Remodeling of Global Transcription Patterns of *Cryptococcus neoformans* Genes Mediated by the Stress-Activated HOG Signaling Pathways

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The ability to sense and adapt to a hostile host environment is a crucial element for virulence of pathogenic fungi, including *Cryptococcus neoformans*. These cellular responses are evoked by diverse signaling cascades, including the stress-activated HOG pathway. Despite previous analysis of central components of the HOG pathway, its downstream signaling network is poorly characterized in *C. neoformans*. Here we performed comparative transcriptome analysis with HOG signaling mutants to explore stress-regulated genes and their correlation with the HOG pathway in *C. neoformans*. In this study, we not only provide important insights into remodeling patterns of global gene expression for counteracting external stresses but also elucidate novel characteristics of the HOG pathway in *C. neoformans*. First, inhibition of the HOG pathway increases expression of ergosterol biosynthesis genes and cellular ergosterol content, conferring a striking synergistic antifungal activity with amphotericin B and providing an excellent opportunity to develop a novel therapeutic method for treatment of cryptococcosis. Second, a number of cadmium-sensitive genes are differentially regulated by the HOG pathway, and their mutation causes resistance to cadmium. Finally, we have discovered novel stress defense and HOG-dependent genes, which encode a sodium/potassium efflux pump, protein kinase, multidrug transporter system, and elements of the ubiquitin-dependent system.

Whether an organism is able to survive and proliferate in certain environmental niches is mainly determined by the ability to sense and adapt to diverse environmental stresses and maintain cellular homeostasis. Cells achieve homeostasis by deploying a series of complex signaling networks. Among these, the p38/Hog1 mitogen-activated protein kinase (MAPK)-dependent signaling pathway plays a pivotal role in regulating a plethora of stress responses in eukaryotic organisms ranging from yeasts to humans (5). The mammalian stress-activated p38 MAPK transduces myriad stress-related signals, governing adaptation to osmotic changes and UV irradiation, programmed cell death, and immune responses by controlling cytokine production and inflammation (10, 32). Comparable stress-sensing signaling cascades have been also uncovered in many fungal species (5, 9). Fungi contain p38-like MAPKs, mostly known as Hog1 MAPKs, to modulate a range of stress responses (5).

The regulatory mechanism of the p38/Hog1 MAPK pathway is widely conserved in many eukaryotic cells. Under unperturbed normal conditions, the p38/Hog1 MAPK remains unphosphorylated, but in response to certain environmental stresses, it is activated by dual phosphorylation of Thr and Tyr residues in the TGY motif via a MAPK kinase (MAPKK) that is activated through phosphorylation by its upstream MAPKK kinase (MAPKKK) (5). Subsequently, the phosphorylated p38/Hog1 MAPKs dimerize and are translocated into the nucleus to trigger activation of transcription factors and induce a plethora of stress defense genes to counteract external stress conditions (see reviews in references 5, 27, 28, 32, and 36).

In spite of the conserved regulatory mechanism of the p38/Hog1 MAPK, fungi and mammals have unique upstream regulatory systems. In particular, fungi employ a two-component-like phosphorelay system, which has been discovered only in bacteria, fungi and plants, but not in mammals. The fungal phosphorelay system consists of three components, including hybrid sensor kinases, a histidine-containing phosphotransfer protein, and response regulators, all of which are absent in mammals and therefore considered as candidate antifungal targets (5, 9).

The basidiomycete *Cryptococcus neoformans*, an opportunistic human-pathogenic fungus causing meningocencephalitis, also utilizes the Hog1 MAPK pathway for adaptation to a wide range of environmental stresses, including osmotic shock, UV irradiation, heat shock, oxidative damage, toxic metabolites, and antifungal drugs (5–8, 35). Compared to other fungal Hog1 MAPK systems, however, the *C. neoformans* Hog1 MAPK pathway is uniquely specialized not only to respond to diverse environmental stresses but also to control production of two virulence factors, the antiphagocytic capsule and antioxidant melanin, and sexual differentiation. Hence, the Hog1 MAPK
may play a pivotal role as a key signaling regulator in C. neoformans that modulates cross talk with other signaling pathways (5–8, 35). Recently, we reported that the Hog1 MAPKs in a number of C. neoformans strains are constitutively phosphorylated under unstressed conditions and in response to osmotic shock rapidly dephosphorylated for activation (6–8, 35), which is in stark contrast to other fungal Hog1 MAPK systems. Dual phosphorylation of the TGY motif in Hog1 requires the Pbs2 MAPK (8). Hog1 MAPK are important to further understand the complex pathway in C. neoformans between the two Saccharomyces cerevisiae (19), Schizosaccharomyces pombe (18), and fission yeast (20). Dual phosphorylation of the TGY motif in Hog1 requires the Pbs2 MAPK (8). Hog1 MAPK are important to further understand the complex pathway in C. neoformans between the two Saccharomyces cerevisiae (19), Schizosaccharomyces pombe (18), and fission yeast (20).

Upstream of the Pbs2-Hog1 pathway, a fungus-specific phosphorylation system has also been discovered in C. neoformans (7). The C. neoformans phosphorylation system comprises seven different sensor hybrid histidine kinases (Tco1 to Tco7), the Ypd1 phosphotransfer protein, and two response regulators (Ssk1 and Skn7) (7). The Pbs2-Hog1 pathway is mainly regulated by Ssk1, but not by Skn7 (7). Among seven Tco proteins, Tco1 and Tco2 play discrete and redundant roles in activating Ssk1 and the Pbs2-Hog1 MAPK pathway (7). However, since Tco1 and Tco2 regulate only a subset of Ssk1- and Hog1-dependent phenotypes, other upstream receptors or sensor proteins remain to be elucidated. More recently, we identified Ssk2 as an interfac ing MAPKKK between the phosphorylation system and the Pbs2-Hog1 MAPK pathway, through comparative analysis of mitotic maps between the serotype D f1 sibling strains, B-3501 and B-3502, which show differential Hog1 phosphorylation patterns (6). Most notably, interchanges of SSK2 alleles between the two C. neoformans strains showing differential Hog1 phosphorylation patterns exchanged the phenotypes governed by constitutive Hog1 phosphorylation (6). Unlike Saccharomyces cerevisiae and Schizosaccharomyces pombe, C. neoformans harbors a single MAPKKK, Ssk2, which is necessary and sufficient to control the Hog1 MAPK (6). Nevertheless, the downstream signaling network of the Hog1 MAPK pathway in C. neoformans was unknown. Identification and characterization of the downstream signaling network of the Hog1 MAPK are important to further understand the complex phosphorylation system and the Hog1 MAPK signaling network.

Here we investigated the downstream signaling network of the HOG pathway by performing genome-wide comparative transcriptome analysis through DNA microarray analysis with the C. neoformans wild-type (WT) strain H99 and hog1Δ, ssk1Δ, and skn7Δ mutant strains responding to high osmotic shock, fludioxonil treatment, and oxidative stress. In this study, we not only gained important insight into global transcriptional remodeling patterns of cryptococcal genes but counteracted external stresses but also elucidated a number of novel characteristics of the HOG pathway and stress-related genes, as well as the Hog1-, Ssk1-, and/or Skn7-dependent genes. Hence this study provides an excellent opportunity to develop a novel therapeutic approach to treat the life-threatening fungal meningitis caused by C. neoformans.

MATERIALS AND METHODS

Strains and growth conditions. The C. neoformans strains used in this study are listed in Table S1 in the supplemental material and were cultured in YPD (yeast extract–peptone–dextrose) medium unless indicated separately. The ssk1Δ (CNAG_06301.2, with the H99 gene identification [ID] no., “CNAG_XXXXX.2,” indicated as by a five-digit number hereafter), ena1Δ (00531), ubc6-2Δ (02214), ubc6Δ (04611), pdr5Δ (08689), pdr5-2A (04098), pdr5-5Δ (06348), and yor1Δ (03503) mutants were obtained from the C. neoformans deletion mutant library (Fungal Genetics Stock Center; http://www.fgsc.net/), which was constructed by the Madhani laboratory (38). As a control WT strain for phenotypic analysis of these mutants we used the H99 isolate CM018, which was used for construction of Madhanis’s C. neoformans deletion mutant library. To verify each mutant recovered from the deletion mutant library, diagnostic PCR was performed with primers listed in Table S1 in the supplemental material to check whether the corresponding genes were disrupted. In addition, the ena1Δ mutant (AI67) and its complemented strains (AI173) were also kindly provided by Alex Idnurm (University of Missouri) (31).

For total RNA isolation used in DNA microarray analysis, the WT H99 strain and hog1Δ (YSB64), ssk1Δ (YSB261), and skn7Δ (YSB349) mutant strains were grown in 50 ml YPD medium at 30°C for 16 h. Then 5 ml of the overnight culture was inoculated into 100 ml of fresh YPD medium and further incubated at 30°C until it approximately reaches an optical density at 600 nm (OD600) of 1.0. For time zero samples, 50 ml of the 100-mI culture was sampled and rapidly frozen in liquid nitrogen. To the remaining 50-ml culture, 50 ml of YPD containing 2 M NaCl, 40 µg/ml fludioxonil (Pestanl; Sigma), or 5 mM H2O2 was added. During incubation, 50 ml of the culture was sampled at 30 and 60 min, pelleted in a tabletop centrifuge, frozen in liquid nitrogen, and lyophilized overnight. The lyophilized cells were subsequently used for total RNA isolation. As biological replicates for DNA microarrays, three to four independent cultures for each strain and growth condition were prepared for total RNA isolation.

