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Journal

Molecular Biology of the Cell, 24(3)

ISSN

1059-1524

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Publication Date

2013-02-01

DOI

10.1091/mbc.e12-06-0477

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Reduced TOR signaling sustains hyphal development in *Candida albicans* by lowering Hog1 basal activity

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ABSTRACT *Candida albicans* is able to undergo reversible morphological changes between yeast and hyphal forms in response to environmental cues. This morphological plasticity is essential for its pathogenesis. Hyphal development requires two temporally linked changes in promoter chromatin, which is sequentially regulated by temporarily clearing the transcription inhibitor Nrg1 upon activation of cAMP/protein kinase A and promoter recruitment of the histone deacetylase Hda1 under reduced target of rapamycin (Tor1) signaling. The GATA family transcription factor Brg1 recruits Hda1 to promoters for sustained hyphal development, and *BRG1* expression is a readout of reduced Tor1 signaling. How Tor1 regulates *BRG1* expression is not clear. Using a forward genetic screen for mutants that can sustain hyphal elongation in rich media, we found *hog1*, *ssk2*, and *pbs2* mutants of the HOG mitogen-activated protein kinase pathway to express *BRG1* irrespective of rapamycin. Furthermore, rapamycin lowers the basal activity of Hog1 through the functions of the two Hog1 tyrosine phosphatases Ptp2 and Ptp3. Active Hog1 represses the expression of *BRG1* via the transcriptional repressor Sko1 as Sko1 disassociates from the promoter of *BRG1* in the *hog1* mutant or in rapamycin. Our data suggest that reduced Tor1 signaling lowers Hog1 basal activity via Hog1 phosphatases to activate *BRG1* expression for hyphal elongation.

Monitoring Editor

Charles Boone
University of Toronto

Received: Jun 25, 2012

Revised: Nov 6, 2012

Accepted: Nov 15, 2012

INTRODUCTION

Candida albicans is a major opportunistic fungal pathogen of humans (Odds, 1988; Calderone and Fonzi, 2001). In healthy individuals *C. albicans* inhabits the oral-pharyngeal, gastrointestinal, and urogenital tracts (Calderone and Fonzi, 2001). However, when the host immune system is compromised, it can cause mucosal infections and life-threatening disseminated infections. Systemic infection is a serious complication for transplant recipients, chemotherapeutic patients, and patients in intensive care. *Candida* ranks fourth in nosocomial bloodstream infections (Wisplinghoff et al., 2004). The

extraordinary ability of *C. albicans* to successfully infect many different anatomical sites of the human host sets it aside from all other pathogenic fungi, making it an important organism to study (Wilson et al., 2009). One critical virulence attribute of *C. albicans* is its morphogenetic plasticity: it is able to grow as yeast, pseudohyphal, and hyphal forms in response to environmental cues. This flexibility is believed to provide the organism with a high degree of phenotypic diversity and adaptability to colonize multiple niches in the host.

Several signal transduction pathways have been shown to regulate hyphal development in response to various environmental signals. These include the cAMP/protein kinase A pathway that is essential for hyphal development and virulence in all conditions (Feng et al., 1999; Bahn and Sundstrom, 2001; Bockmuhl and Ernst, 2001; Bockmuhl et al., 2001; Rocha et al., 2001), mitogen-activated protein kinase (MAPK) pathways (Kohler and Fink, 1996; Leberer et al., 1996; Alonso-Monge et al., 1999), the Rim101 pH-sensing pathway (Porta et al., 1999; Ramon et al., 1999; Davis et al., 2002), the regulation of Ace2 and morphogenesis network (McNemar and Fonzi, 2002; Song et al., 2008), the target of rapamycin (Tor1) signaling pathway (Bastidas et al., 2009; Zacchi et al., 2010; Lu et al., 2011), and more (Uhl et al., 2003; Homann et al., 2009; Blankenship et al., 2010). Despite the importance of these pathways in hyphal

This article was published online ahead of print in MBoc in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E12-06-0477>) on November 21, 2012.

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Abbreviations used: HOG, high osmolarity glycerol; MAPK, mitogen-activated protein kinase; MAPKK/MEK, mitogen-activated protein kinase kinase; RT, reverse transcription; TOR, target of rapamycin; YPD/YEPD, yeast extract peptone dextrose.

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development, how *C. albicans* coordinates information from different signaling pathways in regulating the yeast–hyphal transition remains a major question. We found that temporal coordination of two central cell growth signaling pathways controls hyphal development (Lu *et al.*, 2011). Activation of the cAMP pathway triggers a transient removal of the major transcription repressor of hyphal development, the Nrg1 protein, from promoters of hypha-specific genes for hyphal initiation (Lu *et al.*, 2011). The adenylate cyclase Cyr1 can integrate a diverse range of external signals (Hogan and Muhlschlegel, 2011), such as peptidoglycan fragments (Xu *et al.*, 2008), CO₂ (Klengel *et al.*, 2005), farnesol (Hornby *et al.*, 2001; Davis-Hanna *et al.*, 2008), and elevated temperature (Shapiro *et al.*, 2009), to generate a pulse of cAMP essential for hyphal initiation (Zou *et al.*, 2010). Nrg1 protein can be kept off the promoters of hyphal specific genes if cells are in nutrient-limiting media or in the presence of rapamycin or serum (Lu *et al.*, 2011). The target of rapamycin (TOR) signaling pathway functions as a global regulator of cellular growth in response to nutrient availability from yeast to human and it controls different cellular processes in fungi (Rohde and Cardenas, 2004; Rohde *et al.*, 2008). *C. albicans* has a single TOR kinase, Tor1, which is rapamycin sensitive (Cruz *et al.*, 2001; Bastidas *et al.*, 2009). We find that reduced Tor1 signaling during hyphal initiation leads to the expression of the GATA transcription factor Brg1, which recruits the histone deacetylase Hda1 to the promoters of hypha-specific genes, leading to nucleosome repositioning that blocks Nrg1 from binding to the promoters (Lu *et al.*, 2012). A critical feature of the temporal integration of the two signaling pathways is that Brg1 can bind to the promoters only when Nrg1 is not bound (Lu *et al.*, 2011). This requirement provides a mechanism for why hyphal development requires the cAMP/protein kinase A pathway under all conditions. After hyphal initiation, subsequent commitment to either sustained hyphal development or reversion back to yeast growth is determined by nutrient and growth conditions (Lu *et al.*, 2011).

Downstream effectors of the Tor1 pathway responsible for increased *BRG1* expression and sustained hyphal development are not known. Here we use a forward genetic screen to identify negative regulators that are mediators of Tor1 signaling for hyphal elongation. Unexpectedly, we find that reduced Tor1 signaling lowers the basal activity of the Hog1 MAP kinase for Brg1 expression and hyphal elongation. The HOG (high osmolarity) MAPK pathway plays a central role in stress responses in *C. albicans* (Alonso-Monge *et al.*, 1999; Smith *et al.*, 2004; Arana *et al.*, 2005; Enjalbert *et al.*, 2006). Hog1 is activated by osmotic stress, oxidative stress, and heavy metal stress and is required for the survival of *C. albicans* cells when they encounter the stresses. In contrast to stress-induced rapid Hog1 activation, rapamycin treatment leads to a down-regulation of Hog1 basal activity for a prolonged period of time, and the inactivation of Hog1 is important for sustained hyphal elongation. This places Hog1 under the Tor1 signaling pathway in the regulation of a developmental process for the first time. Hog1 is, therefore, one of the outputs of Tor1 in the temporal and spatial control of cellular growth.

RESULTS

A forward genetic screen identified *hog1*, *pbs2*, and *ssk2* of the HOG MAPK pathway as mutants with the strongest phenotype in sustained hyphal elongation under nutrient-rich conditions

To uncover how Tor1 regulates hyphal elongation, we performed a forward genetic screen for negative regulators of hyphal maintenance. Under a nutrient-rich condition hyphae cells convert to yeast cells due to a high Tor1 activity, as addition of rapamycin to the

| Gene | Description |
|-------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>RBD1</i> | Rhomboid-like protein; similar to putative intramembrane serine proteases; involved in filamentous growth; Gcn4p-regulated; caspofungin repressed |
| <i>HOG1</i> | MAP kinase of osmotic, heavy metal, and core stress response; role in regulation of glycerol, D-arabitol in response to stress; phosphorylated in response to H ₂ O ₂ (Ssk1p dependent) or NaCl; mutant induces protective mouse immune response |
| <i>Orf19.2726</i> | Putative plasma membrane protein; Plc1p regulated |
| <i>GZF3</i> | Putative transcription factor; oxidative stress induced via Cap1p; null mutant exhibits abnormal colony morphology and altered sensitivity to fluconazole, LiCl, and copper |
| <i>Orf19.3720</i> | Open reading frame, uncharacterized |
| <i>Orf19.5406</i> | Protein required for normal filamentous growth; mRNA binds to She3p and is localized to hyphal tips |
| <i>CUP9</i> | Gene upregulated in clinical isolates from HIV ⁺ patients with oral candidiasis; transcription reduced upon yeast-hyphal switch; ketoconazole induced; Plc1p regulated; shows colony morphology-related Ssn6p regulation; biofilm-induced gene |
| <i>PBS2</i> | MAPK kinase (MAPKK); role in osmotic and oxidative stress responses, oxidative stress adaptation; required for stress regulation of Hog1p localization and activity |
| <i>SSK2</i> | MAP kinase kinase kinase (MAPKKK) that regulates Hog1p activation and signaling; transcription is down-regulated in response to treatment with ciclopirox olamine |

TABLE 1: Mutants that could sustain hyphal elongation in YPD medium.

