Regulation of the *Candida albicans* Cell Wall Damage Response by Transcription Factor Sko1 and PAS Kinase Psk1

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The environmental niche of each fungus places distinct functional demands on the cell wall. Hence cell wall regulatory pathways may be highly divergent. We have pursued this hypothesis through analysis of *Candida albicans* transcription factor mutants that are hypersensitive to caspofungin, an inhibitor of beta-1,3-glucan synthase. We report here that mutations in *SKO1* cause this phenotype. *C. albicans* Sko1 undergoes Hog1-dependent phosphorylation after osmotic stress, like its *Saccharomyces cerevisiae* orthologues, thus arguing that this Hog1-Sko1 relationship is conserved. However, Sko1 has a distinct role in the response to cell wall inhibition because 1) *sko1* mutants are much more sensitive to caspofungin than *hog1* mutants; 2) Sko1 does not undergo detectable phosphorylation in response to caspofungin; 3) *SKO1* transcript levels are induced by caspofungin in both wild-type and *hog1* mutant strains; and 4) *sko1* mutants are defective in expression of caspofungin-inducible genes that are not induced by osmotic stress. Upstream Sko1 regulators were identified from a panel of caspofungin-hypersensitive protein kinase-defective mutants. Our results show that protein kinase Psk1 is required for expression of *SKO1* and of Sko1-dependent genes in response to caspofungin. Thus Psk1 and Sko1 lie in a newly described signal transduction pathway.

INTRODUCTION

The fungal cell wall is critical for interaction with the environment and survival. It is the point of contact between the fungus and target surfaces, and processes such as adhesion, dimorphism, and biofilm formation are dependent on a dynamic cell wall (Nobile and Mitchell, 2005; Lesage and Bussey, 2006; Ruiz-Herrera *et al.*, 2006; Dranginis *et al.*, 2007). These processes all contribute to the pathogenicity of *Candida albicans*, the major fungal pathogen of humans. This organism causes superficial, mucosal, and potentially fatal invasive infections (Rangel-Frausto *et al.*, 1999; Rabkin *et al.*, 2000). As a fungal-specific structure, the cell wall is also of interest as a mediator of immunological recognition and evasion (Wheeler and Fink, 2006) and in addition as a target of antifungal drugs such as caspofungin (Letscher-Bru and Herbrecht, 2003). Our interest is in the signaling pathways that govern *C. albicans* cell wall dynamics.

Caspofungin inhibits beta-glucan synthesis to cause cell lysis (Letscher-Bru and Herbrecht, 2003). Caspofungin treatment elicits a broad transcriptional response in the baker's yeast *Saccharomyces cerevisiae* and in *C. albicans* (Reinoso-Martin *et al.*, 2003; Liu *et al.*, 2005; Bruno *et al.*, 2006). The *S. cerevisiae* response is controlled in part by the mitogen-activated protein kinase (MAPK) signaling cascade known as the protein kinase C (PKC) cell wall integrity pathway

(Reinoso-Martin *et al.*, 2003; Levin, 2005; Liu *et al.*, 2005; Bruno *et al.*, 2006). This MAPK pathway is conserved in *C. albicans*, where it also governs cell wall integrity (Navarro-Garcia *et al.*, 1998; Reinoso-Martin *et al.*, 2003). However, there is increasing evidence that the *C. albicans* response to caspofungin has unique features as well. For example, the *C. albicans* response includes induction of numerous secretory genes (Bruno *et al.*, 2006), a gene class that is largely nonresponsive in *S. cerevisiae*. Even more striking is the fact that a major mediator of the *C. albicans* response, transcription factor Cas5, lacks an *S. cerevisiae* orthologue (Bruno *et al.*, 2006). Cas5 is required for induction of genes mainly involved in cell wall biogenesis. Those genes account for a small fraction of caspofungin-responsive genes.

In this study we use a genetic screen to identify new *C. albicans* transcription factors involved in cell wall damage signaling. We also employ a new resource, a set of caspofungin-sensitive protein kinase mutants (Blankenship, Fanning, Hamaker, and Mitchell, unpublished data), to search for upstream signaling components. We uncover a novel cell wall regulatory pathway that includes the transcription factor Sko1 (ORF 19.1032) and the protein kinase Psk1 (ORF 19.7451). In *S. cerevisiae* both ScSko1 and the proteins ScPsk1 and ScPsk2 have been characterized. ScSko1 mediates the adaptive response to osmotic stress via the high-osmolarity glycerol (HOG) pathway. ScSko1 is activated through phosphorylation by the MAP kinase ScHog1 and functions as an activator and repressor of osmotic stress-responsive genes (Proft *et al.*, 2001; Proft and Struhl, 2002). ScSko1 function has not been characterized in the response to cell wall damage. Gene expression studies implicate *C. albicans* Sko1 in the osmotic stress response (Enjalbert *et al.*, 2006), but no *sko1*

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mutant defect has been reported previously (Braun *et al.*, 2001). ScPsk1/2 regulates glucose partitioning for either glucan or glycogen synthesis, and *Scpsk1 Scpsk2* double mutants are sensitive to cell wall damage (Smith and Rutter, 2007). The sole *C. albicans* orthologue Psk1 has not been characterized previously. Our findings define a new regulatory pathway that governs a critical aspect of *C. albicans* growth and survival.

MATERIALS AND METHODS

Media and Growth Conditions

C. albicans cultures were prepared in YPD plus uridine (2% dextrose, 2% bacto peptone, 1% yeast extract, and 80 mg/l uridine) at 30°C with shaking at 200 rpm. Synthetic medium (2% dextrose, 6.7% yeast nitrogen base [YNB] plus ammonium sulfate, and the necessary auxotrophic supplements) was used for selection after transformations. In assays monitoring cell wall damage, cells were plated to YPD + uridine supplemented with 125 ng/ml caspofungin (Merck, Rahway, NJ).

Plasmid Construction

All primers used in this study are listed in Table 1. The *SKO1* complementing plasmid (pRM03) was constructed as follows: Primers SKO1compfwd and SKO1comprev were used to amplify a 2.4-kb fragment containing 993 bp of promoter, the entire open reading frame (ORF), and 220 bp of the 3'UTR. The recently discovered 109 base pairs of intron sequence in the 5'UTR is included the 2.4-kb fragment. The amplicon was ligated to the pGEMT-Easy vector (Promega, Madison, WI) to create pGEMTE-*SKO1* and amplified in *Escherichia coli*. Purified pGEMTE-*SKO1* was digested with NgoMIV and AlwNI and inserted through in vivo recombination in *S. cerevisiae* into a NotI- and EcoRI-digested pDDB78 (Spreghini *et al.*, 2003). The cloned *SKO1* insert was verified by DNA sequencing.

The *HOG1* complementing plasmid (pRM04) was constructed as follows: Primers HOG1compfwd and HOG1deldet were used to amplify a 2.3-kb fragment containing 1 kb of promoter, the entire ORF, and 189 bp of the 3'UTR. The amplicon was ligated to pGEMT-Easy (pGEMTE-*HOG1*) and inserted into pDDB78 as described above to generate pRM04.

Construction of a *SKO1*-V5 epitope-tagged plasmid (pRM05) was performed as follows: Primers SKO1compfwd and SKO1orfrev were used to generate a fragment containing 993 bp of promoter and the entire ORF without the stop codon. The amplicon was inserted into the pYES2.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) to create pYES-*SKO1*-V5. PCR amplification using primers SKO1-V5 fwdpr and CAS5-V5 78 3' with pYES-*SKO1*-V5 as a template was done to amplify a fragment containing the V5 epitope tag, His 6x tag, stop codon, and 209 bp of the *CYC1* terminator region. This fragment was inserted into linearized pDDB78 as described above.