Total RNA preparation. For total RNA isolation, the lyophilized cell pellets were added to a 3-ml volume of sterile 3-mm glass beads, homogenized by shaking, added to 4 ml of TRIZol reagent (Molecular Research Center), and allowed to incubate at room temperature for 5 min. Then 800 µl of chloroform was added, incubated for 3 min at room temperature, transferred to 15-ml round-bottom tubes (SPL), and centrifuged at 10,000 rpm at 4°C for 15 min in a Sorvall SS-34 rotor. Two milliliters of the supernatant was transferred to a new reaction tube, 2 ml isopropanol was added, the tube was mixed for 10 min, and the mixture was allowed to incubate for 10 min at room temperature. Then the mixture was centrifuged at 10,000 rpm at 4°C for 10 min, and the pellet was washed with 4 ml of 75% ethanol diluted with diethylpyrocarbonate (DEPC)-treated water and centrifuged at 8,000 rpm at 4°C for 5 min. The pellet was dried and resuspended with 500 µl DEPC-treated water. The concentration and purity of total RNA samples were calculated by measuring OD260 and gel electrophoresis, respectively. For control total RNA, all total RNAs were prepared from WT and hog1Δ, ssk1Δ, and skn7Δ mutant cells grown under the conditions described above were pooled as reference RNAs.

cDNA synthesis and Cy3 and Cy5 labeling. For cDNA synthesis, the total RNA concentration was adjusted to 1 μg/µl with DEPC-treated water, and 15 µl of the total RNA was added to 1 µl of 5 µg/µl oligo(dt) (5'—TTTTTTTTTTTTTTTTTTTTTTTTTT—5')-pdN6 (Amersham) (1:1 mixture of 10 µg/µl, respectively), incubated at 70°C for 10 min, and on ice for 10 min. Then 15 µl of the following cDNA synthesis mixture was added and incubated at 42°C for 2 hr: 3 µl 0.1 M dithiothreitol, 0.5 µg/µl RNasin (Promega), 0.6 µl aa-dUTP [5-[3-aminoallyl]-2'-deoxyuridine 5'-triphosphate]-dNTPs (a mixture of 6 µl of dTTP, 4 µl aa-dUTP, 10 µl dATP, 10 µl dCTP, and 10 µl dGTP at 100 mM each), 1.5 µl AffinityScript reverse transcriptase (Stratagene), 3 µl AffinityScript buffer, and 7 µl water. This mixture was incubated at 110°C for 1 min, 0.5 M EDTA (pH 8.0) was added, and the mixture was incubated at 65°C for 15 min. After incubation, 25 µl of 1 M HEPES buffer (pH 8.0) and 450 µl of DEPE-tREATED water were added, and the whole mixture was concentrated through a Microcon30 filter (Millipore) and vacuum dried for 1 hr. For Cy3 and Cy5 (Amersham) labeling of the prepared cDNA, Cy3 and Cy5 were dissolved in 10 µl dimethyl sulfoxide, and 1.25 µl of each dye was aliquoted into separate tubes. The cDNAs prepared as described above were added to 9 µl of 0.05 M Na-bicarbonate (pH 8.0) and incubated at room temperature for 15 min. The cDNAs prepared from pooled reference RNAs were mixed with Cy3 as a control, and the cDNAs prepared from each test RNA (each experimental condition) were mixed with Cy5. For a dye-swap experiment, control and test RNAs were labeled oppositely. Each mixture was further incubated at room temperature for 1 hr in the dark and purified with the QIAquick PCR purification kit (Qiagen).

Microarray hybridization and washing. A. C. neoformans serotype D 70-mer microarray slide containing 7,936 probes (Duke University) was prehybridized at 42°C in 60 ml of prehybridization buffer (42.4 ml of sterile distilled water, 2 ml 30% bovine serum albumin, 600 µl 10% sodium dodecyl sulfate [SDS], 15 ml 20× SSC (sodium-sodium citrate, 3 M NaCl, 0.3 M sodium citrate [pH 7.0]), washed with distilled water and isopropanol, and dried by brief centrifugation (110 × g for 2 min). The Cy3- and Cy5-labeled cDNA samples were combined, concentrated through a Microcon30 filter, and vacuum dried. The dried cDNA samples were resuspended with 24 µl of 1× hybridization buffer (250 µl 50% formamide, 125 µl 20× SSC, 5% 10‰ SDS, 120 µl distilled water [dH2O], for a total of 500 µl), added with 1 µl of poly(A) tail DNA (Sigma), further incubated at 100°C for 3 min, and allowed to cool for 5 min at room temperature. The microarray slides were
aligned into the hybridization chamber (DiTech), any dust was removed, and the slides were covered by Liferslips (Erie Scientific). The Cy3- and Cy5-labeled cDNA samples were applied between Liferslips and slides. To prevent slides from drying, 10 µl of 3× SSC buffer was applied to the slides, which were subsequently incubated for 16 h at 42°C. After incubation, the microarray slides were washed with the following three different washing buffers for 2, 5, and 5 min, respectively, on an orbital shaker: wash buffer 1, 10 ml 20× SSC, 600 µl 10% SDS, 184.9 ml dH2O, preheated at 42°C; wash buffer 2, 3.5 ml 20× SSC, 346.5 ml dH2O; and wash buffer 3, 0.88 ml 20× SSC, 349.12 ml dH2O. Three to four independent DNA microarrays with three to four independent biological replicates were performed, including one a dye swap experiment.

Microarray slide scanning and data analysis. After hybridization and washing, the microarray slides were scanned with a GenePix 4000B scanner (Axon Instrument) and the signals were analyzed with GenePix Pro (version 4.0) and gal file (http://geno.wwtul.edu/activity/macro/neoforms). Since total RNAs isolated from serotype A C. neoforms strains were hybridized on the microarray slides printed with the serotype D 70-mer oligonucleotide sequences, the serotype A gene IDs were mapped to those of the serotype D using BLASTN with cutoff E value of E=6. C. neoforms H99 gene sequences that were updated at 24 November 2008 were downloaded from the Broad Institute (http://www.broad.mit.edu/annotation/genome/cryptococcus_neoforms/). The functional category of each C. neoforms H99 gene was assigned using the NCBI KOG database (http://www.ncbi.nlm.nih.gov/COG/grace/shokog.cgi). Using the serotype A gene sequence, each S. cerevisiae gene name or ID listed in the tables in the supplemental material was identified by BLASTP search (E value cutoff, E=6). For hierarchical and statistical analysis, data transported from GenePix software were analyzed with GeneSpring (Agilent) by employing Lowess normalization, reliable gene filtering, hierarchical clustering (standard correlation and average linkage) and zero transformation, and analysis of variance (ANOVA) and Pearson correlation (Microsoft Excel).

Northern hybridization. Northern blot analysis was performed with 10 µg of total RNA from each strain that was used for DNA microarray analysis. Electrophoresis and hybridization were carried out by following the standard protocols previously described (4). Probes for each gene were prepared by PCR amplification with primers listed in Table S1 in the supplemental material, gel extracted, and radiolabeled with the Rediprime II random prime labeling system (Amersham).

Quantitative real-time RT-PCR. Real-time reverse transcription-PCR (RT-PCR) for quantitatively measuring relative expression levels of ERG11 was performed with primers listed in Table S1 in the supplemental material and cDNAs that were generated using the SuperScript II reverse transcriptase kit with total RNAs used in DNA microarray analysis. Relative gene expression was calculated by the threshold cycle (2^-ΔΔCT) method (39). ACT1 was used for normalization of gene expression.

Comparison of stress response gene expression between C. neoforms and other fungi. Protein sequences from C. neoforms H99, S. cerevisiae, S. pombe, and C. albicans were used to perform the BLASTP search against each other. S. cerevisiae sequences were downloaded from Saccharomyces Genome Database (http://www.yeastgenome.org/). S. pombe sequences were downloaded from Schwizosaccharomycetes pombe GeneDB (http://www.sanger.ac.uk/Projects/S_pombe/). C. albicans sequences were downloaded from the Candida Genome Database (http://www.candidagenome.org). Orthologs were selected on the basis of best reciprocal BLAST hit above a cutoff E value of E=6 (see Table S2 in the supplemental material). To compare the expression of stress response genes in four fungi, we used the transcriptome data set from C. neoforms H99 (this study), S. cerevisiae (25), S. pombe (20), and C. albicans (22, 23).

Ergosterol assay. Ergosterol content was measured as previously described (3), but with slight modification. Briefly, each C. neoforms strain was grown in 100 ml YPD medium for 24 h at 30°C. The 100-ml culture was divided into two 50-ml portions for duplicate measurement, and washed three times with water. The cell pellet was frozen in liquid nitrogen and lyophilized overnight. The dried cell pellet was weighed for normalization of ergosterol content, 5 ml of 25% alcoholic potassium hydroxide was added, and the sample was transferred to a sterile borosilicated glass screw-cap tube. Subsequently, the cells were incubated at 80°C for 1 h and allowed to cool to room temperature. Then 1 ml of sterile water and 3 ml of hexane were added, and the mixture was vortexed for 3 min. Then 200 µl of the hexane layer was sampled and mixed with 800 µl of 100% ethanol, and its OD was measured at both 281.5 nm and 230 nm. Ergosterol content was calculated as follows: % ergosterol = [(OD281.5/290) × F] × [weight of F]/[(OD281.5/290)] × [weight of F]. Ergosterol content, where F is the ethanol dilution factor and 281 and 518 are the E values (in percentages per centimeter) determined for crystalline ergosterol and 24(28)dehydroyergosterol, respectively (3).

Stress and antifungal drug sensitivity tests. Each strain was incubated overnight at 30°C in YPD medium, washed, serially diluted (1 to 10^6 dilutions) in dH2O, and spotted (3 µl) onto solid YPD medium containing the indicated concentrations of stress-inducing agents and antifungal drugs as previously described (7, 8). For the osmotic stress sensitivity test, a 0.5 to 1.5 M range of KCl or NaCl was added to YPD or YP agar medium. For the oxidative stress sensitivity test, a range of 2 to 3 mM H2O2 was added to liquefied YPD agar medium prewarmed at 55°C. For antifungal drug sensitivity in a UV crosslinker (UV-PX-2000) at energy levels between 200 and 400 mJ. Then spotted cells were incubated at 30°C for 2 to 4 days and photographed.