nutrient-rich medium sustains hyphal elongation (Lu *et al.*, 2011). If rapamycin functions via Tor1 to inactivate the negative regulator and therefore allows sustained hyphal elongation, deletion of the negative regulator is expected to maintain hyphal elongation even in nutrient-rich conditions regardless of rapamycin. We screened a knockout library of 674 unique genes in *C. albicans* (Noble *et al.*, 2010) for mutants that could sustain hyphal elongation in 2% Bacto peptone, 2% dextrose, 1% yeast extract (YPD) medium. Overnight cultures of the mutants in the knockout collection were diluted into YPD medium at 37°C. Morphology analysis showed that only nine mutants could maintain hyphal elongation in the absence of rapamycin after incubation for 4.5 h (Table 1). All of the mutants grew as yeast form at room temperature. Among the nine mutants, the deletions of *HOG1*, *PBS2* (MAPK kinase [MAPKK, MEK]), and *SSK2* (MAPKK kinase) in the HOG MAPK pathway showed the strongest hyphal elongation under nutrient-rich conditions. Therefore we focused on these three mutants in this study. To confirm the role of the HOG MAPK cascade in hyphal maintenance, we inoculated cells from overnight cultures at room temperature into fresh YPD medium at 37°C for 30 min for hyphal initiation and then incubated the

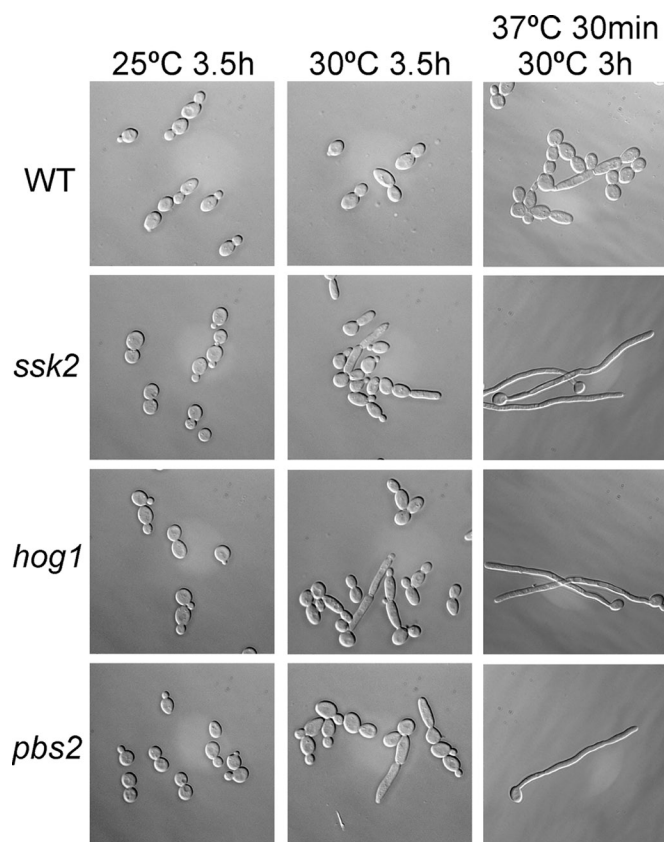


FIGURE 1: Hyphal maintenance in the mutants of the HOG MAPK pathway is independent of rapamycin. Overnight cultures of wild-type and *hog1*, *pbs2*, and *ssk2* mutant strains in the deletion library (Noble et al., 2010) were diluted at 1:100 into YPD medium at 25 or 30°C and incubated for 3.5 h or into prewarmed YPD medium at 37°C for 30 min and then transferred to 30°C for 3 h for cell morphology analysis.

cultures at 30°C for 3 h. As shown in Figure 1, almost all cells of the *hog1* mutant grew as elongated hyphae after 3 h in YPD at 30°C, whereas ~90% of the wild-type cells were in yeast form under the same growth condition. The *pbs2* and *ssk2* mutants exhibited similar phenotypes to that of the *hog1* mutant. Our finding that Hog1 and its upstream kinases Pbs2 and Ssk2 play a repressive role in hyphal elongation is in agreement with previous reports that these mutants have a high tendency to develop filaments (Alonso-Monge et al., 1999; Eisman et al., 2006). Consistent with the previous report, ~10–15% of the *hog1* mutant cells were elongated in an overnight culture grown at 30°C or when grown for 3.5 h at 30°C (Figure 1). Our results demonstrate that the repressive role of Hog1 in hyphal development is during hyphal elongation. Sustained filamentation in *hog1*, *pbs2*, and *ssk2* mutants was only observed after the cells were first grown at 37°C for hyphal initiation. These mutants did not form hyphae when they were grown at room temperature. In addition, hyphal initiation in these mutants seemed similar to that of wild-type cells, as determined by temporal disappearance of Nrg1 during hyphal induction at 37°C (Supplemental Figure S1).

Mutants of the HOG MAPK pathway express *BRG1* in nutrient-rich media in a rapamycin-independent manner

A readout of reduced Tor1 signaling for hyphal elongation in *C. albicans* is *BRG1* expression (Lu et al., 2012). During hyphal initiation when Nrg1 protein is gone, the expression of *BRG1* is activated under reduced Tor1 signaling. Accumulated Brg1 then recruits Hda1

to promoters of hypha-specific genes, leading to obstruction of Nrg1-binding sites and sustained hyphal development (Lu et al., 2012). If Hog1 functions downstream of Tor1 in sustained hyphal development, the *hog1* mutant is expected to express Brg1 during hyphal elongation in nutrient-rich media irrespective of rapamycin. To test this, we examined protein levels of Brg1 in mutants of the HOG MAPK pathway during hyphal induction. Western analysis of Brg1-myc expressed under its endogenous promoter showed that Brg1 was expressed in wild-type cells at 37°C only in the presence of rapamycin, as previously reported (Lu et al., 2012). In contrast, Brg1 was highly expressed in *hog1*, *ssk2*, and *pbs2* mutants at 37°C in a rapamycin-independent manner (Figure 2A). Correlative to its protein levels, *BRG1* expression was maintained at a high level at 37°C independent of rapamycin in *hog1*, *ssk2*, and *pbs2* mutants (Figure 2B). *BRG1* transcription levels remained low at 25°C regardless of rapamycin in *hog1*, *ssk2*, and *pbs2* mutants as in wild-type cells, indicating that removal of Nrg1 by growing cells at 37°C is still required for the activation of *BRG1* expression in these mutants. Our results suggest that the sustained hyphal development in the mutants could be attributed to the sustained activation of *BRG1* expression regardless of rapamycin treatment.

If Hog1 is one of the many downstream targets of the Tor1 signaling, *hog1* mutant is not expected to affect the expression of other genes regulated by the Tor1 signaling. Therefore we wanted to determine whether the expression of other Tor1-regulated genes is affected by the *HOG1* deletion. In *Saccharomyces cerevisiae*, rapamycin induces nuclear localization of the transcription factor Gln3, leading to the transcription of genes regulated by nitrogen catabolite repression (e.g., *DAL5*, *MEP2*, *GAP1*, etc.; Georis et al., 2009). To examine whether *DAL5* is an output of the Tor1 pathway, we performed a quantitative reverse transcription (qRT)-PCR experiment to examine the expression levels of *DAL5* in wild-type and the *hog1* mutant with and without rapamycin. As shown in Figure 2C, the expression of *DAL5* in both the wild-type and *hog1* strains required rapamycin. This is in contrast to *BRG1*, which was expressed in the *hog1* mutant regardless of rapamycin. Therefore the induction of *DAL5* by rapamycin is not regulated through Hog1. In addition, *DAL5* expression required only rapamycin but was independent of temperature, a situation that differed from that of *BRG1*. Our results suggest that the HOG pathway specifically controls the expression of *BRG1* but not other downstream genes of the Tor1 signaling pathway.

Brg1 is required for sustained hyphal elongation in the *hog1* mutant

To determine whether the expression of Brg1 is a major functional output of repressing Hog1 activity for sustained hyphal elongation, we constructed a *hog1 brg1* double mutant. Deletion of *BRG1* in a *hog1* mutant impaired hyphal elongation even in a rapamycin-containing medium at 37°C, and the phenotype of the *hog1 brg1* double mutant resembled the phenotype of the *brg1* single mutant (Figure 3A). In contrast, deletion of *BRG1* had no effect on the hypersensitivity of the *hog1* mutant to osmotic stress, as growth of the *hog1 brg1* double mutant was still sensitive to high salt, similar to that of the *hog1* single mutant (Figure 3B). Taken together, our data show that Brg1 is required for hyphal maintenance in the *hog1* mutant but is not involved in the osmotic response.