The *PSK1*-complementing plasmid (pRM06) was constructed as follows: Primers PSK1compfwd and PSK1comprev were used to generate a 5.3-kb fragment consisting of 965 bp of promoter region, the entire ORF, and 385 bp of the 3'UTR. This fragment was ligated into pGEMT-easy to create pGEMTE-*PSK1* and amplified in *E. coli*. Purified pGEMTE-*PSK1* was digested with NgoMIV and SapI and inserted through in vivo recombination in *S. cerevisiae* into a NotI- and EcoRI-digested pRYS2.

Yeast Strains and Transformation Procedures

C. albicans strains used in this study are listed in Table 2. All strains were derived from strain BWP17 (genotype: *ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*; Wilson *et al.*, 1999). Strain JMR103, the *sko1Δ::ARG4/sko1Δ::URA3* mutant was generated by PCR-directed gene deletion using 120mer oligonucleotides SKO1del5'dr and SKO1del3'dr, respectively, to delete the entire ORF (Wilson *et al.*, 1999). The *SKO1*-complemented strain (JMR109) was generated by transforming JMR103 with NruI-digested pRM03 to direct integration to the *HIS1* locus. JMR103 was brought to His prototrophy through transformation with NruI-digested pDDB78 to create strain JMR104. Strain JMR114, the *hog1Δ::ARG4/hog1Δ::URA3* mutant, was generated using primers HOG1del5'dr and HOG1del3'dr as described

Table 1. Oligonucleotide sequences

Primer	Sequence 5'-3'
SKO1del5'dr	TTTCCGATGTCAATAGTGTGCTACTAGTGGATCATCAATAAATAATGGTAGTTCAGTAATAAACACAA CCTACATATTCCCAACATCTCCAGTGTCAATTTCCCGATCAGCAGCTT
SKO1del3'dr	TATATTTGAGAGCAGAAAAAAGCTACATATATATTCGCTAATATTCTTGATAAAACATACATAGATTAG GATGTATAATTTGCAAAATAACTACAGTTTGTGGAATTGTGAGCGGATA
SKO1compfwd	AGAAACAAATTAAGATAGAGGAGAGAG
SKO1comprev	GGTATATTTGAGAGCAGAAAAAAGC
SKO1orfrev	TGTAGATTAAAGTAGTTGGTATAGTTG
SKO1-V5 fwdpr	GTAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCAGAAACAAATTAAGATAGAGGAGAGAG
CAS5-V5 78 3'	GATATCGAATTCCTGCAGCCCGGGGATCCACTAGTCTAGAGTGAGCTGATACCGCTCG
HOG1del5'dr	TTCAAGTCGTCTTTGAAAAACATACACCGTGAATAATAACAACAACATTTTAAACAAGTTATAGAAAAGA AAATTTTACAAAGATAAAGCATATAAGAAATGTTTCCAGTCACGACGTT
HOG1del3'dr	TCTTCAAAAATACAAGTAGCAATTATAGAAATAAAATTTAAAGTGAAATATGTATTACTATTACTATTA ACTTTACATTATTAATTTTGATTAATATAGTGAATTGTGAGCGGATA
HOG1compfwd	CTTAAAGATTTCATCCAATGATGG
HOG1comprev	CCAAACCCATTTTACCAGATGA
PSK1del5'dr	ATGACTTCAAACCGGCCGCCACCATCACTCCTGTTTTTCATAGAAGACAATCCCACTGCACAACAAC CACAGGAACACCATCAGCAATCCCTTTTAATTTCCAGTCACGACGTT
PSK1del3'dr	TTAGATCTGTAACCATTCATCCTCCATAATATCAGTAATTGTGGGTCTTTCATCCACATCACGAACCAAGAT TTTCTTAATCAAAGTTAACTTGTTCGGTGAATTGTGAGCGGATA
PSK1compfwd	CACATGTTCTACCAACAAGTTACC
PSK1comprev	CTCCAGTTGTCAAATCTATAGGTGAG
SKO1RTfwd	AACCACCACCACCACAAAAT
SKO1Rtrev	CACCACGCAATTCATTTACT
PGA13fwd	ATCACCACCAGTCTGAACA
CRH11fwd	CCAGTTCTTCATCCAGCTCA
CRH11rev	CCAATCAATGCAACAAAGCC
MNN2RTfwd	ATGCAATTTTTACCGAAGG
MNN2Rtrev	TCAGCTGTTTCTTCAACCA
HGT6RTfwd	GGTCCAACCAGAAAACCAGA
HGT6Rtrev	CAAGAAACCCACAACCAGT
SKN1RTfwd	TTATGCTGGTGGACCTTTCC
SKN1Rtrev	TGTTCACCAACAACCAACG
TDH3fwd	ATCCCAACAGGACTGGAGA
TDH3rev	GCAGAAGCTTTAGCAACGTG

Table 2. Yeast strains used in this study

Strain	Genotype	Reference
DAY286	<i>ura3Δ::λimm434/ura3Δ::λimm434, ARG4::URA3::arg4::hisG/ arg4::hisG, his1::hisG/his1::hisG</i>	Davis et al. (2002)
DAY185	<i>ura3Δ::λimm434/ura3Δ::λimm434, ARG4::URA3::arg4::hisG/ arg4::hisG, his1::hisG::pHIS1/his1::hisG</i>	Davis et al. (2002)
JMR104	<i>ura3Δ::λimm434/ura3Δ::λimm434, arg4::hisG/ arg4::hisG, his1::hisG::pHIS1/his1::hisG, sko1::ARG4/ sko1::URA3</i>	This study
JMR109	<i>ura3Δ::λimm434/ura3Δ::λimm434, arg4::hisG/ arg4::hisG, his1::hisG::pHIS1::SKO1/his1::hisG, sko1::ARG4/ sko1::URA3</i>	This study
JMR121	<i>ura3Δ::λimm434/ura3Δ::λimm434, arg4::hisG/ arg4::hisG, his1::hisG::pHIS1/his1::hisG, hog1::ARG4/ hog1::URA3</i>	This study
JMR123	<i>ura3Δ::λimm434/ura3Δ::λimm434, arg4::hisG/ arg4::hisG, his1::hisG::pHIS1::HOG1/his1::hisG, hog1::ARG4/ hog1::URA3</i>	This study
JMR188	<i>ura3Δ::λimm434/ura3Δ::λimm434, arg4::hisG/ arg4::hisG, his1::hisG::pHIS1::PSK1/his1::hisG, psk1::ARG4/ psk1::URA3</i>	This study
JMR192	<i>ura3Δ::λimm434/ura3Δ::λimm434, arg4::hisG/ arg4::hisG, his1::hisG::pHIS1/his1::hisG, psk1::ARG4/ psk1::URA3</i>	This study

above. JMR114 was also brought to His prototrophy through transformation with NruI-digested pDDB78 to create strain JMR121. The *HOG1*-complemented strain (JMR123) was constructed as described above. Strain JMR167, the *psk1Δ::ARG4/psk1Δ::URA3* mutant was generated using primers PSK1del5'dr and PSK1del3'dr as described above. JMR167 was brought to His prototrophy through transformation with SrfI-digested pRYS2 to create strain JMR192. The *PSK1*-complemented strain (JMR188) was constructed as described above. *SKO1*-V5 epitope-tagged strains were generated through transformation of NruI-digested pRM05 as described above. Candidate genes related to the transcription process were described previously (Nobile and Mitchell, 2005). Construction of the insertion mutant strains followed previously described procedures (Davis et al., 2002; Norice et al., 2007).

Cell Wall Susceptibility Assays

Assays followed previously described procedures (Bruno et al., 2006). Briefly, *C. albicans* overnight cultures were diluted to a starting OD_{600 nm} of 3.0. Samples were serially diluted, spotted onto designated plates, incubated at 30°C, and photographed after 1–3 d of growth.

