with an increase in Tec1 protein levels. We also found that osmostress did not affect the nuclear accumulation of a myc6-tagged version of Tec1. Thus, HOG signaling inhibits the ability of Tec1 to associate with its target promoters without affecting its levels or localization.

To gain further insight into how the activity of Tec1 might be

regulated, we tested the possibility that Tec1 or its inhibitor, Dig1, or a related inhibitor, Dig2, might be controlled via direct phosphorylation by Hog1. However, individual or simultaneous mutation of all consensus MAPK sites (with the exception of the site known to be targeted by Fus3 on Tec1, T273) failed to recapitulate the phenotype of the *hog1Δ* mutant with respect to increased expression of FG pathway genes. Also, previous work has shown that consensus MAPK site mutants of Ste12, the combinatorial DNA-binding partner of Tec1, do not show erroneous expression of FG genes. These data suggest that Hog1 most likely inhibits the ability of Tec1 to interact with target promoters indirectly.

Such a model would be consistent with a recent study that demonstrated that a plasma membrane-tethered version of Hog1 that was unable to enter the nucleus upon osmostress retains its ability to suppress cross talk between the HOG and mating signaling pathways (57). Assuming that the mechanism that suppresses cross talk between the HOG and mating pathways is similar to that which suppresses cross talk between the HOG and FG pathways, we suggest that Hog1 targets a cytoplasmic protein whose phosphorylation in some way restricts the ability of Tec1 to bind DNA in the nucleus. This factor could either move into the nucleus, or it could control the movement of other proteins into the nucleus. Such a factor may be either essential or redundant since our screen of the nonessential gene deletion collection for cross talk mutants yielded only strains deleted for *PBS2* and *HOG1*. Since Hog1 also prevents cross talk between the HOG and mating pathways, it is tempting to speculate that such a mechanism also limits activation of the Ste12 activator downstream of the phosphorylation of Fus3 by Ste7.

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