Extent of hyphal elongation in *hog1*, *pbs2*, and *ssk2* mutants is unresponsive to available nutrients

The rapamycin-sensitive Tor1 kinase is a central regulator of cell growth in response to nitrogen and amino acid availability and is known to be activated in nutrient-rich medium (Loewith and Hall,

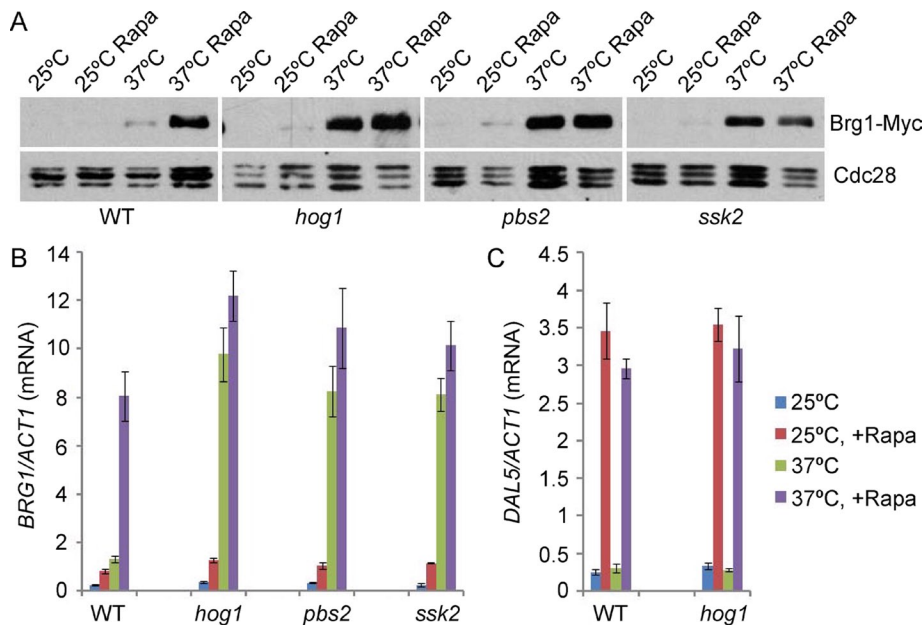


FIGURE 2: Brg1 is highly expressed in the *hog1* mutant at 37°C in a rapamycin-independent manner. (A) Western analysis of Brg1-Myc. Wild-type and indicated mutant cells carrying Brg1-Myc were diluted into YPD medium at 25 or 37°C in the presence or absence of 10 nM rapamycin and cells were collected at 5 h for Western analysis. qRT-PCR analysis of *BRG1* (B) and *DAL5* (C) expression in wild-type cells and indicated mutants. Cells were incubated as described in A. The signals obtained from *ACT1* mRNA were used for normalization. All data show the average of three independent qRT-PCR experiments, with error bars representing the SEM.

2011). This regulation of Tor1 activity by amino acid availability is likely conserved in *C. albicans* (Chen *et al.*, 2012). Because inhibiting Tor1 activity by rapamycin could maintain hyphal development, we hypothesized that hyphal elongation should be blocked in very rich medium, such as 4× yeast extract/peptone/dextrose (YPD), due to enhanced Tor1 activity. As predicted, the hyphal elongation of wild-type cells appeared almost abolished in 4× YEPD at 37°C for 4 h, whereas ~60% of cells grew as elongated filaments in 1× YEPD (Figure 4A). The deficiency of hyphal elongation in 4× YEPD was ascribed to high Tor1 activity, as a sublethal concentration of rapamycin led to sustained hyphal filamentation in 4× YEPD at 37°C (Figure 4B). Unlike the wild-type cells, *hog1*, *ssk2*, and *pbs2* mutants could sustain hyphal elongation under both standard (1× YEPD) and very rich conditions (4× YEPD), and the extent of hyphal elongation in these mutants seemed to be the same in the two media (Figure 4A). This indicates that these mutants could maintain elongation regardless of levels of Tor1 activity. This result is consistent with the model that the HOG MAPK pathway functions downstream of TOR signaling in regulating hyphal elongation.

Rapamycin represses the basal activity of Hog1 during hyphal induction at 37°C

Mutants of *hog1*, *pbs2*, and *ssk2* showed similar extents of hyphal elongation regardless of levels of Tor1 activity, suggesting that Hog1 likely functions downstream of Tor1. If so, rapamycin is expected to reduce the basal activity of Hog1 (defined as the Hog1 activity when cells are not under stresses or starvation). Because Hog1 activity, like that of all other MAPKs, is controlled by dual phosphorylation on two closely spaced residues—a threonine and a tyrosine—by its MEK Pbs2, we could determine whether basal activity of Hog1 is down-regulated in the presence of rapamycin by measuring the levels of phosphorylated Hog1. Hog1 phosphorylation was

monitored by Western blotting with an antibody that recognizes only the active, phosphorylated form of MAPKs (Millar *et al.*, 1995; Smith *et al.*, 2004). Cells were grown in YPD medium in the presence or absence of rapamycin at 25 or 37°C. Upon rapamycin induction at 37°C, we detected a significant decrease in the level of phosphorylated Hog1 after 5 h in comparison to the starting cells (Figure 5A). However, in the medium without rapamycin at 37°C, the level of phosphorylated Hog1 remained largely unchanged. We also showed that the decrease in Hog1 phosphorylation level was temperature dependent, as the phosphorylation level remained almost unchanged at 25°C regardless of rapamycin treatment. As a control, the protein levels of Hog1 did not change under these growth conditions. It has been reported that a significant decrease in the level of phosphorylated Hog1 was observed upon shifting cells from 23°C to either 37 or 42°C, and Hog1 phosphorylation level returned to the basal level after 1 h of incubation (Smith *et al.*, 2004). To further determine the temporal dynamic change in Hog1 activity during hyphal induction, we tracked the levels of phosphorylated Hog1. The level of Hog1 phosphorylation was at the basal level after 1 h of

incubation in YPD medium without rapamycin at 37°C, consistent with the previous study, and remained unchanged for at least 7 h of

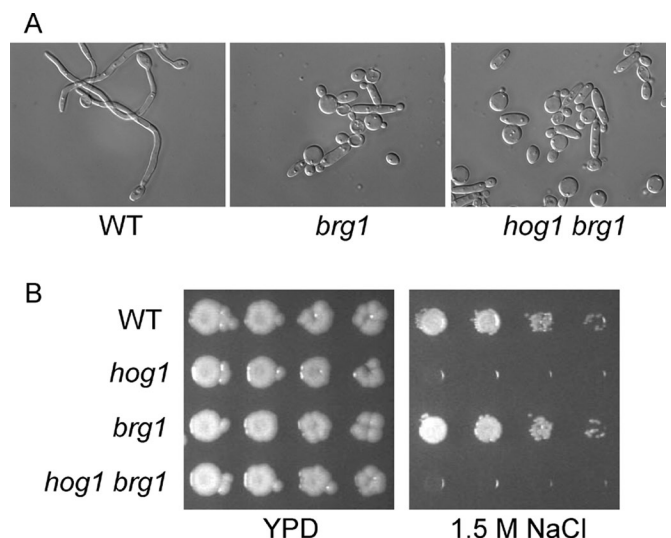


FIGURE 3: Deletion of *BRG1* in the *hog1* mutant completely blocks hyphal elongation. (A) Cells of wild-type, *brg1* single mutant, and *brg1 hog1* double mutant (HLY4095) cells were diluted into YPD with 10 nM rapamycin at 37°C. Photographs were taken after 7 h of incubation. (B) Cells of *brg1 hog1* double mutant are sensitive to osmotic stress. Cells of wild type, single mutants *hog1* and *brg1*, and double mutant *brg1 hog1* were serially diluted fivefold and spotted onto YPD solid medium supplemented with or without NaCl (1.5 M). Photographs were taken after 48 h of growth at 30°C.

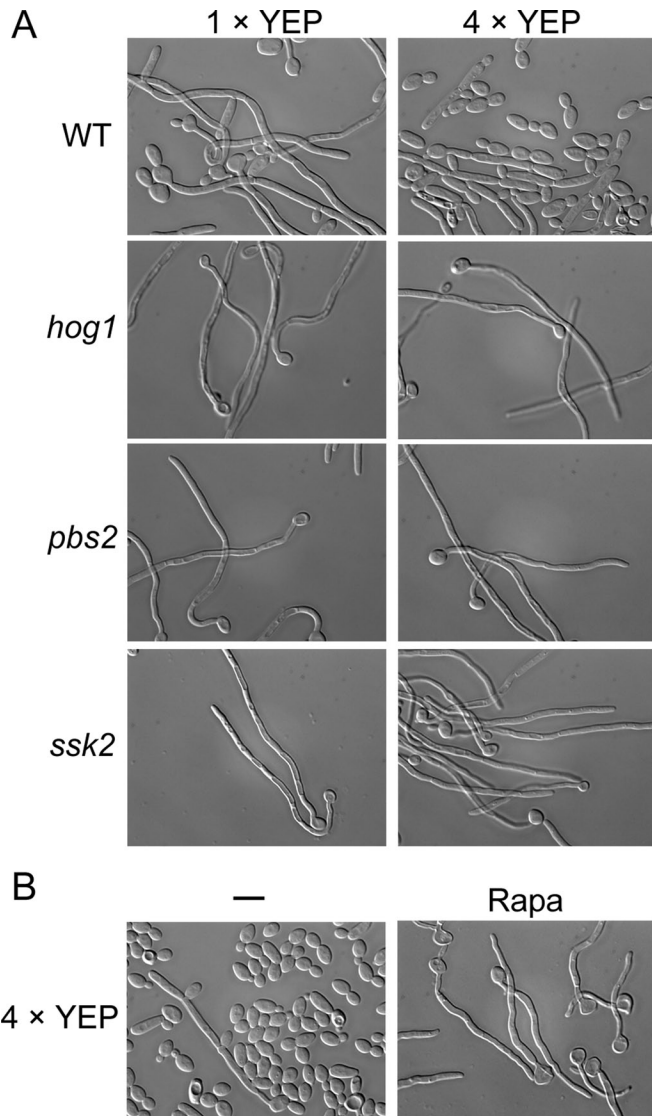


FIGURE 4: Hog1 functions downstream of Tor1 signaling in hyphal maintenance. (A) *hog1*, *pbs2*, and *ssk2* mutant strains in the HOG MAPK pathway could sustain hyphal elongation in rich medium. Overnight cultures of wild-type strain, *hog1*, *pbs2*, and *ssk2* mutant strains were diluted 1:100-fold into 1× YEPD or 4× YEPD medium, and cells were incubated for 4 h at 37°C before photographs were taken. (B) An overnight culture of wild-type cells (SN250) was diluted 1:100-fold into rich medium (4× YEP medium with 2% glucose) at 37°C in the presence or absence of 10 nM rapamycin. Morphology analysis was performed after incubation for 6 h.

incubation. However, in rapamycin-containing medium, the levels of phosphorylated Hog1 decreased after 1 h and did not recover until 7 h of incubation (Figure 5B). It seemed that rapamycin extended the period of low Hog1 activity. To confirm that the effect of rapamycin on Hog1 phosphorylation is mediated through Tor1, we examined Hog1 phosphorylation in the *TOR1-1/TOR1* rapamycin-resistant strain (Cruz et al., 2001). As shown in Figure 5C, rapamycin reduced levels of phosphorylated Hog1 only in the wild-type strain and not in the *TOR1-1/TOR1* strain. From these results, we suggest that rapamycin, by inhibiting Tor1 activity, could repress the basal activity of Hog1 to activate the expression of Brg1 for sustained hyphal elongation.