RNA Isolation and Real-Time PCR Analysis

Overnight cultures of designated *C. albicans* strains were diluted to a starting OD_{600nm} of 0.200 in 100 ml fresh YPD + uridine media. The cultures were incubated with shaking at 30°C to an OD_{600 nm} of 1.0 and split into two 50-ml cultures. A total of 125 ng of caspofungin was added to the experimental culture, and dH₂O was added to the control culture. The cultures were incubated for 30–60 min. Cells were harvested by vacuum filtration and stored at –80°C. For kinetic assays a starter culture of 400 ml was prepared as described above, and after caspofungin treatment, 50-ml samples were collected at each designated time point. Total RNA was isolated using the hot acid phenol method (Nobile and Mitchell, 2005). RNA yield and purity levels were determined spectrophotometrically, and 5 μg of RNA was DNase digested (RQ1 DNase, Promega; or DNaseI, Ambion, Austin, TX). cDNA was synthesized using the Stratascript first strand synthesis kit (Stratagene, La Jolla, CA). As a control for DNA contamination each sample was treated without reverse transcriptase. Primers are listed in Table 1 and were designed using primer 3 input software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR efficiency (E) was determined for all primers through amplification of *C. albicans* genomic DNA. Primer pairs yielding E-values between 99 and 103% were used in subsequent real-time (RT) experiments. RT reactions were prepared in triplicate using iQ SYBR supermix (Bio-Rad), and RT-PCR was performed using the Bio-Rad iCycler thermocycler equipped with an iQ5 multicolor optical unit (Bio-Rad, Richmond, CA), with a program of 95°C for 5 min and then 40 cycles of 95°C for 45 s, followed by 58°C for 1 min. Melt curve analysis confirmed the specificity of the amplification products. Data analysis was conducted using the Bio-Rad iQ5 standard edition optical system software V2.0. Transcript levels were normalized against *TDH3* (which encodes glyceraldehyde-3-phosphate dehydrogenase) expression, and gene expression changes were calculated by the $\Delta\Delta C_T$ method (Kubista et al., 2006). Target gene fold changes for treated or untreated cells were determined by comparison to the wild-type (wt) strain. Significant differences between groups were determined in unpaired *t* tests (<http://graphpad.com/quickcalcs/ttest1.cfm?Format=SD>) with a *p* value of < 0.05 considered to be statistically significant.

Microarray Analysis

Cultures of designated *C. albicans* strains were prepared as described above. Cultures were incubated in the presence of caspofungin for 30 min before harvesting by vacuum filtration. Cells were resuspended in 1.5 ml of ice-cold RNA later (Sigma, St. Louis, MO) to prevent RNA degradation and pelleted. Total RNA was extracted and was DNase treated using the Ribopure yeast kit (Ambion) following manufacturer's instructions. We performed two hybridizations that measured the effects of drug treatment on wt cells, and six hybridizations that compared transcripts from drug-treated mutant cells with drug-treated wt cells. All RNA samples were produced from independent cultures. Transcriptional profiling was performed as previously described (Nantel et al., 2006), and the resulting data were normalized and analyzed in GeneSpring GX version 7.3 (Agilent Technologies, Wilmington, DE). The results of this analysis are listed in Supplementary Dataset 1, which includes significantly modulated genes that exhibited a statistically significant (*t* test; *p* < 0.05) change in transcript abundance of at least 1.5-fold. Gene annotations were determined using the gene ontology term-finder tool for "process" from the *Candida* Genome Database Web page (<http://www.candidagenome.org/cgi-bin/GO/goTermFinder>).

Protein Extraction and In Vivo Sko1 Phosphorylation Assays

C. albicans overnight cultures were collected and diluted to a starting OD_{600 nm} of 0.200 in 100 ml fresh YPD + uridine media. The cultures were incubated with shaking at 30°C to an OD_{600 nm} of 1.0 and split into two 50-ml cultures. The experimental culture was incubated with 1.5 M NaCl for 10 min to induce osmotic shock, and the control culture was treated with dH₂O. For experiments monitoring cell wall damage, the experimental culture was incubated for 1 h with 125 ng caspofungin, and dH₂O was added to the control culture. For kinetic assays a 400-ml starter culture was prepared as described above, and after caspofungin treatment, 50-ml samples were collected at each designated time point. Cells were harvested by vacuum filtration, resuspended in ice cold 20% TCA, and incubated on ice for 30 min. The cells were pelleted at 14,000 rpm for 20 min. A solution of alkaline-buffered acetone was prepared by mixing three parts of 3 M Tris, pH 8.8, to seven parts acetone and was used to wash the pellet twice. The pellet was air-dried and resuspended in 8 M urea. Approximately 100 μl of acid-washed glass beads was added to the cell suspension, and the cells were lysed in a bullet blender (Next Advance, Averill Park, NY). The lysate was pelleted and supernatant was collected. Protein concentration was determined using the Bradford protein assay (Bio-Rad). Cellular lysates were treated with or without calf intestinal phosphatase (New England Biolabs, Beverly, MA) in the presence or absence of phosphatase inhibitors (Sigma). Fifteen micrograms of sample was electrophoresed on 8% SDS polyacrylamide gels, transferred onto PVDF membranes, and stained with Ponceau dye to ensure equal sample loading. Sko1-V5 was probed and detected on immunoblots using anti-V5 monoclonal antibodies conjugated to horseradish peroxidase (Invitrogen) at a 1:2500 dilution and the ECL plus Western blotting chemiluminescent detection system (Amersham, Piscataway, NJ), respectively.

RESULTS

Identification of Caspofungin-hypersensitive Transcription Factor Mutants

To find regulators of the cell wall damage response, we attempted to create homozygous insertion mutants for 67 genes that were related to the transcription process (Table 3).