Microarray data accession number. The whole microarray data generated by this study have been submitted to the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE16692.

RESULTS

DNA microarray analysis of C. neoforms hog1Δ, ssk1Δ, and skn7Δ mutants. To investigate the target genes and downstream signaling network of the Skn7-, Ssk1-, and Hog1-dependent signaling pathway in C. neoforms, we performed comparative transcriptome analysis of the serotype A WT strain (H99) and hog1Δ, ssk1Δ, and skn7Δ mutants under both normal growth conditions and stressed conditions as described in Materials and Methods. For basic validation of our array quality, we monitored expression levels of the HOG1, SSK1, and SKN7 genes and known Hog1-regulated genes, such as GPP1 (glycerol-3-phosphatase) and GPDI (glycerol-3-phosphate dehydrogenase), in our array data. As expected, the relative expression levels of the HOG1, SSK1, and SKN7 genes in each corresponding mutant compared to the WT strain were very low (0.06-0.09, and 0.22-fold changes, respectively) (Fig. 1A). In addition, basal expression levels of the GPDI (glycerol-3-phosphate dehydrogenases; 01745 and 00121) and GPP1 (tri-glycerol-3-phosphate; 01744) homologous genes, which are well-known Hog1-regulated stress defense genes in other fungi, were more than twofold reduced in hog1Δ and ssk1Δ mutants compared to the WT (see Table S2 in the supplemental material). The GPDI and GPP1 genes were more than twofold induced in the WT in response to osmotic shock, whereas their expression levels were substantially lower than those in hog1Δ or ssk1Δ mutants during osmotic shock, further supporting the quality of our array data (see Table S2 in the supplemental material).

Genes regulated by Hog1, Ssk1, and/or Skn7 under unstressed conditions. First we monitored how hog1, ssk1, and skn7 mutations affect gene expression patterns in C. neoforms under unperturbed, unstressed conditions. Among 7,936 probes monitored, 3,858 probes were found to be reliable (Cy3 reference value cutoff of 10 with 100% filtering) (see Tables S3 and S4 in the supplemental material). Supporting previous findings (7, 8), the transcriptional profile of the hog1Δ mutant was markedly similar to that of the ssk1Δ mutant, based on the condition tree analysis (Fig. 1B). A total of 1,697 genes exhibited significantly different expression patterns in hog1Δ, ssk1Δ, or skn7Δ mutants compared to the WT (P < 0.05, ANOVA) (Fig. 1C; and see Tables S4 and S5 in the supplemental material), indicating that a significant portion of the entire C. neoforms.
formans genome could be transcriptionally affected by perturbation of the two-component system and HOG signaling pathways even under unstressed, normal conditions. The change (fold) is illustrated by color (see the color bar scale). (A) Relative expression levels of the SKN7, SSK1, and HOG1 genes in the hog1Δ (YSB64), ssk1Δ (YSB261), and skn7Δ (YSB349) mutant background compared to WT strain H99. (B) Condition tree analysis of global expression profiles in the WT strain and hog1Δ, ssk1Δ, and skn7Δ mutants. Note that the expression profile of the hog1Δ mutant is more closely related to the ssk1Δ mutant than to the WT or skn7Δ mutant. (C) Hierarchical clustering analysis of 1,697 genes which exhibited significantly different expression patterns (P < 0.05, ANOVA) in at least one mutant strain under normal growth conditions (mid-logarithmic growth phase in YPD medium at 30°C). (D) Venn diagram showing Hog1-, Ssk1-, or Skn7-dependent genes. Genes displaying significant upregulation or downregulation of more than twofold change in each mutant compared to the WT (zero transformation in GeneSpring software) in each mutant strain are included.

Several key findings were obtained. First, a majority of the genes (702 genes; 98.3%) were upregulated or downregulated by either Ssk1 or Hog1 under unstressed conditions, while only 86 genes (12%) were regulated by Skn7. Among the Skn7-dependent genes, only 12 genes were found to be Skn7-specific (Fig. 1D). Thus hog1Δ and ssk1Δ mutations alter genome-wide transcription profiles under unstressed conditions to a greater extent than the skn7Δ mutation (Fig. 1D). Second, there was a significantly higher overlap between Ssk1- and Hog1-dependent genes (473 out of 714 genes; 66.2%) than between Skn7-
and Hog1-dependent genes (70 out of 714 genes; 9.8%), further corroborating that Ssk1 is the major upstream regulator of the Hog1 MAPK. Third, regardless of the significant overlap in genes regulated by Ssk1 and Hog1, there were a number of Ssk1-specific genes (153 genes) and Hog1-specific genes (69 genes), strongly suggesting that Ssk1 and Hog1 are not exclusively in a linear pathway and could have other targets or upstream regulators, respectively (Fig. 1D). This explains why the ssh1Δ mutant exhibits slightly different phenotypes (i.e., higher and lower sensitivity to oxidative and osmotic stresses, respectively) compared to hog1Δ mutants and why Hog1 can still be phosphorylated in the absence of the Ssk1 response regulator upon exposure to NaCl (7).

Genes regulated by the two-component system and HOG pathway cover a wide variety of functional categories (see Fig. S1 to S4 in the supplemental material), indicating that active remodeling of various aspects of cellular function could occur simply by perturbation of the pathways even without external stress. When basal expression level changes of signaling components in diverse signal transduction pathways in the ssh1Δ, skn7Δ, and hog1Δ mutants were compared to the WT, several novel findings were apparent (see Fig. S1B and Table S6 in the supplemental material). First, genes required for the antiphagocytic polysaccharide capsule were significantly upregulated in ssh1Δ and hog1Δ mutants, but not in the skn7Δ mutant, compared to the WT, including the CAP10 (1.8- to 2.2-fold), CAP59 (1.6- to ~1.8-fold), CAP60 (1.6 to ~1.9-fold), and CAP64 (1.5- to ~1.7-fold) genes. This may explain why mutation of the HOG pathway increases capsule production in C. neoformans. Second, genes required for melanin biosynthesis were significantly upregulated. The CAP64 (inositol-phosphorylceramide synthase 1), which catalyzes production of diacylglycerol, which activates Pkc1 while genes such as ERG13, ERG10, ID11, HMG2, and ERG8 were upregulated only in the ssh1Δ mutant, and indeed some genes, including the ERG1 and ERG3 genes, were downregulated in the skn7Δ mutant. In contrast, none of genes was significantly upregulated in the skn7 mutant, and indeed some genes, including the ERG1 and ERG3 genes, were downregulated in the skn7Δ mutant. Northern blotting and quantitative real-time RT-PCR showed higher ERG11 expression levels in the hog1Δ and ssh1Δ strains than in the WT and skn7Δ mutant strains, which is in good agreement with the DNA microarray data (Fig. 2C and D).

To further verify the microarray data, we examined whether increased expression levels of some of the ergosterol biosynthesis genes indeed affect cellular ergosterol content in the hog1Δ and ssh1Δ mutants (Fig. 2B). In accordance with the microarray data, cellular ergosterol content was significantly higher in the hog1Δ and ssh1Δ mutants than in the WT strain and the skn7Δ mutant (Fig. 2B), suggesting that increased expression of ergosterol biosynthetic genes leads to enhanced production of cellular ergosterol. Supporting this finding, the ssh1Δ (MAPKKK) and pbs2Δ (MAPKK) mutants in the HOG pathway were also found to contain significantly higher levels of cellular ergosterol than the WT and the skn7Δ mutant (Fig. 2B). Taken together, ergosterol biosynthesis is repressed by the HOG pathway under normal conditions.

**Inhibition of the HOG signaling pathway dramatically increases antifungal activity of amphotericin B against C. neoformans.** The finding that ergosterol biosynthesis is induced by inhibition of the HOG pathway prompted us to investigate the susceptibility of the mutants in the two-component system and the HOG pathway to antifungal drugs that target the ergosterol biosynthetic genes or ergosterol itself. We hypothesized that increased ergosterol content observed in the ssh1Δ, ssh2Δ, pbs2Δ, and hog1Δ mutants could render them hypersensitive to amphotericin B due to the increased number of drug targets. Confirming this hypothesis, the ssh1Δ, ssh2Δ, pbs2Δ, and hog1Δ
FIG. 2. Induction of ergosterol biosynthesis genes and cellular ergosterol contents by perturbation of the HOG signaling pathway. (A) Relative expression profiles of ergosterol biosynthesis genes in hog1Δ, ssk1Δ, and skn7Δ mutants compared to the WT strain. The change (fold) is illustrated by a color (see the color bar scale), and the exact value for each gene is indicated in the table placed to the right side of the hierarchical clustering diagram. "SC gene" indicates S. cerevisiae gene names from the Saccharomyces Genome Database that are homologous to each C. neoformans gene. CoA, coenzyme A. (B) Cellular ergosterol content in the WT strain (H99) and skn7Δ (YSB349), ssk1Δ (YSB261), ssk2Δ (YSB264), and hog1Δ (YSB64) mutants was measured as described in Materials and Methods. Left and right graphs demonstrate the percentage of ergosterol in each strain and the relative increase in ergosterol content compared to that in the WT, respectively. Each bar demonstrates the average from four independent experiments, and error bars indicate the standard deviation. Asterisks indicate that the ssk1Δ, ssk2Δ, pbs2Δ, and hog1Δ mutants contain significantly higher ergosterol levels than the WT (P < 0.05, as analyzed by using the Bonferroni multiple comparison test). (C) Northern blot showing increased expression of ERG11 in the hog1Δ and ssk1Δ mutants. (D) Verification of transcriptional activation of ERG11 in the hog1Δ and ssk1Δ mutants by quantitative real-time RT-PCR. Data obtained from three independent biological replicates with three technical replicates were normalized by using ACT1 as a control. Relative gene expression indicates normalized ERG11 expression levels in each mutant compared to those of the WT strain.
mutants exhibited dramatic hypersensitivity to amphotericin B treatment compared to the WT (Fig. 3A). In contrast, the skn7Δ mutant showed WT levels of susceptibility to amphotericin B (Fig. 3A).