Hog1 phosphorylation in response to stresses is dominant over rapamycin-induced Hog1 dephosphorylation

Signal transduction through the MAPK cascade involves sequential phosphorylation and activation of three distinct kinases: the MAPK kinase kinase, the MAPK kinase (or MEK), and the MAPK itself. Deletion of *PBS2* or *SSK2* sustained hyphal elongation in nutrient-rich conditions, suggesting that phosphorylated Hog1 is repressive to hyphal elongation. Therefore activation of Hog1 in response to osmotic or oxidative stress is expected to block hyphal maintenance. To test this prediction, we examined levels of Hog1 phosphorylation in response to oxidative stress in the absence and presence of rapamycin. As previously shown (Smith et al., 2004), Hog1 was highly phosphorylated after 10 min of exposure to 5 mM H₂O₂, and the level of phosphorylated Hog1 returned to basal level after 120 min (Figure 6A). Shorter exposure time was used for this Western blot than for the blots in Figure 5, so that the signal for phosphorylated Hog1 in response to stresses was not too saturated. The levels of phosphorylated Hog1 between activated state and basal state is very different, which is consistent with the literature (Smith et al., 2004; Figure 6A). Of interest, rapamycin could not lower the basal levels of phosphorylated Hog1 in cells exposed to stresses at 120 min (Figure 6A). Lowering the basal level of Hog1 phosphorylation by rapamycin at 120 min was critical for sustained hyphal elongation, as rapamycin-induced chromatin remodeling could be established only during the time window when Nrg1 was gone from the promoters of hypha-specific genes (Lu et al., 2011, 2012). Adding rapamycin at 120 min after hyphal initiation could not sustain hyphal elongation (Lu et al., 2011). Consistent with the observed levels of Hog1 phosphorylation at 120 min, we found that *C. albicans* cells could not sustain hyphal elongation in the presence of rapamycin when they were exposed to 0.6 M NaCl or 5 mM H₂O₂ (Figure 6B). The defect in hyphal elongation was not likely due to slower cellular growth because the growth rate was not slowed significantly by the stresses used (Supplemental Figure S2). Taken together, our data suggest that Hog1 activation in response to stresses plays a dominant role on Hog1 phosphorylation over the regulation from Tor1.

Reduced Tor1 signaling inhibits the basal activity of Hog1 by promoting the function of Ptp2 and Ptp3, two Hog1 tyrosine phosphatases

How does reduced Tor1 signaling repress the basal activity of Hog1 during hyphal elongation? Inactivation of MAP kinases has been ascribed to the action of phosphatases, including Ser/Thr phosphatases, Tyr phosphatases, and dual-specificity phosphatases (Martin et al., 2005). In *S. cerevisiae*, Hog1 is inactivated by Ptc1, Ptc2, and Ptc3 Ser/Thr phosphatases (Maeda et al., 1994) and Ptp2 and Ptp3 tyrosine phosphatases (Jacoby et al., 1997; Wurgler-Murphy et al., 1997; Zhan et al., 1997). In *C. albicans*, *PTP3* has been reported to be strongly induced after 6 h of culturing in serum-containing medium at 37°C (Nantel et al., 2002). Therefore we first determined the expression levels of *PTP2* (orf19.5045) and *PTP3* upon hyphal induction in rapamycin by qRT-PCR. As shown in Figure 7A, both *PTP2* and *PTP3* were highly induced after incubation at 37°C for 3 h in the presence of rapamycin. However, the expression levels of *PTP2* and *PTP3* did not significantly increase at 37°C in the absence of rapamycin or at 25°C regardless of rapamycin treatment. The patterns of their expression correlated with levels of phosphorylated Hog1 under these growth conditions. In contrast to tyrosine phosphatases, the expression levels of *PTC1* and *PTC2*, two threonine phosphatases of Hog1, were not regulated by temperature or rapamycin. To further determine whether the phosphatases of Hog1

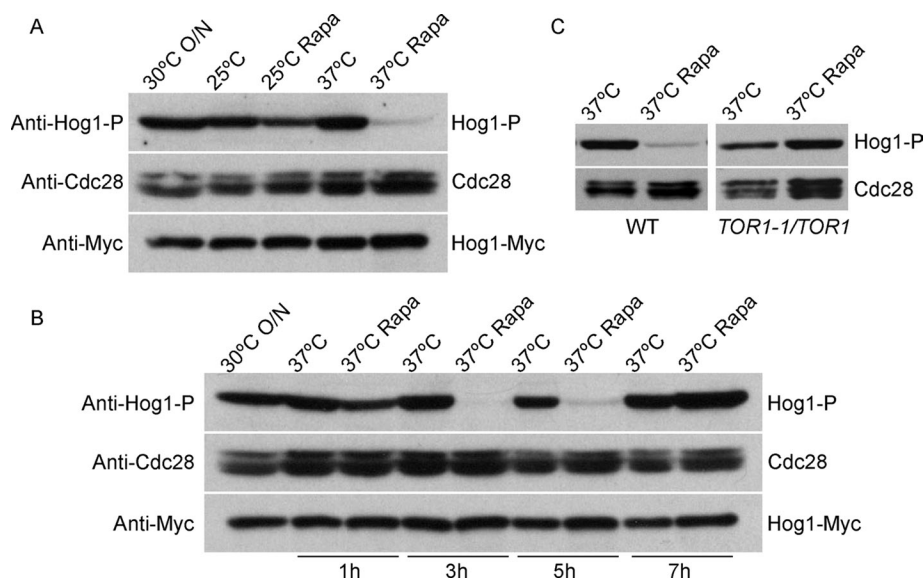


FIGURE 5: The basal level of phosphorylated Hog1 decreases in response to reduced Tor1 signaling. (A) Rapamycin caused a decrease in the basal level of phosphorylated Hog1 at 37°C. Wild-type cells carrying Hog1-Myc (HLY4096) were diluted into YPD medium at 25 or 37°C and incubated for 5 h in the presence or absence of 10 nM rapamycin. (B) A time-course analysis of Hog1 phosphorylation by Western blot. An overnight culture of wild-type strain carrying Hog1-Myc was diluted 1:100-fold into YPD medium at 37°C in the presence or absence of 10 nM rapamycin. Cells were collected at 0, 1, 3, 5, and 7 h for Western analysis. (C). The basal phosphorylation level of Hog1 is not reduced in *TOR1-1/TOR1* rapamycin-resistant strain upon rapamycin induction. Overnight cultures of wild-type and *TOR1-1/TOR1* rapamycin-resistant strains (JRB12) were diluted into YPD medium at 37°C and incubated for 3 h in the presence or absence of 10 nM rapamycin. Western blots in A–C were probed with an anti-phospho-p38 antibody (9216; Cell Signaling), which detects endogenous levels of p38 MAPK only when activated by phosphorylation at threonine 180 and tyrosine 182. Total level of Hog1 protein was determined by probing the blot with an anti-myc antibody.

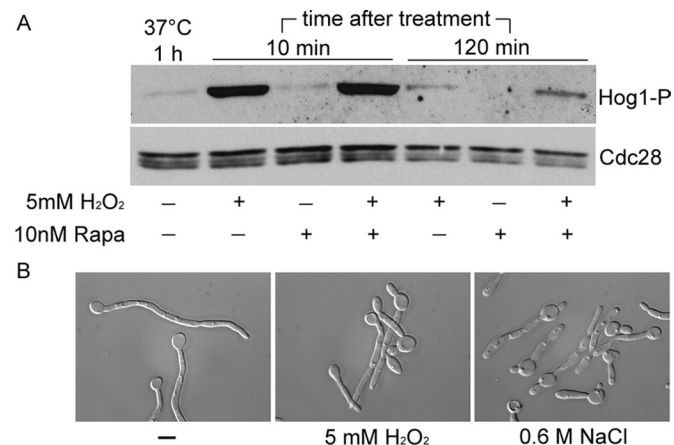


FIGURE 6: Activation of Hog1 in response to stresses impairs hyphal maintenance. (A) An overnight culture of wild-type strain was diluted at 1:100 into prewarmed YPD medium at 37°C. Cells were treated with 10 nM rapamycin, 5 mM H₂O₂, or both after 1 h and collected at 10 and 120 min after treatment for Western analysis. (B) Wild-type (SN250) cells were grown in YPD medium at 30°C overnight and then diluted at 1:100 into prewarmed 37°C YPD medium. After 1 h at 37°C, cells were treated with 5 mM H₂O₂ or 0.6 M NaCl as indicated, and 10 nM rapamycin was added to the samples at the same time. Morphology analysis was performed after incubation for 6 h in the indicated treatment.