Table 3. *C. albicans* insertion mutant summary

ORF	Gene ^a	Clone name ^b	Gene length (nt)	Insertion site ^c (nt)	<i>S. cerevisiae</i> ortholog (best match) ^d	No. of isolates screened ^e	No. Recovered ^f	Mutant strain name	Caspofungin growth	Description
19.1032	<i>SKO1</i>	CAGMJ28	1212	1014	<i>SKO1</i>	24	1	JMR061	–	Putative transcription factor
19.1217	19.1217	CAGJI41	2637	2289	YMR247C	12	2	J141-8	+	Predicted ORF
19.1219	19.1219	CAGJS77	1202	836	(<i>RKR1</i>)	12	0	n/a	n/a	Predicted ORF
19.1354	<i>MSN2</i>	CAGIX50	2718	2338	<i>MSN2</i>	12	0	n/a	n/a	Transcription factor involved in stress response
19.1464	19.1464	CAGJG54	1839	375	None	12	5	JG54-7	+	Predicted ORF
19.1476	19.1476	CAGL856	1632	261	<i>IME4</i>	12	1	JMR090	+	Predicted ORF
19.1574	19.1574	CAGIW50	1767	1274	<i>TAF7</i>	12	0	n/a	n/a	Predicted ORF
19.166	<i>ASG1</i>	CAGJ631	2973	1979	<i>ASG1</i>	12	0	n/a	n/a	Predicted zinc finger transcription factor
19.1685	<i>ZCF7</i>	CAGN980	1350	808	(YPR196W)	12	4	JMR049	+	Putative transcription factor
19.1973	<i>HAP5</i>	CAGNV37	1047	815	(<i>HAP5</i>)	12	8	JMR053	+	Putative transcription factor
19.2012	<i>NOT3</i>	CAGHL11	2397	1705	(<i>NOT3</i>)	12	11	HL11-1	+	Putative transcription factor
19.2105	19.2105	CAGK273	651	159	<i>CWC2</i>	12	1	JMR091	+	Predicted ORF
19.232	19.232	CAGNJ15	915	506	<i>MED7</i>	12	0	n/a	n/a	Putative transcription factor
19.2381	19.2381	CAGLR79	939	156	None	24	0	n/a	n/a	Predicted ORF
19.2580	<i>HST2</i>	CAGOP19	996	758	<i>HST2</i>	12	0	n/a	n/a	Predicted ORF
19.2646	<i>ZCF13</i>	CAGIS08	3729	2651	(<i>HAP1</i>)	24	2	IS08-7	–g	Predicted zinc finger transcription factor
19.2654	<i>RMS1</i>	CAGLP16	1659	926	<i>SET7</i>	24	0	n/a	n/a	Predicted ORF
19.2736	19.2736	CAGKZ07	441	399	<i>BUR6</i>	12	3	JMR074	+	Predicted ORF
19.2747	<i>RGT1</i>	CAGHK21	3090	2850	(<i>RGT1</i>)	12	8	HK21-1	+	Putative transcription factor
19.2748	<i>ARG83</i>	CAGLE67	2925	484	(<i>ARG81</i>)	12	2	JMR085	+	Predicted zinc finger transcription factor
19.2808	<i>ZCF16</i>	CAGJ869	3237	1778	(<i>CAT8</i>)	12	2	J869-9	+	Predicted zinc finger transcription factor
19.2963	19.2963	CAGJ120	921	32	(<i>HST1</i>)	12	2	J120-1	+	Predicted ORF
19.3012	<i>ARO80</i>	CAGJA54	3198	2429	<i>ARO80</i>	12	2	JA54-3	+	Predicted ORF
19.3035	19.3035	CAGHK81	4233	2931	<i>CHD1</i>	12	6	HK81-2	+	Predicted ORF
19.3130	19.3130	CAGLQ69	996	225	YLR243W	12	0	n/a	n/a	Predicted ORF
19.3242	19.3242	CAGJX76	735	450	<i>TAF10</i>	12	0	n/a	n/a	Predicted ORF
19.3833	19.3833	CAGLP47	4201	2667	<i>TFC3</i>	12	0	n/a	n/a	Putative transcription factor
19.391	<i>UPC2</i>	CAGOF87	2139	874	<i>UPC2</i>	12	2	JMR038	+	Transcription factor regulating ergosterol synthesis
19.3912	<i>GLN3</i>	CAGKJ50	2049	1237	<i>GLN3</i>	12	0	n/a	n/a	Transcription factor regulating filamentation
19.4194	19.4194	CAGN140	1092	24	<i>TFB4</i>	12	0	n/a	n/a	Putative transcription factor
19.4420	19.4420	CAGL823	2403	981	(<i>RRN6</i>)	12	0	n/a	n/a	Predicted ORF
19.4524	<i>ZCF24</i>	CAGHM72	2185	776	(<i>ASG1</i>)	12	8	HM72-5	+	Predicted zinc finger transcription factor
19.4545	<i>SWI4</i>	CAGJP77	3000	284	(<i>SWI4</i>)	12	0	n/a	n/a	Putative transcription factor
19.4628	19.4628	CAGNF22	1497	13	<i>MPE1</i>	12	0	n/a	n/a	Predicted ORF
19.4722	19.4722	CAGP462	825	269	<i>RTG1</i>	12	1	JMR089	yes	Predicted ORF
19.4775	<i>CTA8</i>	CAGQK08	2286	1429	(<i>HSF1</i>)	12	2	JMR044	+	Putative transcription factor
19.4814	19.4814	CAGQE66	336	256	None	12	4	JMR040	+	Predicted ORF
19.4851	<i>TFA1</i>	CAGQS80	1185	280	<i>TFA1</i>	12	0	n/a	n/a	Putative transcription factor
19.4853	<i>HCM1</i>	CAGP438	1740	1030	<i>HCM1</i>	12	0	n/a	n/a	Putative transcription factor
19.4882	19.4882	CAGNN71	855	627	<i>TFA2</i>	20	1	JMR087	+	Putative transcription factor
19.5097	<i>CAT8</i>	CAGM111	3171	38	<i>CAT8</i>	24	0	n/a	n/a	Putative transcription factor

Continued

Table 3. Continued

ORF	Gene ^a	Clone name ^b	Gene length (nt)	Insertion site ^c (nt)	<i>S. cerevisiae</i> ortholog (best match) ^d	No. of isolates screened ^e	No. Recovered ^f	Mutant strain name	Caspofungin growth	Description
19.5268	19.5268	CAGJM31	513	142	<i>NUT2</i>	12	0	n/a	n/a	Predicted ORF
19.5377	<i>HOS2</i>	CAGK036	1365	1068	<i>HOS2</i>	12	1	K036-9	+	Predicted ORF
19.5501	<i>YAF9</i>	CAGLJ16	765	28	<i>YAF9</i>	12	0	n/a	n/a	Predicted ORF
19.5552	19.5552	CAGL213	2208	1315	(<i>CRT10</i>)	12	1	JMR088	0	Predicted ORF
19.5558	<i>RBF1</i>	CAGIR07	1605	654	None	12	0	n/a	n/a	Transcription factor involved in filamentous growth and pathogenesis
19.5666	19.5666	CAGO538	441	159	<i>SUB1</i>	12	3	JMR035	+	Predicted ORF
19.5680	19.5680	CAGNU89	1176	1156	None	12	0	n/a	n/a	Predicted ORF
19.5846	19.5846	CAGKB46	1485	1173	<i>TFB2</i>	12	0	n/a	n/a	Predicted ORF
19.5871	19.5871	CAGJC25	2093	253	<i>SNF5</i>	12	3	JC25-6	+	Predicted ORF
19.5910	19.5910	CAGMJ07	2280	13	<i>NTO1</i>	12	6	JMR062	+	Predicted ORF
19.5917	<i>STP3</i>	CAGJ322	1311	113	(<i>STP2</i>)	12	0	n/a	+	Transcription factor regulating <i>SAP2</i> & <i>OPT3</i>
19.5992	<i>WOR2</i>	CAGKZ68	1341	303	(<i>LYS14</i>)	12	0	n/a	n/a	Predicted zinc finger transcription factor
19.6109	<i>TUP1</i>	CAGKQ96	1545	349	<i>TUP1</i>	12	4	JMR069	n/a	Transcriptional corepressor
19.6393	19.6393	CAGLA20	1251	776	<i>GTS1</i>	12	0	n/a	n/a	Predicted ORF
19.6414	19.6414	CAGHQ12	1923	205	None	12	7	HQ12-3	+	Predicted ORF
19.6649	<i>BRF1</i>	CAGKG39	1662	1306	<i>BRF1</i>	12	0	n/a	n/a	Putative transcription factor
19.6753	19.6753	CAGJL50	450	27	YBR062C	12	0	n/a	n/a	Predicted ORF
19.6849	<i>ELC1</i>	CAGR528	303	269	<i>ELC1</i>	24	3	JMR046	+	Predicted ORF
19.7017	19.7017	CAGK324	1008	413	<i>YOX1</i>	12	3	JMR092	+	Predicted ORF
19.7046	<i>MET28</i>	CAGKQ61	522	118	None	12	0	n/a	n/a	Predicted ORF
19.705	19.705	CAGQ353	1350	1330	<i>CEN5</i>	12	0	n/a	n/a	Predicted ORF
19.7234	19.7234	CAGKV55	1686	698	<i>RSC8</i>	8	0	n/a	n/a	Predicted ORF
19.7317	<i>UGA33</i>	CAGKZ33	1450	717	(<i>UGA3</i>)	12	8	JMR077	+	Predicted zinc finger transcription factor
19.7372	<i>ZCF36</i>	CAGIU75	3327	3164	(<i>HAP1</i>)	12	4	IU75-1	+	Predicted zinc finger transcription factor
19.7381	<i>ZCF37</i>	CAGJ793	1875	1144	(<i>LYS14</i>)	12	5	J793-2	+	Predicted zinc finger transcription factor
19.861	19.861	CAGJM52	531	267	(<i>YAP6</i>)	12	0	n/a	n/a	Putative transcription factor
19.9780	<i>BDF1</i>	CAGKO55	2199	2000	<i>BDF1</i>	12	0	n/a	n/a	Putative transcription factor

^a ORF assignments and gene designations are taken from the *Candida* Genome Database; <http://www.candidagenome.org/>.