We also monitored amphotericin B susceptibility of C. neoformans strains having mutations of hybrid sensor kinases (Tco1 to Tco7, except for Tco6), which act upstream of the Ssk1 response regulator. Previously we have shown that Tco1 and Tco2 play redundant and distinct roles in sensing and responding to MAPKKK that abolishes Hog1 phosphorylation (6). According to previous studies, the Tco2Δ mutant in the H99 strain, mutation of the skn7Δ mutant is less than that in the H99 strain, exhibited reduced susceptibility to amphotericin B than the Tco2Δ mutant in the H99 strain, in which Hog1 is constitutively phosphorylated, albeit to a lesser extent than in the H99 strain, exhibited reduced susceptibility to amphotericin B than JEC21 (Fig. 3C). Similar to the H99 strain, mutation of the Ssk2 MAPKKK that abolishes Hog1 phosphorylation (6) increased amphotericin B sensitivity (6–8). To test this hypothesis, we examined amphotericin B sensitivity of other C. neoformans strains, such as JEC21 and B3501-A, showing differential Hog1 phosphorylation levels (6). In support of the second hypothesis, we examined amphotericin B sensitivity of other C. neoformans strains, such as JEC21 and B3501-A, showing differential Hog1 phosphorylation levels (6). In support of the second hypothesis, the JEC21 strain, in which Hog1 is not constitutively phosphorylated (6), was even more hypersensitive to amphotericin B than the ssk2Δ mutant in the H99 strain background (Fig. 3C). In the JEC21 strain background, mutations of the SSK2, PBS2, and HOG1 genes did not affect sensitivity to amphotericin B (Fig. 3C). In contrast, the B3501 strain, in which Hog1 is constitutively phosphorylated, albeit to a lesser extent than in the H99 strain, exhibited reduced susceptibility to amphotericin B than JEC21 (Fig. 3C). Similar to the H99 strain, mutation of the SSK2 MAPKKK that abolishes Hog1 phosphorylation (6) increased amphotericin B sensitivity (Fig. 3C). Taken together, these data strongly indicate that constitutively phosphorylated Hog1 represses the ergosterol biosynthetic pathway under normal conditions.

To further support this finding, we also examined the susceptibility of the mutants toazole drugs, including triazoles (fluconazole and itraconazole) and imidazole (ke-
FIG. 5. Inhibition of the HOG pathway affects expression levels of a number of cadmium-responsive genes and increases resistance to cadmium in *C. neoformans*. (A) Relative expression profiles of 71 putative cadmium-responsive genes in the hog1Δ, ssk1Δ, and skn7Δ mutants compared to the WT strain. Putative cadmium-responsive genes in *C. neoformans* listed here were selected from 1,697 genes described in Fig. 1 and exhibited...
toconazole), which inhibit the fungal cytochrome P450 en-
zyme 14α-demethylase and prevent conversion of lanosterol
to ergosterol. We had expected that the ssk1Δ and hog1Δ
mutants having increased expression of many ergosterol bio-
synthesis genes, particularly including ERG11, would show
higher resistance to azole compounds. The ssk1Δ, ssk2Δ,
pbs2Δ, and hog1Δ mutants all exhibited increased resistance to
fluconazole and ketoconazole but not to itraconazole (Fig. 4).
Interestingly, the skn7Δ mutant also showed higher resistance to
fluconazole and ketoconazole than the WT for unknown
reasons (Fig. 4). The fact that the skn7Δ mutant exhibited WT
levels of ERG11 expression (Fig. 2) strongly suggested that the
fluconazole resistance observed in the skn7Δ mutant is an
ERG11-independent phenomenon. Interestingly, none of the
hybrid sensor kinases was found to be differentially involved in
resistance to fluconazole and ketoconazole, further indicating
that differential responses of the HOG mutants to polyene and
azole drugs is not receptor or sensor mediated. In conclusion,
inactivation of the HOG pathway increases ergosterol content
by induction of ergosterol biosynthesis genes and therefore
congers synergistic effects with amphotericin B treatment but
antagonistic effects with fluconazole and ketoconazole.

The HOG pathway negatively modulates resistance to heavy
metal stress. Another key finding revealed by this array analy-
ysis is that a number of genes involved in cadmium sensitivity
were differentially regulated in the hog1Δ and ssk1Δ mutants
(Fig. 5A). Among the 1,697 genes exhibiting different expression
patterns in hog1Δ, ssk1Δ, or skn7Δ mutants, 71 genes were
orthologous to genes whose mutation increases sensitivity to
cadmium in either S. cerevisiae or S. pombe (Fig. 5A) (33, 45).
Previously it has been reported that perturbation of the HOG
pathway in C. albicans, Candida lusitaniae, and S. pombe in-
creases cadmium sensitivity (12, 19, 33). Among the 71 genes
identified in our array, however, half (36 genes) were indeed
induced more than 1.5-fold in either C. neoformans hog1Δ or
ssk1Δ mutants compared to the WT, while only 12 genes were
reduced more than 1.5-fold in the mutants (Fig. 5A). This
suggested the possibility that inhibition of the HOG pathway
could cause cadmium tolerance in C. neoformans by activating
transcription of cadmium-responsive genes. To address this
model, we have examined the cadmium sensitivity of the HOG
mutants in C. neoformans. Interestingly, the mutants of the
HOG pathway, including the ssk1Δ, ssk2Δ, pbs2Δ, and hog1Δ
mutants, showed higher resistance to cadmium sulfate than the
WT strain and the skn7Δ mutant (Fig. 5B). Among hybrid
sensor kinases, the tco2Δ mutant was also more resistant to
cadmium, albeit to a lesser extent than the HOG pathway
mutants, than the WT, indicating that Tco2 is involved in
cadmium sensitivity with a positive relationship with other
HOG signaling components, similar to the amphotericin B
susceptibility. With the exception of Tco2, none of the Tco
sensor kinases was involved in susceptibility to cadmium.
Taken together, the HOG pathway negatively regulates resis-
tance to heavy metal stress in C. neoformans.

ESR and CSR genes in C. neoformans. To investigate how
the HOG pathway controls stress responses against environ-
mental cues, genome-wide transcription patterns of the WT
and hog1Δ, ssk1Δ, and skn7Δ mutant strains were monitored in
response to osmotic shock, oxidative stress, and antifungal
drug treatment (fluconoxil). A total of 2,218 genes in the WT
were found to be more than twofold up- or downregulated at
any time point (30 or 60 min) in response to at least one of the
stress conditions (P < 0.05, ANOVA) and were named ESR
(environmental stress regulated) genes as described previously
(see Table S7 in the supplemental material) (20). Several in-
teresting observations emerged. First, global gene expression
patterns in response to H2O2 were clearly distinguishable from
those in response to osmotic stress and fluconoxil treatment
(Fig. 6A). Second, a much greater number of genes were dif-
ferentially regulated in response to H2O2 (1,719 genes) than
osmotic stress (580 genes) and fluconoxil treatment (510
genases). Only a small portion of genes (125 out of 2,218 genes;
5.6%) were found to be commonly regulated in response to all
stresses tested, while the majority (1,947 out of 2,218 genes;
87.8%) were stress-specifically regulated (SSR) at a twofold-
change cutoff (Fig. 6B). This implies that diverse signaling
regulators may work to respond to each environmental cue.

Among 2,218 ESR genes, 125 genes were found to be co-
drate regulated (48 genes) or downregulated (77 genes)
in response to all stresses and were named CSR (common
stress regulated) genes (Fig. 6C; and see Table S8 in the supple-
mental material). We also defined CSR extended (CSRE)
genases (394 genes) as those upregulated (179 genes) or down-
regulated (215 genes) in response to at least two stresses (Fig.
6B; and see Table S8 in the supplemental material). CSR and
CSRE genes cover groups of genes involved in diverse cellular
functions, indicating that the overall physiological status of C.
neoformans is reorganized to adapt to any external stress and
maintain normal cellular physiology (see Fig. S5 in the supple-
mental material). Furthermore, a significant proportion of
CSR genes seemed to be modulated by Hog1 and Skk1, but not
by Skn7 (Fig. 6C), indicating that the HOG signaling pathway
in conjunction with the two-component system is the major
controller of the common stress response in C. neoformans.

Upregulated CSR or CSRE genes were overrepresented
among those involved in inorganic ion transport and metabo-
ilism and secondary metabolite biosynthesis, transport, and
catabolism. Among downregulated CSR or CSRE genes, genes
involved in amino acid transport/metabolism and energy pro-
duction/conversion were most downregulated (9.9% each),
indicating that cells lower energy production during adaptation
to environmental stresses (see Fig. S5 in the supplemental

significant homology to S. cerevisiae and S. pombe cadmium-responsive genes by BLAST search (33, 45). The change (fold) is illustrated by color
(see the color bar scale), and the exact value for each gene was indicated in the table placed to the right side of the hierarchical clustering diagram.