are required for rapamycin-induced hyphal maintenance, we constructed *ptc1 ptc2* and *ptp2 ptp3* double mutants and the *ptp2* single mutant. Other mutants, *ptc1*, *ptc2*, and *ptp3*, are from Noble et al. (2010). No dramatic defect in hyphal maintenance was observed in any of the single mutants and the *ptc1 ptc2* double mutant. Only the *ptp2 ptp3* double mutant showed impaired hyphal elongation in either serum- or rapamycin-containing medium at 37°C (Figure 7B). Cell length was measured, and the average length of hyphae in wild-type and *ptp2 ptp3* mutant strains was graphed (Figure 7B). Wild-type cells formed long hyphae after 3.5 h of growth in YPD plus serum ($84.2 \pm 11.8 \mu\text{m}$, $n = 28$; mean \pm SD) or 7 h of growth in rapamycin-containing medium ($73.3 \pm 12.7 \mu\text{m}$, $n = 49$; mean \pm SD) at 37°C. Hyphae length of the *ptp2 ptp3* double mutant was shorter in YPD plus serum ($45.7 \pm 12.5 \mu\text{m}$; $n = 59$; mean \pm SD; $p < 0.05$) and in rapamycin-containing medium ($48.1 \pm 11.7 \mu\text{m}$; $n = 87$; mean \pm SD; $p < 0.05$). Consistent with the defective hyphal maintenance in the *ptp2 ptp3* mutant, the level of *BRG1* expression did not increase dramatically in YPD plus rapamycin at 37°C compared with that in wild-type cells (Figure 7C and unpublished data). As expected, *BRG1* was highly induced by rapamycin at 37°C in *ptc1*, *ptc2*, *ptp2*, and *ptp3* single mutants and the *ptc1 ptc2* double mutant as in wild-type cells (Figure 7C and unpublished data). Taken together, those data show that rapamycin-

or serum-induced hyphal elongation is mediated through promoting functions of the two tyrosine phosphatases Ptp2 and Ptp3.

We next examined whether the function of Ptp2 and Ptp3 in hyphal elongation was to inactivate Hog1. If so, deletion of *HOG1* is expected to bypass the requirement of Ptp2 and Ptp3 in hyphal elongation. Indeed, we found that deletion of *HOG1* in *ptp2 ptp3* double mutant restored its defects in hyphal maintenance (Figure 7D) and *BRG1* expression (Figure 7C). We also examined the level of phosphorylated Hog1 in the *ptp2 ptp3* double mutant upon rapamycin induction and found that the basal level of phosphorylated Hog1 was not reduced by rapamycin (Figure 7E). Taken together, our data suggest that reduced Tor1 signaling represses the basal activity of Hog1 during hyphal elongation by promoting the function of Ptp2 and Ptp3, two tyrosine phosphatases of Hog1. However, overexpression of *PTP2* or *PTP3* could not bypass the requirement for rapamycin in hyphal maintenance and *BRG1* expression (our unpublished data), indicating that the transcriptional up-regulation of *PTP2* and *PTP3* is not sufficient for rapamycin-induced hyphal elongation. The Tor1 signaling pathway may regulate the phosphatases at a posttranslational level as well.

Basal Hog1 activity is required for the promoter association of Sko1, a transcription repressor of *BRG1* expression

How does Hog1 regulate *BRG1* expression? In *S. cerevisiae*, Hog1 becomes intimately linked with promoter regions during stress

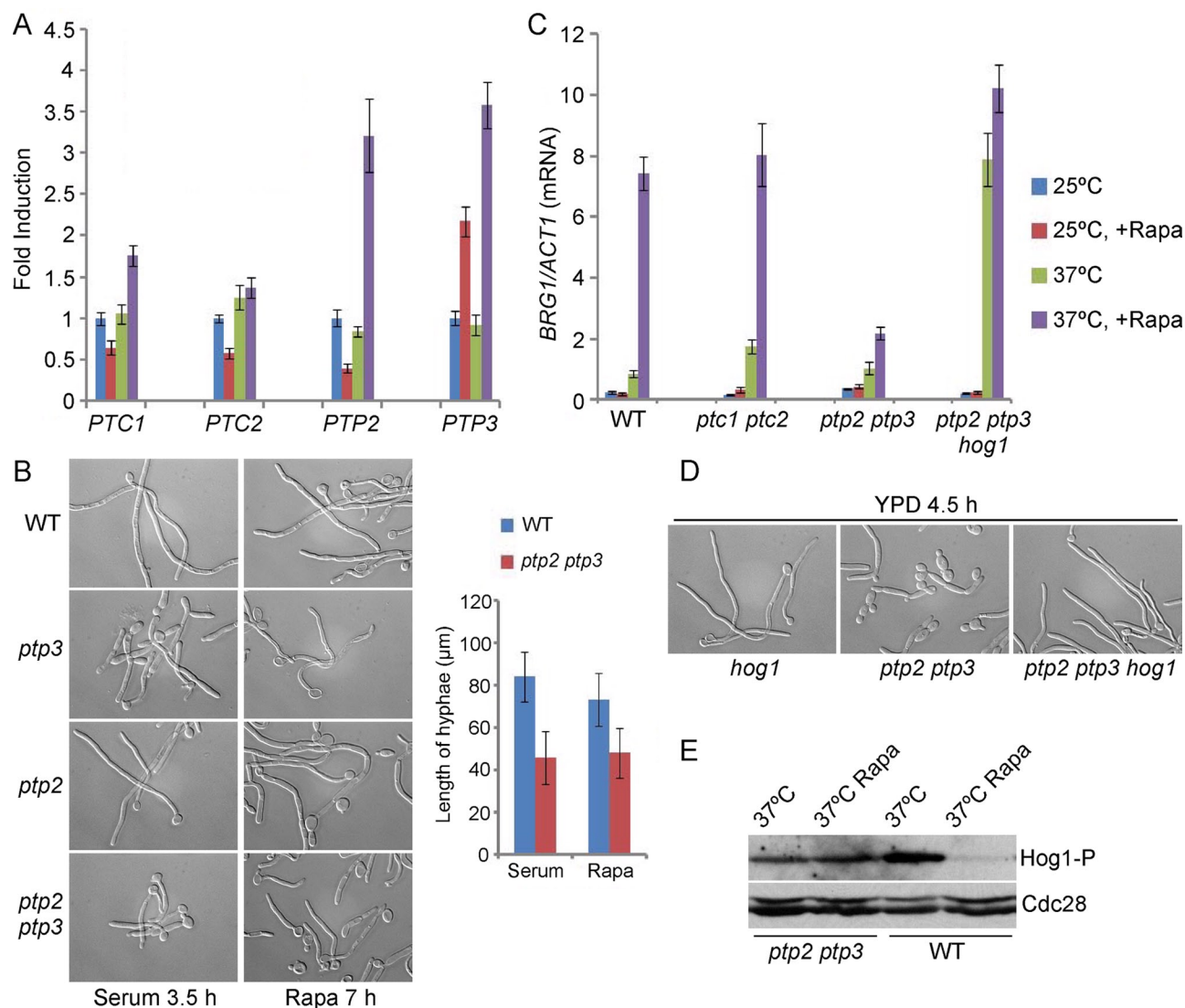


FIGURE 7: The expression levels of *PTP2* and *PTP3*, two Hog1 tyrosine phosphatases, are up-regulated during hyphal induction in response to rapamycin, and deletion of both *PTP2* and *PTP3* impairs hyphal maintenance. (A) Wild-type cells (SN250) were diluted into YPD medium at 25 or 37°C and incubated for 3 h in the presence or absence of 10 nM rapamycin. Expression levels of indicated genes were quantified by qRT-PCR and normalized with *ACT1*. The normalized value in YPD at 25°C was set to be 1.00. (B) Cell morphology analysis of wild-type and indicated mutant strains. An overnight culture of cells was diluted into YPD with either 10% serum or 10 nM rapamycin at 37°C. Hyphal lengths in the wild-type and *ptp2 ptp3* double mutant cells after hyphal induction were measured and are shown. (C) qRT-PCR analysis of *BRG1* expression in wild-type (SN250), double mutants *ptc1 ptc2* (HLY4106) and *ptp2 ptp3* (HLY4101), and triple mutant *ptp2 ptp3 hog1* (HLY4105). Overnight cultures of wild-type and indicated mutant strains were diluted into YPD medium at 25 or 37°C and incubated for 5 h in the presence or absence of 10 nM rapamycin. The signals obtained from *ACT1* mRNA were used for normalization. The data showed the average of three independent qRT-PCR experiments with error bars representing the SEM in A and C. (D). Cells of the *hog1* single mutant, *ptp2 ptp3* double mutant, and *ptp2 ptp3 hog1* triple mutant were diluted into YPD at 37°C. Observations were made 4.5 h after dilution. (E) The basal phosphorylation level of Hog1 is not reduced in the *ptp2 ptp3* double mutant upon rapamycin induction. Overnight cultures of wild-type and *ptp2 ptp3* double mutant strains were diluted into YPD medium at 37°C and incubated for 3 h in the presence or absence of 10 nM rapamycin. Western analysis was performed as described in Figure 5.