^b Clone name refers to the insertion clone used to make each mutant (see Materials and Methods).

^c Insertion site is the distance from the ATG initiator of the ORF to the transposon insertion site.

^d *S. cerevisiae* orthologues or closest homologs (in parentheses) are taken from the *Candida* Genome Database; <http://www.candidagenome.org/>.

^e No. of screened refers to the number of independent transformants from which Arg+Ura+ segregants were derived to screen for homozygotes.

^f No. recovered is the number of independent homozygotes identified among the Arg+Ura+ segregants screened.

^g Only one isolate with this insertion allele was caspofungin hypersensitive; additional isolates behaved similarly to control strain DAY286.

We were unable to create mutants in 34 of these genes, some of which may be essential. We note that the *S. cerevisiae* orthologues of 13 of these genes are essential, but homozygous *C. albicans* mutants for another six of these genes have been made previously by other methods. We screened the mutants we recovered in 33 genes for altered growth on caspofungin medium and found a caspofungin-sensitive strain with an insertion in *SKO1* (Table 3).

Sko1 is orthologous to the *S. cerevisiae* transcription factor ScSko1, which functions in the osmotic stress response. To verify that Sko1 governs caspofungin sensitivity in *C. albi-*

cans, we constructed a *sko1Δ/Δ* deletion mutant. Growth of the *sko1Δ/Δ* mutant was drastically reduced on caspofungin plates compared with nutrient YPD plates (Figure 1). Similar results were observed using another independent *sko1Δ/Δ* mutant (derived from an independent heterozygote; data not shown). The caspofungin-hypersensitive phenotype of both mutants was complemented by introduction of a wt copy of *SKO1* (Figure 1 and data not shown), indicating that the *sko1Δ* mutation is the cause of caspofungin hypersensitivity. These findings show that *SKO1* is required for normal caspofungin sensitivity.

Figure 1. Caspofungin sensitivity assays. Overnight cultures of prototrophic *C. albicans* strains were serially diluted and spotted onto nutrient YPD medium or YPD supplemented with caspofungin (125 ng/ml). The wild-type *C. albicans* reference strain (DAY185), null mutant (Δ/Δ), and complemented ($\Delta/\Delta+$) strains are shown. Panels A and B show two different plates.

Regulation of SKO1 Expression by Cell Wall Damage

Transcription factors are often induced under conditions that require their biological activity. Thus, we hypothesized that caspofungin treatment may induce *SKO1* expression. We measured *SKO1* transcript levels by RT-PCR after caspofungin treatment. *SKO1* was up-regulated sixfold in wt cells treated with caspofungin (Figure 2A). *SKO1* expression was

Figure 2. *SKO1* Expression Analysis. (A) *SKO1* expression was monitored using real-time (RT) PCR analysis in the reference strain DAY185 and prototrophic *hog1Δ/Δ* strain (JMR114) with or without 125 ng caspofungin. (B) RT-PCR analysis of *SKO1* expression in the reference strain DAY185 and prototrophic *sko1Δ/Δ* mutant (JMR104) and *sko1Δ/Δ* $\pm$ -complemented strains (JMR109), with or without 125 ng caspofungin. Transcript levels were normalized to *TDH3* expression, and fold changes between strains were normalized to the wt reference strain adjusted to value of 1.0. (C) Wild-type cells (strain JMR143) carrying *SKO1-V5* were treated for various times with caspofungin ($t = 0, 30, 60$, and 90 min). Sko1-V5 was detected in an immunoblot.

not detected in the *sko1Δ/Δ* deletion mutant, thus confirming primer specificity, and was restored to wt levels in the *sko1Δ/Δ* $\pm$ -complemented strain (Figure 2B). To monitor Sko1 protein levels, we constructed a strain carrying a functional epitope-tagged Sko1-V5 (Figure 1). Consistent with our gene expression results, Western blotting analysis showed that there was an increase in the amount of Sko1-V5 protein levels after caspofungin treatment (Figure 2C). We conclude that caspofungin induces *SKO1* gene expression and protein accumulation.

Role of SKO1 in the Transcriptional Response to Cell Wall Damage

We considered the possibility that Sko1 may be required for expression of caspofungin-responsive genes. Alternatively, Sko1 may be required for expression of osmotic stress response genes that promote survival after cell wall damage.

Figure 3. Gene expression response to caspofungin in wt and *sko1Δ/Δ* strains. Kinetic analysis of *PGA13* (A), *GPD2* (B), and *RHR2* (C) expression after caspofungin treatment in reference strain DAY185 strain (solid line with black squares), the *sko1Δ/Δ* mutant strain (dashed line with gray circles), and the *sko1Δ/Δ* $\pm$ -complemented strain (solid line with white diamonds, only in A). Transcript levels were normalized to *TDH3* expression.

Figure 4. Verification of *Sko1* target genes identified through microarray analysis. RT-PCR expression analysis of *SKO1* array target genes *CRH11* (A), *MNN2* (B), *SKN1* (C), and *HGT6* (D) with or without caspofungin treatment in reference strain DAY185, the *sko1Δ/Δ* mutant strain (JMR104), and the *sko1Δ/Δ/+*-complemented strain (JMR109). Transcript levels were normalized to *TDH3* expression, and fold changes between strains were normalized to the reference strain, adjusted to value of 1.0. **p* < 0.05 compared with the reference strain.

To test these hypotheses, we monitored expression of the caspofungin-responsive gene *PGA13* and the osmotic stress response genes *RHR2* and *GPD2*. *PGA13* specifies a cell wall protein and is induced in response to cell wall damage (Bruno *et al.*, 2006) but not in response to osmotic stress (Enjalbert *et al.*, 2006). *Rhr2* and *Gpd2* catalyze the synthesis of glycerol, which is critical in adaptation to osmotic stress (Fan *et al.*, 2005; Enjalbert *et al.*, 2006). We observed that *PGA13* was induced in the wt and *sko1Δ/Δ/+*-complemented strains, but not in the *sko1Δ/Δ* mutant (Figure 3A). On the other hand, *GPD2* and *RHR2* expression was similar in the wt strain and *sko1Δ/Δ* mutant (Figure 3, B and C). Therefore, although caspofungin treatment induces two osmotic stress-responsive genes, this response is independent of *Sko1* function. In contrast, induction of the cell wall protein gene *PGA13* depends on *Sko1* function.

To define *Sko1*-dependent genes in broader terms, we performed microarray comparisons of the wt strain and *sko1Δ/Δ* mutant treated with caspofungin (Supplementary Dataset 1, Worksheet 1 and 2). We found that *Sko1* regulates 79 caspofungin-responsive genes, including several cell wall biogenesis genes (Supplemental Dataset 1, Worksheet 3). RT-PCR analysis confirmed the reduced expression of cell wall biogenesis genes *CRH11*, *MNN2*, and *SKN1* in the *sko1Δ/Δ* mutant treated with caspofungin (Figure 4, A–C). Gene expression levels were restored to wt in the *sko1Δ/Δ/+*-complemented strain (Figure 4, A–C). Therefore, *Sko1* is necessary for expression of many caspofungin-responsive genes.

We noted that carbohydrate metabolic genes, such as the glucose transporter gene *HGT6*, were significantly overexpressed in the *sko1Δ/Δ* mutant (Supplementary Table S1, Worksheets 1 and 2). These genes are not induced by caspofungin. RT-PCR assays showed that *HGT6* is overexpressed in the *sko1Δ/Δ* mutant with or without caspofungin treatment (Figure 4D). These findings indicate that *Sko1* is a negative regulator of carbon metabolic genes.