(B) Each C. neoformans strain—including the WT (H99) and hog1Δ (YSB64), pbs2Δ (YSB123), ssk2Δ (YSB264), ssk1Δ (YSB261), skn7Δ
(YSB349), tco1Δ (YSB278), tco2Δ (YSB281), tco1Δ tco2Δ (YSB324), tco3Δ (YSB284), tco4Δ (YSB417), tco5Δ (YSB286), and tco7Δ (YSB348)
mutants—was grown overnight at 30°C in liquid YPD medium, 10-fold serially diluted (1 to 10^6 dilutions), and spotted (3 μl of dilution) onto YPD
agar containing the indicated concentrations of cadmium sulfate (CdSO₄). Cells were incubated at 30°C for 72 h and photographed.
FIG. 6. ESR and CSR genes in *C. neoformans*. (A) ESR genes in *C. neoformans*. The ESR genes were defined as genes for which expression was induced or repressed by more than a twofold change in at least one time point (30 and 60 min) under any one of the following stress conditions: 1 M NaCl (Os), 20 μg/ml fludioxonil (Fx), or 2.5 mM H$_2$O$_2$ (Ox). The change (fold) is illustrated by a color (see the color bar scale). The hierarchical clustering of the 2,218 ESR genes that were selected by ANOVA (*P* < 0.05) with GeneSpring software was demonstrated. (B) Venn diagram showing osmolarity-regulated (OsR), fludioxonil-regulated (FxR), and oxidative stress-regulated (OxR) genes. Genes displaying significant upregulation or downregulation (>2-fold) under each stress condition are included. (C) CSR genes in *C. neoformans*. The CSR genes were defined as genes for which expression was induced or repressed more than twofold in at least one time point under all three stress conditions. The change (fold) is illustrated by a color (see the color bar scale). Hierarchical clustering of the expression profiles of 125 CSR genes in the WT and *hog1Δ, ssk1Δ*, and *skn7Δ* mutants is illustrated.
Among upregulated CSR or CSRE genes, the ENA1 gene (00531) encoding a putative P-type ATPase sodium pump and the NHA1 (01678) gene encoding a Na\(^+\)/H\(^+\) antiporter were highly upregulated (more than threefold induction) in response to exposure to 1 M NaCl. In S. cerevisiae, Nha1 and Ena1 are required for an immediate and long-term adaptation, respectively, to high-salt conditions (43). Interestingly, however, our array data showed that expression of the C. neoformans ENA1 and NHA1 genes was also highly induced in response to H\(_2\)O\(_2\) in both WT and skn7\(^+/+\) mutants, but not in ssk1\(^+/+\) and hog1\(^+/+\) mutants, strongly suggesting that Ena1 plays a role in osmotic response under the glucose starvation condition. However, the ena1\(^+/+\) mutant was as resistant to H\(_2\)O\(_2\) as the WT (Fig. 7B), indicating Ena1 does not play a major role in oxidative stress response. Two ena1\(^+/+\) mutants independently constructed by the Madhani and Idnurm laboratories exhibited identical phenotypes (Fig. 7B).

Among other transporter genes, a gene (02455) showing the highest homology to the S. cerevisiae high-affinity choline/ethanolamine transporter Hnm1 was also upregulated in response to common stress (34). In contrast, genes involved in carbohydrate transport were significantly downregulated in response to common stresses (particularly for osmotic and fludioxonil treatment), including GAL2 (galactose permease), HXT5, HXT13, HXT5, and HXT17 (Fig. 7A; and see Table S8 in the supplemental material). Furthermore, the group of genes involved in iron transport and metabolism, including CFO1 and FRE2 (06821), was commonly upregulated, indicating that these genes also play important roles in adaptation to various other stresses besides maintaining iron homeostasis.

SSR genes in C. neoformans. As mentioned above, the majority of ESR genes were SSR in C. neoformans, suggesting that a unique set of stress defense genes is transcrip-
tionally regulated in a stress-specific manner (Fig. 8 and 9 and see Fig. 11).

Osmotic stress (NaCl SSR genes). A total of 1,641 genes were found to be differentially regulated under osmotic stress conditions (1 M NaCl) in WT (P < 0.05, ANOVA). Among these, 580 genes (283 upregulated, 299 downregulated, with 2 genes upregulated at one time point and downregulated at another time point) were transcriptionally regulated with more than a twofold change. Half of the genes (289 genes) were osmotic SSR genes (Fig. 8), named as OsSR genes, and listed in Table S9 in the supplemental material, while the other half were included in the CSR and CSRE genes as described above.

Among the upregulated OsSR genes, genes involved in transport and metabolism of various metabolites, including amino acids, nucleotides, coenzymes, inorganic ions, and secondary metabolites, were most notably overrepresented (see Fig. S6 in the supplemental material), indicating that transporter and permease genes may play a role in counteracting external osmotic changes by transporting diverse osmolytes. These include DUR3 (07448; plasma membrane transporter).
for both urea and polyamines, MEP2/AMT2 (04758; ammonium permease), STL1 (01683; glycerol symporter), AQY1 (01742; aquaporin water channel), PHO84 (02777; high-affinity inorganic phosphate transporter), and QDR1 (02050; multidrug transporter of the major facilitator superfamily), which all belong to the group of genes showing the highest induction among OsSR genes (see Table S9 in the supplemental material). The downregulated OsSR genes include the following categories of genes, such as cytoskeleton, signal transduction mechanisms, and intracellular trafficking/secretion/vesicular transport (see Fig. S6 in the supplemental material).

Generally, expression profiles of the OsR genes were greatly affected by mutation of either the SSK1 or HOG1 gene (Fig. 8). Among them, the four clusters indicated in Fig. 8 were notable, although the functions for a majority of the genes are unknown. In cluster II, where upregulated OsR genes were notably downregulated by mutation of the SSK1 and HOG1 genes, AQY1 is evident. In S. cerevisiae, aquaporin (Aqy1) is required for prolonged survival under rapid changes in osmolarity (13). Therefore, it is likely that C. neoformans induces expression of the AQY1 gene upon exposure to high osmotic conditions to maintain intracellular water balance. Basal and induced expression levels of AQY1 were more than 10-fold decreased in both sklΔ and hog1Δ mutants.

**Fludioxonil stress (fludioxonil SSR genes).** C. neoformans undergoes genome-wide remodeling of transcriptional profiles by fludioxonil treatment in a similar pattern to osmotic stress (Fig. 6A). A total of 1,215 genes were found to be differentially regulatedunder fludioxonil treatment in the WT (ANOVA; P < 0.05). Among them, 210 genes (240 upregulated and 272 downregulated, with 2 genes that were upregulated at one time point and downregulated at another time point) were transcriptionally regulated with more than twofold changes (see Table S10 in the supplemental material). Also similar to NaCl stress, 37.8% of genes (193 genes) were fludioxonil SSR (Fig. 9A) and named “FxSR genes.”

Among upregulated FxSR genes, groups of genes involved in posttranslational modification, protein turnover, and lipid transport and metabolism were overrepresented (see Fig. S7 in the supplemental material). Furthermore, similar to OsSR genes, a group of genes involved in the secondary metabolite biosynthesis, transport, and metabolism were notably overrepresented in the FxSR genes. The most notable groups of genes overrepresented in downregulated FxSR genes include those involved in transport and metabolism of carbohydrates, nucleotides, lipid, and some secondary metabolites (see Fig. 7 in the supplemental material).

Among the upregulated FxSR genes, several genes encoding putative membrane ATP binding cassette (ABC) transporters were most evident. In S. cerevisiae, the ABC-type multidrug transporters, including Pdr5, Pdr15, Snaq2, and Yor1l, play a critical role in cellular detoxification and pleiotropic drug resistance (PDR) (48). Our array data clearly showed that PDR5/15 (00869, 04098, and 06348; here named PDR5, PDR5-2, and PDR5-3, respectively), YOR1 (03503), and SNO1 (06338) homologues were highly upregulated (up to 57-fold changes for PDR5) specifically upon exposure to fludioxonil treatment, indicating that these proteins may enhance efflux of fludioxonil (see Table S10 in the supplemental material). More interestingly, expression of these genes was even more upregulated (up to 164-fold changes for PDR5) by mutation of the HOG1 gene, which may also explain the resistance of the hog1Δ mutant to the drug treatment.

To address the role of ABC multidrug transporters, we have monitored the drug sensitivity of pdr5Δ, pdr5-2Δ, pdr5-3Δ, and yor1Δ mutants to various stress and drug treatments (Fig. 10A). All of these mutants showed WT levels of sensitivity against various stresses, such as osmotic and salt shock, UV irradiation, oxidative stress, and cadmium stress, indicating that these ABC multidrug transporters are not involved in general stress response. However, the pdr5Δ mutant, but not other pdr5-2Δ, pdr5-3Δ, and yor1Δ mutants, exhibited slightly increased sensitivity to fludioxonil and fluconazole compared to the WT, indicating that Pdr5 may be involved in efflux of antifungal drug for detoxification in agreement with our microarray data showing striking expression-level changes of PDR5 during fludioxonil exposure. Other Pdr5 homologues and Yor1 may play redundant roles in drug efflux, and therefore single mutations may not generate any discernible phenotypes. Since C. neoformans contains a number of Pdr5- or Pdr15-like ABC efflux pumps in the genome, multiple deletions of the ABC efflux pump genes may generate more readily discernible phenotypes.

Expression profiles for almost half of the FxSR genes were perturbed by mutation of HOG1 and SSK1 (Fig. 9A). Some of the upregulated FxSR genes (indicated as clusters I and II in Fig. 9) were clearly downregulated in either sklΔ or hog1Δ mutants. In contrast, some of the downregulated FxSR genes were upregulated in the HOG mutants (indicated as cluster IV in Fig. 9). Interestingly, the PKA1 gene (00396), encoding a cyclic AMP (cAMP)-dependent protein kinase A (PKA) catalytic subunit, was found to be upregulated in response to fludioxonil in a HOG-dependent manner (Fig. 9A; and see Table S10 in the supplemental material). To address whether the cAMP/PKA signaling pathway is involved in fludioxonil sensitivity, we measured the fludioxonil sensitivity of various cAMP/PKA mutants in C. neoformans (Fig. 9B). The pka1Δ mutant and other cAMP mutants (the gpa1Δ, cac1Δ, pka2Δ, and pka1Δ/pka2Δ mutants), however, did not show any differential sensitivity to fludioxonil, indicating that the cAMP pathway is not directly involved in adaptation to fludioxonil. In contrast, the acalΔ mutant was more sensitive to fludioxonil than the WT strain (Fig. 9B), suggesting that Acal is involved in response to fludioxonil independent of the cAMP pathway.