responses (Alepuz *et al.*, 2001). Hog1 could function directly on the promoter of the *BRG1* gene to regulate its transcription as in *S. cerevisiae*. By chromatin immunoprecipitation (ChIP) of C-terminal Myc-tagged Hog1, we could not detect Hog1 on the promoter of *BRG1* regardless of temperature or rapamycin (unpublished data), excluding a direct role of Hog1 in *BRG1* transcription. The Sko1 transcription repressor has been shown to regulate certain Hog1-dependent genes in *S. cerevisiae* (Proft and Serrano, 1999; Proft

et al., 2001; Rep *et al.*, 2001). Recently Sko1 was found to repress the yeast-to-hypha transition in *C. albicans* (Alonso-Monge *et al.*, 2010). Therefore it is likely that Hog1 acts through the downstream transcription factor Sko1 to regulate the expression of *BRG1*. As predicted, the protein levels of Brg1 in *sko1* mutant cells were high at 37°C regardless of rapamycin as in the *hog1* mutant (Figure 8A). Therefore, Sko1 is required to repress *BRG1* expression in the absence of rapamycin at 37°C. We next determined whether Sko1

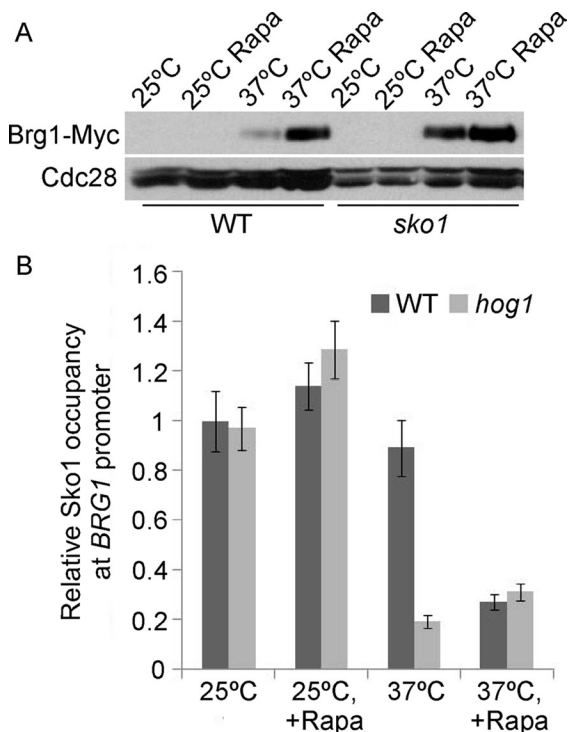


FIGURE 8: Sko1 disassociates from *BRG1* promoter to activate its expression during hyphal induction under reduced Tor1 signaling in a Hog1-dependent manner. (A) Western analysis of Brg1-Myc in wild-type strain and *sko1* mutant strain. Wild-type and *sko1* mutant cells carrying Brg1-Myc were diluted 1:100-fold into YPD medium at 25 or 37°C and incubated for 5 h in the presence or absence of 10 nM rapamycin. (B) Sko1 could not bind to the *BRG1* promoter when Hog1 was absent at 37°C in YPD medium. Overnight cultures of the wild-type strain and a *hog1* mutant strain carrying Sko1-Myc were diluted into YPD medium at 25 or 37°C in the presence or absence of 10 nM rapamycin and incubated for 5 h. ChIP DNA was quantitated as described (Lu et al., 2012) by qPCR with primers at –1415 to –1088 base pairs of the *BRG1* promoter. The value for wild-type cells in YPD at 25°C was set to be 1.00. The ChIP data showed the average of three independent qPCR experiments, with error bars representing the SEM.

functions directly on the promoter of *BRG1* by a ChIP experiment of Sko1-Myc. Sko1 was found at the promoter of *BRG1* under a yeast growth condition and remained at the promoter in YPD medium at 37°C without rapamycin but was not at the promoter at 37°C in the presence of rapamycin (Figure 8B). Therefore rapamycin caused the dissociation of Sko1 from the *BRG1* promoter at 37°C. The rapamycin-induced dissociation of Sko1 was regulated through the reduction in Hog1 basal activity, as Sko1 was not bound to the promoter of *BRG1* in the *hog1* mutant irrespective of rapamycin. Conditions for Sko1 dissociation correlate well with that of *BRG1* expression, being rapamycin dependent in wild-type cells and constitutive in the *hog1* mutant. Taken together, our data show that reduced Tor1 signaling leads to lower basal activity of Hog1, which causes the repressor Sko1 to disassociate from the promoter of *BRG1*, leading to *BRG1* expression and sustained hyphal elongation.

DISCUSSION

In this study we report that reduced Tor1 signaling down-regulates Hog1 basal activity to activate the expression of Brg1 for hyphal elongation (Figure 9). Several lines of evidence suggest that Hog1 functions downstream of Tor1 in hyphal development. First, the

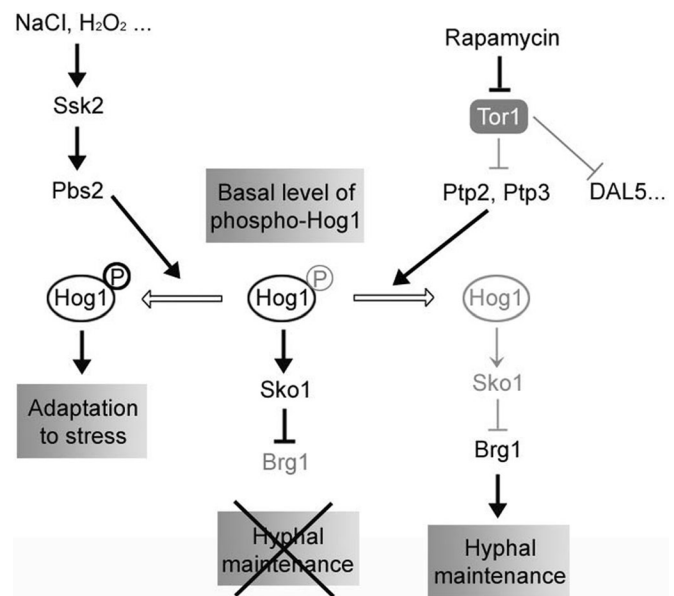


FIGURE 9: A schematic diagram depicting the mechanism of how the Tor1 signaling and HOG MAPK pathway integrate to sustain hyphal elongation. Rapamycin reduces Tor1 activity, which in turn represses the basal activity of Hog1 via two tyrosine phosphatases, Ptp2 and Ptp3. Inactive Hog1 fails to keep the repressor Sko1 on the promoter of *BRG1*, allowing the expression of Brg1 and hyphal elongation. Black lines represent active regulatory relationships; gray lines represent relationships that are inactive.

hog1 mutant is able to express Brg1 and sustain hyphal elongation regardless of nutrient status or presence of rapamycin in the media. Second, rapamycin reduces Hog1 basal activity, as measured by its phosphorylation. Third, deletion of *HOG1* activates the expression of only *BRG1* and not other rapamycin-inducible genes. We further show that reduced Tor1 lowers Hog1 activity via the function of two Hog1 tyrosine phosphatases, Ptp2 and Ptp3. Transcript levels of both phosphatases are up-regulated in response to rapamycin. The *ptp2 ptp3* double mutant blocks most of the rapamycin-induced *BRG1* expression and hyphal elongation and cannot reduce the basal level of phosphorylated Hog1 in response to rapamycin. Of importance, deletion of *HOG1* in the *ptp2 ptp3* double mutant completely bypasses the requirement of Ptp2 and Ptp3 in Brg1 expression and hyphal elongation. Therefore Tor1 functions through the Hog1 tyrosine phosphatases Ptp2 and Ptp3 to lower Hog1 basal activity. This places Hog1 under the regulation of the nutrient-sensing Tor1 signaling pathway for the first time in *C. albicans*. In the fission yeast *Schizosaccharomyces pombe*, TOR signaling regulates mitotic commitment by activating the same stress MAP kinase via the down-regulation of a MAPK tyrosine phosphatase (Petersen and Nurse, 2007). So far, Hog1 is mostly known for its rapid activation/phosphorylation and the importance of active Hog1 for cellular adaptation and cell cycle arrest in response to environmental stresses. Here we provide the first case for a down-regulation of the basal level of phosphorylated Hog1 and the importance of this regulation for a developmental program.

Many signaling pathways can regulate hyphal development in *C. albicans*. This study provides a few hubs for integrating nutritional and stress signals from different pathways or sensors in hyphal development (Figure 9). One point of signal integration is at the level of phosphorylated Hog1 in a cell. In *C. albicans* Hog1 is activated by various stresses, including osmotic stress, oxidative stress, and heavy

metals (Alonso-Monge et al., 1999; Smith et al., 2004; Arana et al., 2005; Enjalbert et al., 2006). Here we show that Hog1 phosphorylation by Pbs2 is epistatic to the action of rapamycin, as wild-type cells in the presence of both rapamycin and osmotic or oxidative stress cannot undergo hyphal elongation. Another point of signal integration is at the level of Ptp2 and Ptp3 activity. In addition to rapamycin, serum and alkaline pH likely sustain hyphal elongation through Ptp2 and Ptp3 as well. *PTP3* expression is up-regulated during hyphal induction in serum (Nantel et al., 2002) or alkaline pH (Bensen et al., 2004). The *hog1* mutant can form hyphae even in acidic pH (Eisman et al., 2006). We show here that the *ptp2 ptp3* double mutant blocks the effect of serum on hyphal elongation. In addition, both serum and alkaline pH require Hda1-mediated chromatin remodeling for hyphal elongation (Lu et al., 2011). Beside Hog1 and its phosphatases, TOR is a key integrator of multiple upstream signals and a regulator of diverse functions for metabolism and cell growth in many organisms. Tor1 regulation is likely conserved in *C. albicans* (Cruz et al., 2001; Zacchi et al., 2010; Chen et al., 2012), although the number of publications on how Tor1 activity is regulated in *C. albicans* is still limited. Orf19.2726 identified in this study may be a Tor1 regulator or function in the Tor1 pathway. Other than the three mutants of the HOG pathway, deletion of Orf19.2726 is the only mutant from the screen that expressed Brg1 regardless of rapamycin. The orthologous gene for Orf19.2726 in *S. cerevisiae* is Sfk1. Sfk1 is a plasma membrane protein that is suggested to act together with or upstream of Stt4 to generate normal levels of the phospholipid phosphatidylinositol 4-phosphate (Audhya and Emr, 2002). Based on the suggested function for Sfk1 in *S. cerevisiae* and the function of Orf19.2726 in the regulation of Brg1 expression, Orf19.2726 is a candidate for Tor1 regulation or function.