Identification of Upstream Regulators of *SKO1* Expression

To identify upstream regulators of *Sko1* activity, we first considered the *S. cerevisiae* paradigm. The protein kinase ScHog1 activates ScSko1 by phosphorylation in response to osmotic shock, thereby causing a change in ScSko1 electrophoretic mobility (Proft *et al.*, 2001). Thus, we considered that *C. albicans* Hog1 may be a regulator of *Sko1* in response to caspofungin treatment. Prior studies have shown that the *C. albicans* the HOG pathway is important for cell wall biosynthesis and stability (Eisman *et al.*, 2006; Enjalbert *et al.*, 2006; Munro *et al.*, 2007). However, we observed that a *hog1Δ/Δ* mutant was only slightly hypersensitive to caspofungin compared with the *sko1Δ/Δ* mutant (Figure 1), and it

Figure 5. Hog1-dependent phosphorylation of *Sko1* after osmotic stress. (A) *Sko1*-V5 was visualized on an immunoblot of wt cells (strain JMR143) or *hog1Δ/Δ* cells, with or without 1.5 M NaCl treatment for 10 min. (B) Total protein extracts were collected and treated with 50 U of calf alkaline phosphatase in the presence or absence of phosphatase inhibitors as indicated. *Sko1*-V5 was detected on an immunoblot.

Figure 6. SKO1 Expression in caspofungin-hypersensitive protein kinase mutants. SKO1 expression was monitored by RT-PCR in reference strain DAY286 and in the 17 protein kinase insertion homozygotes indicated. All strains were treated with caspofungin for 60 min. SKO1 transcript levels were normalized as described in the Figure 4 legend.

expressed SKO1 normally (Figure 2A). Protein analysis from wt cells treated with caspofungin showed that Sko1 does not undergo an electrophoretic shift (Figure 2C). On the other hand, we observed a Sko1 electrophoretic shift after osmotic shock in wt cells but not in the *hog1Δ/Δ* mutant strain (Figure 5A). The Sko1 electrophoretic shift was sensitive to phosphatase treatment (Figure 5B). These results suggest that Hog1 phosphorylates Sko1 after osmotic stress, but argue that the HOG pathway does not regulate Sko1 after caspofungin-induced cell wall damage.

We have recently identified insertion mutants in several protein kinase-related genes that are hypersensitive to caspofungin (Blankenship, Fanning, Hamaker, and Mitchell, unpublished data). Those protein kinases are additional candidate SKO1 regulators. We found that SKO1 expression was similar to wt in eight mutants, reduced about twofold in four mutants, and increased about twofold in four mutants. We note that SKO1 expression was increased in all mutants of the PKC-signaling pathway (Figure 6). SKO1 expression was most severely reduced in the *psk1-/-* mutant (Figure

6). Indeed, several independent *psk1Δ/Δ* deletion strains were hypersensitive to caspofungin (Figure 1 and data not shown), a phenotype that was complemented by a wt *PSK1* allele (Figure 1). SKO1 was expressed at its uninduced level in three independent *psk1Δ/Δ* mutant deletion mutants, regardless of caspofungin treatment (Figure 7A and data not shown). Therefore, Psk1 is a positive regulator of SKO1 expression in caspofungin-treated cells.

Our observations predict that a *psk1Δ* mutation will affect expression of Sko1 target genes. RT-PCR assays showed reduced expression of *PGA13* and *MNN2* and the increased expression of *HGT6* in *psk1Δ/Δ* cells, compared with wt or complemented strains (Figure 8, A and B). Interestingly, *HGT6* was overexpressed in the *psk1Δ/Δ* mutant only after caspofungin treatment (Figure 8C), the circumstance in which the mutant has reduced expression of SKO1 (Figure 7). These results support the model that Psk1 is required for functional expression of SKO1 in response to caspofungin.

DISCUSSION

The fungal cell wall has vital roles in growth, survival, morphogenesis, and pathogenicity. Critical for the coordination of these activities is the dynamic nature of the cell wall, its ability to respond to external and internal stimuli. We propose that the distinct evolutionary paths of each fungal species may be reflected in unique cell wall regulatory pathways. Our identification of a *C. albicans* Psk1-Sko1 pathway (Figure 9) lends support to this idea. Inhibition of cell wall biogenesis by caspofungin causes an increase in SKO1 expression. This increase is dependent on the protein kinase Psk1 and culminates in the expression of diverse genes that are necessary for cell wall stability. Although aspects of Sko1 and Psk1 function are conserved in *S. cerevisiae*, the connections among Sko1, Psk1, and cell wall perturbation may be unique to *C. albicans*.

Conservation of the Hog1-Sko1 Relationship

Our findings argue that Sko1 functions in the Hog1-dependent osmotic stress response, a relationship well established in *S. cerevisiae* (Proft *et al.*, 2001; Rep *et al.*, 2001). This role

Figure 7. Psk1 Requirement for SKO1 expression. (A) RT-PCR analysis of SKO1 expression in reference strain DAY185, the prototrophic *psk1Δ/Δ* mutant strain (JMR192), and the *psk1Δ/Δ/+*-complemented strain (JMR188) with or without caspofungin treatment. SKO1 transcript levels were normalized as described in the Figure 4 legend. **p* < 0.05 compared with the reference strain.

Figure 8. Expression of *SKO1* target genes in *psk1Δ/Δ* mutants. RT-PCR expression analysis of *SKO1* target genes *PGA13* (A), *MNN2* (B), and *HGT6* (C) with or without caspofungin treatment in reference strain DAY185, the prototrophic *psk1Δ/Δ* mutant strain (JMR192), and the *psk1Δ/Δ*+ complemented strain (JMR188) with or without caspofungin treatment. Transcript levels were normalized as described in the Figure 4 legend. **p* < 0.05 compared with the reference strain.

was foreshadowed by microarray analysis (Enjalbert *et al.*, 2006), which revealed that *SKO1* expression is induced 1.5-fold by osmotic stress, dependent on *HOG1*. Our results point to a second aspect of this relationship: Sko1 undergoes Hog1-dependent phosphorylation after osmotic stress. Hog1 may phosphorylate Sko1 directly, as known for the *S. cerevisiae* orthologues, because the ScSko1 phosphoacceptor sequence is well conserved in *C. albicans* Sko1 (Krantz *et al.*, 2006). Indeed, *sko1Δ/Δ* mutants are slightly sensitive to osmotic stress (our unpublished results), so these modes of Sko1 regulation may be functionally significant. Therefore, aspects of the Hog1–Sko1 relationship are conserved in the *C. albicans* osmotic stress response.

Role of *SKO1* in the Cell Wall Damage Response

Our findings establish that Sko1 is necessary for the cell wall damage response. In principle, the caspofungin hypersensi-

Figure 9. Model for the *C. albicans* Psk1-Sko1 signaling pathway. Cell wall damage induced by caspofungin treatment activates Psk1. Psk1 acts on an unidentified transcription factor, leading to elevated *SKO1* expression. The Psk1 target is depicted as an activator, but could equally well be a repressor. Sko1 activates downstream target genes to restore integrity of the cell wall.

tivity of the *sko1Δ/Δ* mutant might have reflected an aberrant osmotic stress response. This response is induced by cell wall perturbation in both *S. cerevisiae* (Boorsma *et al.*, 2004) and, as we show here, in *C. albicans*. However, two *C. albicans* osmotic stress genes are induced by caspofungin independently of Sko1. Furthermore, the major Sko1-dependent genes that are induced by caspofungin, such as *CRH11*, *PGA13*, and *MNN2*, are not induced by osmotic stress (Enjalbert *et al.*, 2006). The fact that *SKO1* is induced by caspofungin in both wt and *hog1Δ/Δ* strains, along with our failure to detect caspofungin-induced Sko1 phosphorylation, further underscore the independence of Sko1 and Hog1 activities after cell wall perturbation. Therefore, the Hog1–Sko1 paradigm does not account for the role of Sko1 in the cell wall damage response.