**Oxidative-stress (H2O2 SSR genes).** C. neoformans remodels genome-wide expression profiles in response to H2O2 in much more unique and dramatic patterns than in response to osmotic shock and fludioxonil treatment (Fig. 6A). First, the number of H2O2-regulated genes is much greater. A total of 2,700 genes were found to be differentially regulated in response to H2O2 exposure in the WT (P < 0.05, ANOVA). Among them, 1,719 genes (864 upregulated and 861 downregulated, with 5 genes that were both up- or downregulated depending on time points) were more than twofold regulated in at least one time point (Fig. 11A; and see Table S11 in the supplemental material). Second, a greater number of stress-specific genes were found in response to H2O2. Notably, 84.9% of genes (1,459 genes out of 1,719 genes) were named OxSR (oxidative stress specifically regulated) genes, indicating that C.
C. neoformans uniquely remodels genome-wide expression profiles in response to oxidative stress. The following categories of genes were overrepresented in upregulated OxSR genes: signal transduction, inorganic ion transport and metabolism, posttranslational modification, transcription, and amino acid transport and metabolism (see Fig. S8 in the supplemental material). Expectedly, genes encoding putative or known oxidative defense proteins were highly upregulated. These include \textit{TRR1} (05847; cytoplasmic thioredoxin reductase, 23- to 60-fold induction), \textit{TSA1} (03482; thioredoxin peroxidase, 8- to 18-fold induction), \textit{CPP1} (7- to 11-fold induction), \textit{GRX3} (02950; glutathione-dependent oxidoreductase, 2-fold induction), and \textit{GPX2} (02503; phospholipid hydroperoxide glutathione peroxidase, 2.6-fold induction). Induction of \textit{TRR1}, \textit{TSA1}, \textit{CPP1}, and \textit{GPX2} was dependent upon Skn7, Ssk1, and Hog1, further corroborating that both Skn7- and Ssk1-Hog1 signaling pathways are involved in oxidative stress response.

Among genes involved in posttranslational modification and protein turnover, a number of genes encoding ubiquitin-conjugating enzymes were notable, including \textit{UBI4} (01920; ubiquitin, 6.0-fold), \textit{UBC4} (05696 and 01084; ubiquitin-conjugating enzyme, 2.4- to 5.1-fold), \textit{UBC6} (02214 and 05765; ubiquitin-conjugating enzyme, 19-fold and 2.5-fold, respectively), \textit{UBC7} (06592; ubiquitin-conjugating enzyme, 2.1-fold), and \textit{UBC8} (04611; ubiquitin-conjugating enzyme, 7.2-fold). (Note that 05765, named \textit{Ubc6}, shows much higher

**Fig. 9.** Fludioxonil stress-specific response genes in \textit{C. neoformans}. (A) Hierarchical clustering of the expression profiles of fludioxonil stress-specific response (FxSR) genes in the WT and \textit{hog1Δ} (YSB64) mutant; the control H99 WT strain CMO18 (WT-M); and the \textit{ubc6-2Δ} (02214), \textit{ubc8Δ} (04611), \textit{pdr5Δ} (00869), \textit{pdr5-2Δ} (04098), \textit{pdr5-3Δ} (06348), and \textit{yor1Δ} (03503) mutants—was grown overnight at 30°C in liquid YPD medium, 10-fold serially diluted (1 to 10⁴ dilutions), and spotted (3 μl of dilution) onto YPD agar containing the indicated concentrations of H₂O₂, CdSO₄, fludioxonil, amphotericin B (AmpB), and fluconazole. To measure osmotic stress response, YP agar medium containing either NaCl or KCl was used. UV sensitivity was measured as described in Materials and Methods. Cells were incubated at 30°C for 72 h and photographed.

**Fig. 10.** Role of multidrug efflux pump genes and ubiquitin-conjugating enzymes in stress response of \textit{C. neoformans}. (A and B) Each \textit{C. neoformans} strain—including the WT (H99) and \textit{hog1Δ} (YSB64) mutant; the control H99 WT strain CMO18 (WT-M); and the \textit{ubc6-2Δ} (02214), \textit{ubc8Δ} (04611), \textit{pdr5Δ} (00869), \textit{pdr5-2Δ} (04098), \textit{pdr5-3Δ} (06348), and \textit{yor1Δ} (03503) mutants—was grown overnight at 30°C in liquid YPD medium, 10-fold serially diluted (1 to 10⁴ dilutions), and spotted (3 μl of dilution) onto YPD agar containing the indicated concentrations of H₂O₂, CdSO₄, fludioxonil, amphotericin B (AmpB), and fluconazole. To measure osmotic stress response, YP agar medium containing either NaCl or KCl was used. UV sensitivity was measured as described in Materials and Methods. Cells were incubated at 30°C for 72 h and photographed.
FIG. 11. Oxidative stress-specific response genes in C. neoformans. (A) Hierarchical clustering of the expression profiles of the oxidative stress-specific response (OxSR) gene group in the WT and hog1Δ, ssk1Δ, and skn7Δ mutants is illustrated. The right side of the diagram indicates groups of genes whose expression is regulated by the HOG pathway and have orthologs in either S. cerevisiae or S. pombe. CoA, coenzyme A. (B) Each graph illustrate induction or repression levels of SCH9 in our array analysis in the WT (H99; ○) and skn7Δ (●), ssk1Δ (□), and hog1Δ

| Serotype A | S.cer | S.pom | Description |
|-----------|-------|-------|-------------|
| CAGN_0067 | D2P2 | rnt1 | conserved hypothetical protein |
| CAGN_0871 | UV55 | succ | UV-endonuclease UVE-1 |
| CAGN_01744 | OCH1 | α-1,3-mannosyltransferase |
| CAGN_01412 | PTH1 | rad51 | nuclear elongation and deformation protein 1 |
| CAGN_08533 | ADP1 | rad52 | ATP-dependent permease |
| CAGN_05811 | PER4 | swa1 | endopeptidase |
| CAGN_08502 | PGR8 | ipl4 | serine-type endopeptidase |
| CAGN_07903 | RGP3 | RhoGTP |
| CAGN_08328 | RAO2 | rad51 | single-stranded DNA-specific endodeoxyribonuclease |
| CAGN_04461 | FAA1 | rad52 | long-chain-fatty-acid-CoA ligase (162m0253) |
| CAGN_04485 | FAA1 | rad52 | long-chain-fatty-acid-CoA ligase (162m0409) |
| CAGN_02312 | TGF3 | rad52 | palmitoleoyl-phosphatidylcholine domain-containing protein |
| CAGN_02357 | MOK2 | per1 | STE/STE7 protein kinase |
| CAGN_04354 | PPY1 | azoxysporphamide |
| CAGN_06889 | SncyT | zf-C3H4 C3H4 type zinc finger |
| CAGN_02662 | ATG4 | pep7 | peptidase family C45 protein |
| CAGN_02601 | VPY7 | par7 | nkt GTPhex activator |
| CAGN_06762 | MNN2 | par7 | conserved hypothetical protein |
| CAGN_02034 | ATG28 | vdgl | UDP-glucose starch glucosyltransferase |
| CAGN_06732 | ATG2 | alg2 | conserved hypothetical protein |
| CAGN_04206 | MUP105 | alg2 | conserved hypothetical protein |
| CAGN_05015 | CTT1 | cif1 | catalase 4 |
| CAGN_03635 | FLR1 | cif1 | SCF1 protein |
| CAGN_02375 | GCP1 | cif1 | adh1 keto reductase |
| CAGN_06840 | PRC1 | cif1 | carboxypeptidase C |
| CAGN_02186 | GCA1 | cif1 | 6-phosphogluconolactonase |
| CAGN_03557 | TCA2 | cif1 | transcription initiation factor l small chain |
| CAGN_01186 | JYN1 | cif1 | pumili domain-containing protein c |
| CAGN_02805 | VCK1 | cif1 | calcium/hydrogen antiporter |

Clusters:
- Cluster 1 contains genes involved in amino acid synthesis.
- Cluster 2 contains genes involved in carbohydrate metabolism.
- Cluster 3 contains genes involved in lipid metabolism.
- Cluster 4 contains genes involved in cofactor metabolism.

Gene Ontology:
- OxSR genes are involved in oxidative stress response.