Temporal coordination of different signaling pathways provides a mechanism for integrating multiple external signals. We previously showed that a pulse of cAMP/protein kinase A activation leads to the transient removal of Nrg1 protein from promoters of hypha-specific genes, a step essential for hyphal initiation (Lu et al., 2011). Sustained hyphal elongation requires reduced Tor1 signaling for the expression of Brg1 in time for chromatin remodeling (Lu et al., 2011, 2012). Here we show that rapamycin lowers Hog1 activity to express Brg1 only during hyphal elongation from 1 to 7 h in YPD. Consistent with this time frame of Hog1 inactivation, the *hog1* mutant starts to convert to yeast cells after 8 h of growth in YPD at 37°C. Similarly, hyphae of wild-type cells also start to convert to yeast cells after 8 h in YPD with serum. What regulates sustained hyphal growth beyond 8 h? We showed that Ume6 functions after Brg1 and Hda1 (Lu et al., 2012). Therefore regulators of Ume6 expression could function after the Hog1–Brg1 regulation. Five genes (*RBD1*, *GZF3*, *CUP9*, orf19.3720, and orf19.5406) identified from the screen in this study may function after Brg1. Our previous screen also uncovered many genes required for hyphal elongation in rapamycin but not required for Hda1 recruitment (Lu et al., 2012). Some of these genes may function after Brg1/Hda1 in hyphal development. In support of a sequential regulation of hyphal development, Eed1 is shown to be required for hyphal elongation and *UME6* expression (Martin et al., 2011). *UME6* overexpression can suppress the *eed1* mutant in hyphal extension (Martin et al., 2011). We find that Eed1 is not required for the recruitment of Hda1 to hypha-specific promoters (our unpublished data), suggesting that Eed1 functions after Brg1–Hda1 in the regulation of *UME6* expression.

In this study, we present the first case of how a basal level of phosphorylated Hog1 can regulate a developmental program. Sko1 is a basic leucine zipper transcription factor and forms a complex with Tup1p and Cyc8p to both activate and repress transcription

(Vincent and Struhl, 1992; Proft and Struhl, 2002). In response to osmotic stress, phosphorylation by active Hog1 triggers a switch of Sko1–Cyc8–Tup1 from a repressing to an activating state (Proft and Struhl, 2002). In *C. albicans*, Sko1 is both a repressor of hyphal development and an activator of genes induced by stresses (Rauceo et al., 2008; Alonso-Monge et al., 2010). As in *S. cerevisiae*, Sko1 is probably a direct target of Hog1 kinase and is similarly regulated in *C. albicans*. Because activation of the Hog1 MAPK pathway is essential for stress adaptation and cell cycle arrest in response to stresses, a high level of phosphorylated Hog1 is required for its downstream functions, such as converting Sko1 from a repressor to an activator of stress-activated genes. In contrast, only a basal level of phosphorylated Hog1 is required to maintain Sko1 on the promoter of *BRG1* to repress the hyphal transcriptional program (Figure 9). Sko1 dissociates from the promoter when levels of phosphorylated Hog1 is lowered (by rapamycin) or abolished (*HOG1* deletion; Figure 9). Therefore the exact mechanism of Sko1 regulation by Hog1 is likely gene specific and requires different levels of active Hog1. Our study provides a unique case of Hog1 function. Such regulation of Sko1 by only a basal level of active Hog1 might also exist in other organisms.

MATERIALS AND METHODS

Media and growth conditions

C. albicans strains were routinely grown at 30°C in YPD. Transformants were selected on synthetic medium (2% dextrose, 0.17% Difco yeast nitrogen base without ammonium sulfate, 0.5% ammonium sulfate, and auxotrophic supplements). Strains were grown in YPD at 25°C for yeast form and in YPD plus 10% serum (Sigma-Aldrich, St. Louis, MO) or 10 nM rapamycin at 37°C for hyphal induction.

Screening for mutants that could sustain hyphal elongation in YPD medium

The deletion mutant library affecting 674 genes of *C. albicans* (Noble et al., 2010) and the wild-type reference strain SN250 were grown overnight in liquid YPD at 30°C. Then cells were diluted 1:100-fold into prewarmed YPD at 37°C. Fourteen mutants could sustain hyphal elongation after incubation for 4.5 h. Among them, five mutants gave elongated cells to various extents in overnight cultures at 30°C.

Plasmid and strain construction

SC5314 genomic DNA was used as the template for all PCR amplifications of *C. albicans* genes. The *C. albicans* strains used in the study are listed in Table 2. The primers used for PCR amplification are listed in Table 3. Strains *hog1*, *pbs2*, *ssk2*, and *sko1* (Homann et al., 2009; Noble et al., 2010) were streaked on 5-fluoroorotic acid-containing medium to generate Ura[–] strains and were subsequently transformed with *SpeI*-digested pPR673-BRG1 (Lu et al., 2012) to express the Brg1-13Myc fusion protein under the endogenous promoter. A 1.02-kb PCR product (primers 1 and 2) containing the C-terminal *HOG1* coding region was inserted into the *Bam*HI-*Mlu*I site of pPR673 (Lu et al., 2008). The resulting plasmid was digested with *Pml*I to target the integration into its own locus to express Hog1-13Myc. pPR673-SKO1 was constructed by inserting a 1.84-kb PCR fragment (primers 3 and 4) containing the *SKO1* coding sequence into the *Bam*HI-*Mlu*I site of pPR673. *PstI*-digested pPR673-SKO1 was introduced into strains BWP17 and *hog1* to express the Sko1-Myc fusion protein under the endogenous promoter.

Gene deletions in this work were based on PCR recombination by the method of Wilson et al. (1999) and SAT1-flipping strategy (Reuss et al., 2004). Primers 5 and 6 were used to amplify *C. albicans*

| Strain | Genotype | Source |
|---------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------|
| SN250 | <i>hisΔ1/his1Δ, leu2Δ::C.dubliniensis HIS1/leu2Δ::C.maltosa LEU2, arg4Δ/arg4Δ, URA3/ura3Δ::imm⁴³⁴, IRO1/iro1Δ::imm⁴³⁴</i> | Noble et al. (2010) |
| BWP17 | <i>ura3Δ::imm⁴³⁴/ura3Δ::imm⁴³⁴, his1Δ::hisG/his1Δ::hisG, arg4Δ::hisG /arg4Δ::hisG</i> | Wilson et al. (1999) |
| CAI4 | <i>ura3Δ::imm⁴³⁴/ura3Δ::imm⁴³⁴</i> | Fonzi and Irwin (1993) |
| HLY4095 | <i>hisΔ1/his1Δ, leu2Δ/ leu2Δ, arg4Δ/arg4Δ, ura3Δ/ura3Δ::imm⁴³⁴, iro1/iro1Δ::imm⁴³⁴, brg1Δ::HIS1/brg1Δ::LEU2, hog1Δ::ARG4/hog1Δ::URA3</i> | This study |
| HLY4096 | <i>ura3Δ::imm⁴³⁴/ura3Δ::imm⁴³⁴, HOG1/HOG1-13MYC-URA3</i> | This study |
| HLY4097 | <i>hisΔ1/his1Δ, leu2Δ/ leu2Δ, arg4Δ/arg4Δ, ura3Δ/ura3Δ::imm⁴³⁴, iro1/iro1Δ::imm⁴³⁴, hog1Δ::C.dubliniensis HIS1/hog1Δ::C.maltosa LEU2 BRG1/BRG1-13MYC-URA3</i> | This study |
| HLY4098 | <i>hisΔ1/his1Δ, leu2Δ/ leu2Δ, arg4Δ/arg4Δ, ura3Δ/ura3Δ::imm⁴³⁴, iro1/iro1Δ::imm⁴³⁴, pbs2Δ::C.dubliniensis HIS1/pbs2Δ::C.maltosa LEU2 BRG1/BRG1-13MYC-URA3</i> | This study |
| HLY4099 | <i>hisΔ1/his1Δ, leu2Δ/ leu2Δ, arg4Δ/arg4Δ, ura3Δ/ura3Δ::imm⁴³⁴, iro1/iro1Δ::imm⁴³⁴, ssk2Δ::C.dubliniensis HIS1/ssk2Δ::C.maltosa LEU2 BRG1/BRG1-13MYC-URA3</i> | This study |
| HLY4082 | <i>ura3Δ::imm⁴³⁴/ura3Δ::imm⁴³⁴, BRG1/BRG1-13MYC-URA3</i> | Lu et al. (2012) |
| HLY4100 | <i>ura3Δ::imm⁴³⁴/ura3Δ::imm⁴³⁴, his1Δ::hisG/his1Δ::hisG, arg4Δ::hisG /arg4Δ::hisG, ptp2Δ::HIS1/ptp2Δ::ARG4</i> | This study |
| HLY4101 | <i>hisΔ1/his1Δ, leu2Δ/ leu2Δ, arg4Δ/arg4Δ, ura3Δ/ura3Δ::imm⁴³⁴, iro1/iro1Δ::imm⁴³⁴, ptp3Δ::C.dubliniensis HIS1/ptp3Δ::C.maltosa LEU2 ptp2Δ::URA3/ptp2Δ::ARG4</i> | This study |
| HLY4102 | <i>hisΔ1/his1Δ, leu2Δ/ leu2Δ, arg4Δ/arg4Δ, ura3Δ/ura3Δ::imm⁴³⁴, iro1/iro1Δ::imm⁴³⁴, sko1Δ::HIS1/sko1Δ::LEU2, BRG1/BRG1-13MYC-URA3</i> | This study |
| HLY4103 | <i>ura3Δ::imm⁴³⁴/ura3Δ::imm⁴³⁴, his1Δ::hisG/his1Δ::hisG, arg4Δ::hisG /arg4Δ::hisG, SKO1/SKO1-13MYC-URA3</i> | This study |
| HLY4104 | <i>hisΔ1/his1Δ, leu2Δ/ leu2Δ, arg4Δ/arg4Δ, ura3Δ/ura3Δ::imm⁴³⁴, iro1/iro1Δ::imm⁴³⁴, hog1Δ::C.dubliniensis HIS1/hog1Δ::C.maltosa LEU2 SKO1/SKO1-13MYC-URA3</i> | This study |
| HLY4105 | <i>hisΔ1/his1Δ, leu2Δ/ leu2Δ, arg4Δ/arg4Δ, ura3Δ/ura3Δ::imm⁴³⁴, iro1/iro1Δ::imm⁴³⁴, ptp3Δ::C.dubliniensis HIS1/ptp3Δ::C.maltosa LEU2 ptp2Δ::ura3Δ/ptp2Δ::ARG4, hog1Δ::URA3/hog1Δ::FRT</i> | This study |
| HLY4106 | <i>hisΔ1/his1Δ, leu2Δ/ leu2Δ, arg4Δ/arg4Δ, ura3Δ/ura3Δ::imm⁴³⁴, iro1/iro1Δ::imm⁴³⁴, ptc1Δ::C.dubliniensis HIS1/ptc1Δ::C.maltosa LEU2 ptc2Δ::URA3/ptc2Δ::ARG4</i> | This study |
| JRB12 | SC5314 TOR1-1/TOR1 | Cruz et al. (2001) |