Our hypothesis is that the caspofungin-inducible genes that depend upon Sko1 for full expression contribute to the *sko1Δ/Δ* mutant's caspofungin hypersensitivity. We have identified 26 genes of this class in our experiments. This number includes 25 genes that were induced by caspofungin in the wt strain, as detected (≥ 1.5 -fold) with our current array platform, as well as *PGA13*, for which induction was detected only by RT-PCR (Supplemental Dataset, Worksheet 4). (Based on the caspofungin-inducible gene set defined by Bruno *et al.* (2006) with a different array platform, there are 14 genes of this class, as summarized in the Supplemental Dataset Worksheet 5). For example, *KRE1*, *SKN1*, *PHR1*, *CRH11*, *PGA13*, *PGA31*, and *MNN2* have all been implicated in cell wall biogenesis (Boone *et al.*, 1991; Mio *et al.*, 1997; Popolo and Vai, 1998; De Groot *et al.*, 2003; Pardini *et al.*, 2006). In addition, we have observed that *mn2* and *pga13* homozygous insertion mutants are caspofungin hypersensitive (our unpublished data). These observations suggest that

Sko1-dependent induction of these genes may be critical for an effective response to cell wall damage.

It seems likely that additional Sko1-regulated genes may also influence the *sko1Δ/Δ* mutant's caspofungin hypersensitivity. Most Sko1-regulated genes are not induced by caspofungin under our treatment conditions. A major subset of these genes is involved in carbohydrate metabolism ($p = 6.51 \times 10^{-5}$ for 46/447 genes; <http://www.candidagenome.org/cgi-bin/GO/goTermFinder>), such as *PFK2* (glycolysis), *PCK1* (gluconeogenesis), and *REG1* (carbon regulation). Several hexose transporter genes, such as *HGT6*, are also regulated by Sko1. The cell wall is composed mainly of glucose polymers, so altered flux through carbon metabolic pathways may have significant consequences for cell wall biogenesis. Thus we suggest that multiple classes of Sko1-regulated genes impact the integrity of the cell wall.

Upstream Regulators of SKO1

Although several studies have revealed that transcription factors have been rewired in *C. albicans* compared with *S. cerevisiae* (Kadosh and Johnson, 2001; Khalaf and Zitomer, 2001; Ihmels *et al.*, 2005; Martchenko *et al.*, 2007; Banerjee *et al.*, 2008), seldom have the relevant upstream regulators been identified. Here we have identified protein kinase Psk1 as a regulator of *SKO1* expression. Psk1 is a PAS-domain protein, and PAS-domain proteins of prokaryotes and eukaryotes regulate diverse physiological processes (Rutter *et al.*, 2001; Gilles-Gonzalez and Gonzalez, 2004). The *S. cerevisiae* PAS protein kinases ScPsk1 and ScPsk2 control glucose partitioning. *S. cerevisiae* ScPsk1/2 phosphorylates the enzyme UDP-glucose pyrophosphorylase to stimulate the formation of UDP-glucose, the precursor for glycogen and glucan synthesis (Smith and Rutter, 2007). Thus, *Scpsk1/2Δ* double mutants are sensitive to cell wall-perturbing agents (Smith and Rutter, 2007). In this context, it is not surprising that the *C. albicans psk1Δ/Δ* mutant is caspofungin-hypersensitive. However, its connection to Sko1 is unexpected.

Our conclusion that Psk1 acts upstream of Sko1 is based on two lines of evidence. First, we found that that *psk1* insertion and deletion homozygotes express *SKO1* RNA at its basal level, even after caspofungin treatment. Thus Psk1 is required specifically for the induction of *SKO1* by caspofungin. Second, we observed that two Sko1-dependent cell wall genes, *PGA13* and *MNN2*, are expressed at reduced levels in *psk1Δ/Δ* mutants. In addition, the Sko1-repressed gene *HGT6* is expressed at elevated levels in *psk1Δ/Δ* mutants. Interestingly, the altered regulation of *HGT6* in *psk1Δ/Δ* mutants argues that the induction of *SKO1* by the cell wall damage has functional consequences: *HGT6* is expressed at normal levels in *psk1Δ/Δ* mutants in the absence of caspofungin, when *SKO1* is expressed at its basal level. However, *HGT6* is overexpressed in *psk1Δ/Δ* mutants in the presence of caspofungin, when *SKO1* induction is defective. We suggest that caspofungin treatment increases the demand for Sko1 activity, which is limiting in *psk1Δ/Δ* mutants. Limitation of Sko1 activity may partially recapitulate a *sko1Δ/Δ* mutant phenotype, resulting in elevated *HGT6* expression. This observation, along with the caspofungin hypersensitivity of the *psk1Δ/Δ* mutant, argues that Psk1-dependent induction of *SKO1* is critical for an effective response to cell wall perturbation.

The Psk1-Sko1 relationship represents a new cell wall damage signaling pathway. Our gene expression data provide some insight into the outputs of the pathway, though we have not yet distinguished direct Sko1 target genes. Two key aspects of the pathway remain to be discovered. One is

the mechanism by which Psk1 regulates Sko1 RNA accumulation. A simple possibility is that Psk1 phosphorylates and activates another transcription factor, which in turn activates *SKO1* expression. Transcription factor mutant screens, as reported here and in Bruno *et al.* (2006), may identify this component. A second area for future analysis is the mechanism by which Psk1 may sense cell wall perturbation. The similarity of overall Psk1 biological function in *S. cerevisiae* and *C. albicans* may indicate that upstream signaling components are conserved, so that gene discovery strategies carried out in both organisms may converge upon these genes. Finally, our results argue that Sko1 lies at the intersection of two *C. albicans* stress response pathways, defined by Hog1 and Psk1. An interesting possibility is that Sko1 may coordinate these responses.

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REFERENCES

- Banerjee, M., Thompson, D. S., Lazzell, A., Carlisle, P. L., Pierce, C., Monteagudo, C., Lopez-Ribot, J. L., and Kadosh, D. (2008). UME6, a novel filament-specific regulator of *Candida albicans* hyphal extension and virulence. *Mol. Biol. Cell* 19, 1354–1365.
- Boone, C., Sdicu, A., Laroche, M., and Bussey, H. (1991). Isolation from *Candida albicans* of a functional homolog of the *Saccharomyces cerevisiae* KRE1 gene, which is involved in cell wall beta-glucan synthesis. *J. Bacteriol.* 173, 6859–6864.
- Boorsma, A., de Nobel, H., ter Riet, B., Bargmann, B., Brul, S., Hellingwerf, K. J., and Klis, F. M. (2004). Characterization of the transcriptional response to cell wall stress in *Saccharomyces cerevisiae*. *Yeast* 21, 413–427.
- Braun, B. R., Kadosh, D., and Johnson, A. D. (2001). NRG1, a repressor of filamentous growth in *C. albicans*, is down-regulated during filament induction. *EMBO J.* 20, 4753–4761.
- Bruno, V. M., Kalachikov, S., Subaran, R., Nobile, C. J., Kyratsous, C., and Mitchell, A. P. (2006). Control of the *C. albicans* cell wall damage response by transcriptional regulator Cas5. *PLoS Pathog* 2, e21.
- Davis, D., Edwards, J. E., Jr., Mitchell, A. P., and Ibrahim, A. S. (2000). *Candida albicans* RIM101 pH response pathway is required for host-pathogen interactions. *Infect. Immun.* 68, 5953–5959.
- Davis, D. A., Bruno, V. M., Loza, L., Filler, S. G., and Mitchell, A. P. (2002). *Candida albicans* Mds3p, a conserved regulator of pH responses and virulence identified through insertional mutagenesis. *Genetics* 162, 1573–1581.
- De Groot, P. W., Hellingwerf, K. J., and Klis, F. M. (2003). Genome-wide identification of fungal GPI proteins. *Yeast* 20, 781–796.
- Dranginis, A. M., Rauceo, J. M., Coronado, J. E., and Lipke, P. N. (2007). A biochemical guide to yeast adhesins: glycoproteins for social and antisocial occasions. *Microbiol. Mol. Biol. Rev.* 71, 282–294.
- Eisman, B., Alonso-Monge, R., Roman, E., Arana, D., Nombela, C., and Pla, J. (2006). The Cek1 and Hog1 mitogen-activated protein kinases play complementary roles in cell wall biogenesis and chlamydospore formation in the fungal pathogen *Candida albicans*. *Eukaryot. Cell* 5, 347–358.
- Enjalbert, B., Smith, D. A., Cornell, M. J., Alam, I., Nicholls, S., Brown, A. J., and Quinn, J. (2006). Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen *Candida albicans*. *Mol. Biol. Cell* 17, 1018–1032.
- Fan, J., Whiteway, M., and Shen, S. H. (2005). Disruption of a gene encoding glycerol 3-phosphatase from *Candida albicans* impairs intracellular glycerol accumulation-mediated salt-tolerance. *FEMS Microbiol. Lett.* 245, 107–116.
- Gilles-Gonzalez, M. A., and Gonzalez, G. (2004). Signal transduction by heme-containing PAS-domain proteins. *J. Appl. Physiol.* 96, 774–783.
- Ihmels, J., Bergmann, S., Gerami-Nejad, M., Yanai, I., McClellan, M., Berman, J., and Barkai, N. (2005). Rewiring of the yeast transcriptional network through the evolution of motif usage. *Science* 309, 938–940.