Gene Expression:
- WT (H99):
  - Control:
  - YP (- glucose):
  - YP + 1M NaCl:
  - YP + 1M KCl:
- hog1Δ:
  - Control:
  - YP (- glucose):
  - YP + 1M NaCl:
  - YP + 1M KCl:
- ssk1Δ:
  - Control:
  - YP (- glucose):
  - YP + 1M NaCl:
  - YP + 1M KCl:
- skn7Δ:
  - Control:
  - YP (- glucose):
  - YP + 1M NaCl:
  - YP + 1M KCl:

Gene Knockout:
- WT (H99)
  - Control:
  - YP (- glucose):
  - YP + 1M NaCl:
  - YP + 1M KCl:
  - Fludioxonil 2.5 µg/mL:
  - Fludioxonil 3 µg/mL:
  - Fludioxonil 5 µg/mL:
  - Fludioxonil 7.5 µg/mL:
  - Fludioxonil 10 µg/mL:
- hog1Δ
  - Control:
  - YP (- glucose):
  - YP + 1M NaCl:
  - YP + 1M KCl:
  - Fludioxonil 2.5 µg/mL:
  - Fludioxonil 3 µg/mL:
  - Fludioxonil 5 µg/mL:
  - Fludioxonil 7.5 µg/mL:
  - Fludioxonil 10 µg/mL:
- ssk1Δ
  - Control:
  - YP (- glucose):
  - YP + 1M NaCl:
  - YP + 1M KCl:
  - Fludioxonil 2.5 µg/mL:
  - Fludioxonil 3 µg/mL:
  - Fludioxonil 5 µg/mL:
  - Fludioxonil 7.5 µg/mL:
  - Fludioxonil 10 µg/mL:
- skn7Δ
  - Control:
  - YP (- glucose):
  - YP + 1M NaCl:
  - YP + 1M KCl:
  - Fludioxonil 2.5 µg/mL:
  - Fludioxonil 3 µg/mL:
  - Fludioxonil 5 µg/mL:
  - Fludioxonil 7.5 µg/mL:
  - Fludioxonil 10 µg/mL:
homology to *S. cerevisiae* Ubc6 than 02214, named Ubc6-2.) A recent study shows that ubiquitin-conjugating systems required for protein degradation are one of the four group of genes that are commonly induced in response to oxidative stress in eukaryotic organisms, including humans, plants, and fission and budding yeasts (46). In fact, the *ubc4*Δ mutant exhibits hypersensitivity to H$_2$O$_2$ in *S. cerevisiae* (21). Furthermore, the ubiquitin-proteasome system negatively regulates the two-component system by selective degradation of Ssk1 in *S. cerevisiae* (44).

To address any involvement of the ubiquitin-dependent system in stress responses in *C. neoformans*, we monitored stress sensitivity of strains having mutation in genes encoding two ubiquitin-conjugating enzymes, including *UBC6*-2 and *UBC8*, since they showed greatest induction in response to oxidative stress (19.2- and 7.2-fold induction) (Fig. 10B). Our results demonstrated that the ubiquitin-proteasome system is involved in diverse stress responses. Although the *ubc6-2*Δ mutant did not exhibit any increased stress sensitivity to osmotic and oxidative stress, it showed slightly increased sensitivity to cadmium and fluoroconil. Interestingly, the *ubc6-2*Δ mutant showed increased sensitivity to amphotericin B but increased resistance to fluconazole, similar to the *hog1*Δ mutant, although to a lesser extent, indicating that *ubc6-2*Δ may be involved in ergosterol biosynthesis. In contrast, the *ubc8*Δ mutants show WT levels of susceptibility to most general stresses and antifungal drugs. Interestingly, however, the *ubc8*Δ mutant is hypersensitive to H$_2$O$_2$ compared to the WT strain, indicating that Ubc8 appeared to be involved in oxidative stress response. These results indicated that the ubiquitin-dependent system appears to be involved in certain stress response of *C. neoformans* by employing different components of the Ubc proteins.

Two categories of genes were overrepresented in downregulated OsR genes. One group of genes is involved in translation, ribosomal structure, and biogenesis and the other is involved in energy production and conversion. Particularly the former was most notable (23.5% versus 5.7% random occurrence) (see Fig. S8 in the supplemental material). A number of ribosomal component genes were significantly downregulated upon exposure to H$_2$O$_2$, including more than 90 ribosomal protein genes (see Table S11 in the supplemental material). However, the repression of ribosomal protein genes was not observed in the *hog1*Δ mutant, indicating that Hog1 MAPK is involved in ribosome biosynthesis. Previous genome-wide transcriptome analysis of *S. cerevisiae* and *S. pombe* also demonstrated that groups of ribosome biosynthesis genes are significantly downregulated in response to oxidative stresses (H$_2$O$_2$ or menadione) (14, 20, 25), indicating that inhibition of protein synthesis in response to oxidative stress is a general phenomenon in fungi.

Among the OxSR genes, a significant number of genes appear to be Hog1 dependent, as indicated as clusters I to VI in Fig. 11. Interestingly, genes in clusters I, II, V, and VI were differentially regulated in the *hog1*Δ mutant, but not in the *ssk1*Δ mutant, further indicating that Ssk1 is not the only upstream regulator of the Hog1 MAPK particularly in oxidative stress response. Genes in OxSR clusters III and IV are both Ssk1- and Hog1-dependent genes. Interestingly, the Sch9 protein kinase (06301) in OxSR cluster III, whose expression is induced only in response to oxidative stress, was differentially regulated in the *hog1*Δ and *ssk1*Δ mutant compared to the WT (Fig. 11B). In *S. cerevisiae*, Sch9 kinase plays an important role in adaptation to osmotic and oxidative stresses by being recruited to promoters of osmotress-responsive genes through physical interaction with the Sko1 transcription factor and Hog1 MAPK (41). Although a Sko1-like transcription factor appears to be absent in *C. neoformans*, it is still possible that the Sch9 kinase could be required for adaptation to osmotic and oxidative stresses of *C. neoformans* in association with Hog1 and/or other unknown transcription factors. To address this possibility, we have tested the stress susceptibility of the *sch9*Δ mutant in *C. neoformans* (Fig. 11C). The *sch9*Δ mutant exhibited hypersensitivity to oxidative stress response compared to the WT, similar to the HOG mutants (Fig. 11C). However, Sch9 kinase appeared to be controlled by multiple signaling pathways besides the HOG pathway due to the following reasons. First, the *sch9*Δ mutant was as resistant to UV as the WT. Second, the *sch9*Δ mutant was more hypersensitive to fluoroconil and cadmium than the WT, which is in stark contrast to the *hog1*Δ mutant showing resistance to both agents. Third, Sch9 was not involved in susceptibility to amphotericin B and fluconazole, unlike the HOG pathway mutants (Fig. 11C). Fourth, the *sch9*Δ mutant showed hypersensitivity to sodium salt (Na$^+$), but not to potassium salt (K$^+$), whereas the *hog1*Δ mutant showed hypersensitivity to both salts. Taken together, Sch9 is involved in regulation of a subset of HOG-dependent phenotypes.

**Comparison of stress-regulated genes between fungal species.** Finally, we have compared stress-regulated genes of *C. neoformans* with those of other pathogenic (*C. albicans*) and nonpathogenic (*S. cerevisiae* and *S. pombe*) fungi as described in Materials and Methods. We did not find any gene whose expression is commonly upregulated or downregulated under hyperosmotic conditions (OsR genes) in all four fungi (see Fig. S9A and Table S12 in the supplemental material). However, four *C. neoformans* OsR genes, including *PRM10, STL1, ENA1,* and *ALD5*, were also differentially regulated in at least two other fungal species, implying that these genes could play an evolutionarily conserved role in adaptation to osmotic stress.

In contrast to the OsR genes, a greater number of oxidative

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(□) mutants, upon osmotic shock (Os), fluoroconil treatment (Fx), and oxidative stress (Ox). (C) Each *C. neoformans* strain—including the WT (H99) and *ssk1*Δ (YSB251) and *hog1*Δ (YSB64) mutants; the control H99 WT strain CMO18 (WT-M); and the *sch9*Δ mutant—was grown overnight at 30°C in liquid YPD medium, 10-fold serially diluted (1 to 10$^4$ dilutions), and spotted (3 μl of dilution) onto YPD agar containing the indicated concentrations of fluoroconil, hydrogen peroxide, amphotericin B (AmpB), and fluconazole. To measure osmotic stress response, YP agar media containing either NaCl or KCl were used. UV sensitivity was measured as described in Materials and Methods. Cells were incubated at 30°C for 72 h and photographed.
stress-regulated (OxR) genes were commonly regulated between all four fungal species (see Fig. S9B in the supplemental material). Among these, 13 *C. neoformans* OxR genes were also differentially regulated in all other fungi. These include upregulated OxR genes, such as *FLR1, GPX2, RAD16, TSA1, ISU1, UBC8, and TRR1*, and downregulated OxR genes, such as *RL11, UTP22, RPC40, FEN1, RPS7B*, and *UTP18*. Furthermore, 152 (50 upregulated and 102 downregulated) *C. neoformans* OxR genes were also differentially regulated in at least two other fungi, indicating that regulatory mechanisms are much more shared between fungi for oxidative stress response than for osmotic stress response.

When CSR genes (oxidative and osmotic stresses) were compared between fungi, almost none of the CSR genes were commonly found in at least three out of four fungi, indicating that each fungal species contains diverse stress response and defense systems.

**DISCUSSION**

The major goals of this study were to characterize the genome-wide transcriptional remodeling patterns in the human pathogen *C. neoformans* in response to diverse environmental stresses and to elucidate the downstream network of the two-component system and HOG signaling pathway during regulation of normal growth and stress responses of *C. neoformans*. Through this study, we have identified novel target genes controlled by the HOG pathway and also discovered a number of unique characteristics of the HOG signaling pathway in *C. neoformans*, which were not apparent in our previous studies (5–8), as summarized in Fig. S10 in the supplemental material.

Generally summarizing our array data, *C. neoformans* expresses not only a group of genes commonly responding to diverse environmental stresses, such as osmotic shock, oxidative stress, and antifungal agents, but also a subset of genes specifically modulated by each stress named the “SSR genes.” Particularly, the remodeling of global gene expression profiles was found to be mainly controlled by the Hog1 MAPK and Skk1 response regulator, but not by the Skn7 response regulator, further corroborating that the Skk1-dependent Hog1 MAPK signaling pathways play central roles in stress responses. Furthermore, the Ssk1-Hog1 signaling pathway not only controls stress-induced responses but also plays important roles in maintaining a normal cellular homeostasis under unstressed conditions. A number of genes were differentially regulated by mutation of the SSK1 and HOG1 genes, but not SKN7, even under unstressed growth conditions. Under both unstressed and stressed conditions, transcriptome profiles of the hog1Δ mutant were much more similar to those of the ssk1Δ mutant than the skn7Δ mutant, further confirming that Hog1 is mostly in the linear pathway with the Ssk1 response regulator, but not with the Skn7 response regulator. However, it should be noted that a number of genes were found to be either Hog1 specific or Ssk1 specific, revealing that Hog1 and Ssk1 are not absolutely interdependent.