TABLE 2: *C. albicans* strains used in this study.

URA3 and ARG4 from plasmids pGEM-URA3 and pRS-ARG4ΔSpel, respectively. The first copy of *HOG1* was disrupted by the transformation of ARG4 into the *brg1* mutant (Ura[−]) strain (Lu et al., 2012). *C. albicans* URA3 was used to replace the second copy of *HOG1*. To delete *PTP2* in wild-type strain and in the *ptp3* mutant, primers 7 and 8 were used to amplify *C. albicans* *HIS1*, *URA3*, and *ARG4* from plasmids pGEM-HIS1, pGEM-URA3, and pRS-ARG4ΔSpel, respectively. Wild-type strain BWP17 was transformed with ARG4 to produce *PTP2/ptp2* heterozygotes. The *ptp2/ptp2* homozygote was screened out by subsequent transformation of *HIS1*. Strain *ptp3* was streaked on 5-fluoroorotic acid-containing medium to generate the Ura[−] strain and transformed with URA3 to substitute the first copy of *PTP2*. The second copy of *PTP2* in the *ptp3* mutant was disrupted by the transformation of ARG4. The resulting *ptp2 ptp3* double mutant was streaked on 5-fluoroorotic acid-containing medium to generate the Ura[−] strain and transformed with a PCR product (primers 5 and 6) amplified from pGEM-URA3 to disrupt the first copy of *HOG1*. Upstream (primers 19 and 20) and downstream (primers 21 and 22) sequences of *HOG1* were cloned as *Apal*-*XhoI* and *NotI*-*SacI* fragments, respectively, on both sides of the *SAT1* flipper cassette to obtain the plasmid pSFS2-HOG1 for *HOG1* disruption. The deletion construct containing the *SAT1* flipper cassette digested

from pSFS2-HOG1 with *Apal* and *SacI* was transformed into *ptp2/ptp2 ptp3/ptp3 HOG1/hog1* to replace the second copy of *HOG1*. Primers 23 and 24 were used to delete *PTC2* in the *ptc1* mutant strain as described for constructing the *ptp2 ptp3* double mutant. All deletions were confirmed by PCR (unpublished data).

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described with modifications (Lu et al., 2008). DNA was sheared by sonication six times for 20 s at high power on a Bioruptor (Diagenode, Denville, NJ) with 40-s intervals on ice. A 10-μl amount of anti-Myc (SC-789; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies was used for ~4 mg of chromatin proteins in an immunoprecipitation volume of 200 μl.

Quantitative PCR expression analysis

Methods for RNA isolation were carried out as previously described (Lane et al., 2001). We DNase treated 10 μg of total RNA at 37°C for 1 h using the RNase-free kit (Qiagen, Valencia, CA), synthesized cDNA using the SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA), and performed qPCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) using the primers 9 and 10 for *PTC1*, primers 11 and 12 for *PTC2*, primers 13 and 14 for *PTP2*, primers 15 and

| Primer | Sequence (5'–3') ^a | Purpose and features |
|--------|-----------------------------------------------------------------------------------------------------------------|----------------------|
| 1 | CGGGATCCCGTGCTCAGCCGTTGATAG | pPR673-HOG1 |
| 2 | GGCGACGCGTCGAGCTCCGTTGGCGGAATC | |
| 3 | CGGGATCCCATGTCCAGTGACGTAAGTATA | pPR673-SKO1 |
| 4 | GGCGACGCGTCGTGTAGGATTTAAAGTAGTTGG | |
| 5 | CAACATTTTAAACAAGTTATAGAAAGAAAATTTTACAAAGATAAAGCATATAAGAAAATTGT- GGAATTGTGAGCGGATA ^b | HOG1 disruption |
| 6 | ATAGTAATACATATTTCACTTTTAAATTTATTTCTATAATTGCTAGCTTGATTTTTGAATTTT- CCAGTCACGACGTT ^b | |
| 7 | CTATTACATTACTTATCTTATTGTTCTACGCGTGTGTTACTTCCCCACTTCTGTTTTCACCAT- TGTGGAATTGTGAGCGGATA ^b | PTP2 disruption |
| 8 | GAATATTTATACTTGATTCTGTTGATAATCTTTTTCATGATTAATTAATAAATTT- TATAAAAATTTTATAAATTTTCCAGTCACGACGTT ^b | |
| 9 | TCTGACAGGTGCTAATGACTC | PTC1-qRT PCR |
| 10 | GGCAATGTATGTGTGGACATC | |
| 11 | AAGCTGCTATTTTCACTGGC | PTC2-qRT PCR |
| 12 | GCACATCCACTATCATCATC | |
| 13 | ACCTTGTTGTATGTCATG | PTP2-qRT PCR |
| 14 | GTTTGTAGAAGCTGTTAG | |
| 15 | GATATTAGACCATCTGCTC | PTP3-qRT PCR |
| 16 | CAGCAAATCTATCTCTCTG | |
| 17 | CGAGGTAGACAGTGTTTCAG | DAL5-qRT PCR |
| 18 | CCAAGCACTGACGCATAAC | |
| 19 | CTAGGGCCCCCTCAGCTAGTAACACTACTG | pSFS2-HOG1 |
| 20 | GTA ^{CTCGAG} CTCCATCTGCAGACATTTTC | |
| 21 | CTAGCGGCCGCCAACGGAGCTTAATCTTC | |
| 22 | TCCCCGCGGGGAACCTGATGTTAGTTG | |
| 23 | CAATAATAATAGTAATAATAATAGATTCAATTCAGTTTCAGTTTCAATTTCAATTTCAATTTCAAT TTGTGGAATTGTGAGCGGATA ^b | PTC2 disruption |
| 24 | CAAAATCAAAATTAATAATCAAAATTCATTCCGTTACTTAAAAATTTTATTTTTTTTTTAAT- TCTTTCCAGTCACGACGTT ^b | |

^aRestriction sites are underlined.

^bBoldface sequences in primers are segments that anneal to plasmids pGEM-HIS1, pGEM-URA3, and pRS-ARG4ΔSpeI for amplification of disruption cassettes.

TABLE 3: Primers used in this study.

16 for *PTP3*, and primers 17 and 18 for *DAL5*. The primers for *BRG1* and *ACT1* were used as previously described (Lu *et al.*, 2012).

Detection of Hog1 phosphorylation by Western blot

Cells of each sample were pelleted at 3000 × g for 5 min at 4°C and resuspended in 0.35 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP40) plus protease inhibitor cocktail (Roche, Indianapolis, IN). Total protein was extracted using a Fast-Prep system (FastPrep-24; MP Biomedicals, Solon, OH). Equal amounts of proteins were loaded onto each lane, based on OD₂₈₀ measurement. Proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Hybond; GE Healthcare, Piscataway, NJ). Western blot detection of Hog1 phosphorylation was carried out as described by the manufacturer's instructions for the anti-phospho-p38 antibody 9216 of Cell Signaling (Beverly, MA). After blocking, the membrane was incubated with the anti-phospho-p38 antibody at 4°C overnight. Secondary antibody (goat anti-mouse; Bio-Rad) was used at 1:500 dilution and incu-

bated for 1 h at room temperature. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA) was used for detection.

ACKNOWLEDGMENTS

We are grateful to S. M. Noble and A. D. Johnson for constructing deletion mutants and making the collection available to the *C. albicans* research community. We thank J. Heitman for the TOR1-1 strain. This work was supported by National Institutes of Health Grants R01 GM055155 and R01 AI099190 to H.L.

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