- Kadosh, D., and Johnson, A. D. (2001). Rfg1, a protein related to the *Saccharomyces cerevisiae* hypoxic regulator Rox1, controls filamentous growth and virulence in *Candida albicans*. *Mol. Cell. Biol.* 21, 2496–2505.
- Khalaf, R. A., and Zitomer, R. S. (2001). The DNA binding protein Rfg1 is a repressor of filamentation in *Candida albicans*. *Genetics* 157, 1503–1512.
- Krantz, M., Becit, E., and Hohmann, S. (2006). Comparative analysis of HOG pathway proteins to generate hypotheses for functional analysis. *Curr. Genet.* 49, 152–165.
- Kubista, M. *et al.* (2006). The real-time polymerase chain reaction. *Mol. Aspects Med.* 27, 95–125.
- Lesage, G., and Bussey, H. (2006). Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* 70, 317–343.
- Letscher-Bru, V., and Herbrecht, R. (2003). Caspofungin: the first representative of a new antifungal class. *J. Antimicrob. Chemother.* 51, 513–521.
- Levin, D. E. (2005). Cell wall integrity signaling in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* 69, 262–291.
- Liu, T. T., Lee, R. E., Barker, K. S., Lee, R. E., Wei, L., Homayouni, R., and Rogers, P. D. (2005). Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. *Antimicrob. Agents Chemother.* 49, 2226–2236.
- Martchenko, M., Levitin, A., Hogues, H., Nantel, A., and Whiteway, M. (2007). Transcriptional rewiring of fungal galactose-metabolism circuitry. *Curr. Biol.* 17, 1007–1013.
- Mio, T., Yamada-Okabe, T., Yabe, T., Nakajima, T., Arisawa, M., and Yamada-Okabe, H. (1997). Isolation of the *Candida albicans* homologs of *Saccharomyces cerevisiae* KRE6 and SKN1, expression and physiological function. *J. Bacteriol.* 179, 2363–2372.
- Munro, C. A., Selvaggini, S., de Bruijn, I., Walker, L., Lenardon, M. D., Gerssen, B., Milne, S., Brown, A. J., and Gow, N. A. (2007). The PKC, HOG and Ca²⁺ signalling pathways co-ordinately regulate chitin synthesis in *Candida albicans*. *Mol. Microbiol.* 63, 1399–1413.
- Nantel, A., Rigby, T., Hogues, H., and Whiteway, M. (2006). Microarrays for studying pathology in *Candida albicans*. Hoboken, NJ: Wiley Press.
- Navarro-Garcia, F., Alonso-Monge, R., Rico, H., Pla, J., Sentandreu, R., and Nombela, C. (1998). A role for the MAP kinase gene MKC1 in cell wall construction and morphological transitions in *Candida albicans*. *Microbiology* 144(Pt 2), 411–424.
- Nobile, C. J., and Mitchell, A. P. (2005). Regulation of cell-surface genes and biofilm formation by the *C. albicans* transcription factor Bcr1p. *Curr. Biol.* 15, 1150–1155.
- Norice, C. T., Smith, F. J., Jr., Solis, N., Filler, S. G., and Mitchell, A. P. (2007). Requirement for *C. albicans* Sun41 in biofilm formation and virulence. *Eukaryot. Cell* 6, 2046–2055.
- Pardini, G., De Groot, P. W., Coste, A. T., Karababa, M., Klis, F. M., de Koster, C. G., and Sanglard, D. (2006). The CRH family coding for cell wall glycosylphosphatidylinositol proteins with a predicted transglycosidase domain affects cell wall organization and virulence of *Candida albicans*. *J. Biol. Chem.* 281, 40399–40411.
- Popolo, L., and Vai, M. (1998). Defects in assembly of the extracellular matrix are responsible for altered morphogenesis of a *Candida albicans* phr1 mutant. *J. Bacteriol.* 180, 163–166.
- Proft, M., Pascual-Ahuir, A., de Nadal, E., Arino, J., Serrano, R., and Posas, F. (2001). Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress. *EMBO J.* 20, 1123–1133.
- Proft, M., and Struhl, K. (2002). Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. *Mol. Cell* 9, 1307–1317.
- Rabkin, J. M., Orolloff, S. L., Corless, C. L., Benner, K. G., Flora, K. D., Rosen, H. R., and Olyaei, A. J. (2000). Association of fungal infection and increased mortality in liver transplant recipients. *Am. J. Surg.* 179, 426–430.
- Rangel-Frausto, M. S. *et al.* (1999). National epidemiology of mycoses survey (NEMIS): variations in rates of bloodstream infections due to *Candida* species in seven surgical intensive care units and six neonatal intensive care units. *Clin. Infect. Dis.* 29, 253–258.
- Reinoso-Martin, C., Schuller, C., Schuetzer-Muehlbauer, M., and Kuchler, K. (2003). The yeast protein kinase C cell integrity pathway mediates tolerance to the antifungal drug caspofungin through activation of Slf2p mitogen-activated protein kinase signaling. *Eukaryot. Cell* 2, 1200–1210.
- Rep, M., Proft, M., Remize, F., Tamas, M., Serrano, R., Thevelein, J. M., and Hohmann, S. (2001). The *Saccharomyces cerevisiae* Sko1p transcription factor mediates HOG pathway-dependent osmotic regulation of a set of genes encoding enzymes implicated in protection from oxidative damage. *Mol. Microbiol.* 40, 1067–1083.
- Ruiz-Herrera, J., Elorza, M. V., Valentin, E., and Sentandreu, R. (2006). Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. *FEMS Yeast Res.* 6, 14–29.
- Rutter, J., Michnoff, C. H., Harper, S. M., Gardner, K. H., and McKnight, S. L. (2001). PAS kinase: an evolutionarily conserved PAS domain-regulated serine/threonine kinase. *Proc. Natl. Acad. Sci. USA* 98, 8991–8996.
- Smith, T. L., and Rutter, J. (2007). Regulation of glucose partitioning by PAS kinase and Ugp1 phosphorylation. *Mol. Cell* 26, 491–499.
- Spreghini, E., Davis, D. A., Subaran, R., Kim, M., and Mitchell, A. P. (2003). Roles of *Candida albicans* Dfg5p and Dcw1p cell surface proteins in growth and hypha formation. *Eukaryot. Cell* 2, 746–755.
- Wheeler, R. T., and Fink, G. R. (2006). A drug-sensitive genetic network masks fungi from the immune system. *PLoS Pathog* 2, e35.
- Wilson, R. B., Davis, D., and Mitchell, A. P. (1999). Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. *J. Bacteriol.* 181, 1868–1874.