 Among a number of novel discoveries made in this study, the findings that most of ergosterol biosynthesis genes were upregulated and the actual ergosterol content was increased by mutation of the HOG pathway were the most striking and unexpected results since these phenomena have not been observed in other fungal species reported thus far. Comparative DNA microarray analysis recently performed in *C. albicans* by Enjabert and coworkers revealed that the expression levels of ergosterol biosynthesis genes are indeed generally decreased in the hog1Δ mutant compared to the WT (23). Particularly, levels of expression of the ERG11 and ERG1 genes were 1.7- and 1.9-fold decreased, respectively, compared to that of the WT (23). In agreement with this result, the *C. albicans* hog1Δ mutant does not show any synergistic effects with most known antifungal drugs (1). In *C. neoformans*, however, our study clearly demonstrated that ERG11 expression levels were enhanced in both hog1Δ and ssk1Δ mutants, but not in the skn7Δ mutant, explaining why the HOG pathway mutants were highly resistant to fluconazole and ketoconazole but hypersensitive to amphotericin B. In contrast, azole drug resistance observed in the skn7Δ mutant appears to be unrelated to the ergosterol biosynthesis since the skn7Δ mutant showed WT levels of ERG11 expression and amphotericin B susceptibility. It is probable that drug efflux and/or influx systems may be altered in the *C. neoformans* skn7Δ mutant, as exemplified by other azole-resistant fungal strains (40).

Our discovery provides a novel antifungal therapeutic method against cryptococcosis as follows: treatment of patients by combining amphotericin B and a HOG inhibitor followed by combination therapy with azole drugs and a HOG activator. Our data strongly implicate that potent inhibitors of the HOG pathway, especially the Ssk1 response regulator or Tco2 hybrid sensor kinase, whose orthologs are not observed in humans, will have strong synergistic effects with amphotericin B to treat cryptococcosis. Our study could provide a strong case for supporting the value of genome-wide transcriptome analysis using microarray analysis by directly providing an approach for development of novel therapeutic method.

Among genes differentially regulated by the HOG pathway under normal conditions, a group of 71 genes involved in cadmium resistance were notable since involvement of the HOG pathway in heavy metal stress had not been addressed before in *C. neoformans*. Heavy metals, such as cadmium, affect various aspects of cellular responses, including cell cycle regulation, growth, differentiation, apoptosis, and oxidative stress response (11, 26). Recently a number of cadmium-responsive genes have been identified in both *S. cerevisiae* and *S. pombe* (33, 45). The discovery that the ssk1Δ and hog1Δ mutants exhibit increased resistance to cadmium compared to the WT and skn7Δ mutants is a somewhat unexpected result based on findings in other fungi. In *S. pombe*, the spec1Δ (Hog1 homolog), wis4Δ (Ssk22 homolog), and mcs4Δ (Ssk1 homolog) mutants all show hypersensitivity to both cadmium and hydrogen peroxide (33). In *C. albicans*, the hog1Δ mutant does not show any significant hypersensitivity to cadmium compared to the WT (2).

This study provides further insights into the downstream network of the HOG pathway for regulation of virulence factor production and sexual differentiation of *C. neoformans*. It has been reported that the CAP10, CAP59, CAP60, and CAP64 genes were essential for capsule biosynthesis in *C. neoformans*, although their biochemical properties remain to be elucidated (15–18). For melanin production, two laccase genes, *LAC1* and *LAC2*, were found to exist in *C. neoformans*. Between these, Lac1 is the predominant laccase since deletion of the *LAC1*
gene alone, but not the LAC2 gene, abolishes melanin production in C. neoformans (49). Our array data demonstrated that all four of the capsule synthesis genes and the LAC1 gene were upregulated in the ssk1Δ and hog1Δ mutants, indicating that these genes are directly or indirectly regulated by the HOG pathway. In the skn7Δ mutant, only the LAC1 gene, but not the capsule genes, was upregulated, further corroborating that Skn7 is negatively involved in melanin, but not capsule production (7). Our array data showing upregulation of SXX1 and GPA2 by the hog1Δ and ssk1Δ mutations may provide a possible answer for the previous question of how Hog1 and Skn1 negatively regulate pheromone production and sexual reproduction (7, 8). It has been recently reported that C. neoformans Gpa2 physically interacts with Ste3α, Gpb1, and Crg1 and therefore promotes the pheromone response MAPK pathway for mating (29, 37). As expected, overexpression of dominant active GPA2Δ203L strikingly activates pheromone expression and mating (29). Therefore, our array data strongly indicate that Hog1 represses the Gpa2-mediated pheromone response pathway under normal conditions, and inactivation of the HOG pathway drastically induces GPA2 expression, which subsequently increases pheromone production and mating.

Induction of the ENA1 gene in response to osmotic stress is somewhat expected based on studies performed in other fungi. The osmoadaptation mechanism has been well characterized in S. cerevisiae. Immediately after osmotic shock, Hog1 is directly recruited to and interacts with the Nha1 Na+/H+ antiporter and the Tok1 potassium channel (to a lesser extent) to rapidly counteract increased ion concentrations in the nucleus and restore the ability of most DNA binding proteins to reassociate with the chromatin (43). After the immediate adaptation to high-salt conditions, Hog1 induces the Ena1 Na+ extrusion pump for a longer-term adaptation to high-salt conditions (43). Our phenotypic analysis of the ena1Δ mutant demonstrated that Ena1 is required for conflerring resistance to osmotic stress, particularly under carbon starvation conditions (Fig. 7B). Recently, Idnurm et al. identified ENA1 as a major virulence gene via signature-tagged insertional mutagenesis (31). Interestingly, the ena1Δ mutant exhibited increased sensitivity to high pH, indicating that Ena1 is required for counterbalancing the decreased H+ concentration in the environment (31). It is not known if Hog1 is similarly recruited to an Nhal antipporter and Tok1 potassium channel for an immediate salt adaptation of C. neoformans at this point. Interestingly, however, NHA1 appears to be transcriptionally induced by osmotic stress dependent on the HOG pathway (see Table S9 in the supplemental material), which is rather unexpected since activation of Nhal is not dependent on transcriptional activation by Hog1 but depends on a physical interaction with Hog1 in S. cerevisiae (42). The detailed mechanism of molecular interaction between Nhal and Hog1 remains to be elucidated.

Our array study revealed novel features of the C. neoformans Sch9 protein kinase previously reported by Wang et al. (47). The prior study demonstrated that the sch9Δ mutant has increased capsule production and thermostolerance and defective mating capability (47). Regardless of the enhanced capsulation and thermostolerance that could increase pathogenicity of C. neoformans, the sch9Δ mutant is attenuated in virulence (47). Our array data and biological analysis of the sch9Δ mutant may provide an answer for its reduced virulence. The sch9Δ mutant was found to be hypersensitive to both oxidative and osmotic stress (Fig. 11B), indicating that it is unlikely to survive in the hostile host environment and would be more susceptible to host defense mechanisms. Interestingly, both basal and induced expression levels of SCH9 were significantly decreased in ssk1Δ and hog1Δ mutants, indicating that Sch9 is one of the target kinases modulated by the HOG pathway in C. neoformans. In fact, Wang et al. previously proposed that Sch9 is mainly independent of the cAMP signaling pathway, which is another major signaling pathway controlling capsule production, mating, and virulence of C. neoformans. It is possible that increased capsule production of the hog1Δ and ssk1Δ mutants (6, 8) may also result from decreased expression of SCH9 under normal conditions. The functional correlation between Sch9 and the HOG pathway has been suggested in S. cerevisiae, where mutation of the SCH9 gene also increased susceptibility to osmotic and oxidative stresses (41).

A final important discovery of our transcriptome analysis is the potential implication of the ubiquitin-proteasome system in regulation of stress responses, which was first suggested in C. neoformans. In S. cerevisiae, the pheromone-responsive MAPK pathway is tightly controlled by ubiquitin-dependent Ste11 degradation during pheromone induction (24). Furthermore, the S. cerevisiae two-component system is negatively regulated through targeted degradation of the Ssk1 response regulator by Ubc7/Qri8, an endoplasmic reticulum (ER)-associated ubiquitin-conjugating enzyme (44). Ubiquitination for targeted protein degradation by the proteasome is mediated by three classes of enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2 or Ubc), and ubiquitin-protein ligases (E3). The ubiquitin-proteasome system is involved in endoplasmic reticulum associated protein degradation (ERAD), which contributes to selective removal of misfolded proteins, or unassembled subunits of multimeric complexes. Therefore, it is conceivable that external stress, such as oxidative damage, may increase the number of misfolded or damaged proteins inside the cell, and this accumulation could be prevented by activation of the ubiquitin-proteasome system. Our study shows that the putative ubiquitin system in C. neoformans is involved not only in stress response, but also in defending against antifungal drugs (Fig. 10B). However, functions of different components of the ubiquitin-proteasome system in stress responses and their potential connection with the HOG pathway remain to be further elucidated in future studies.

In conclusion, our study highlights the importance of genome-wide comparative transcriptome analysis in human fungal pathogens for not only elucidating previously undiscovered features and target genes of the two-component system and HOG pathway but also directly suggesting a novel therapeutic approach for effective treatment of cryptococcosis. A number of features and target genes for the stress-activated two-component system and HOG pathway identified by our analysis are coincident with those obtained from other fungi, and yet several novel features uncovered by our study further confirm the unique specialization of the HOG pathway in C. neoformans. Further exploitation of the molecular mechanism between signaling components, the downstream network, and feedback regulatory mechanisms of the HOG pathway in C. neoformans.
will provide an unprecedented opportunity to develop a novel anticytomicocele therapy.

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