Cryptococcus neoformans Histone Acetyltransferase Gcn5 Regulates Fungal Adaptation to the Host

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Cryptococcus neoformans is an environmental fungus and an opportunistic human pathogen. Previous studies have demonstrated major alterations in its transcriptional profile as this microorganism enters the hostile environment of the human host. To assess the role of chromatin remodeling in host-induced transcriptional responses, we identified the C. neoformans Gcn5 histone acetyltransferase and demonstrated its function by complementation studies of Saccharomyces cerevisiae. The C. neoformans gcn5Δ mutant strain has deficits in high-temperature growth and capsule attachment to the cell surface, in addition to increased sensitivity to FK506 and oxidative stress. Treatment of wild-type cells with the histone acetyltransferase inhibitor garcinol mimics cellular effects of the gcn5Δ mutation. Gcn5 regulates the expression of many genes that are important in responding to the specific environmental conditions encountered by C. neoformans inside the host. Accordingly, the gcn5Δ mutant is avirulent in animal models of cryptococcosis. Our study demonstrates the importance of chromatin remodeling by the conserved histone acetyltransferase Gcn5 in regulating the expression of specific genes that allow C. neoformans to respond appropriately to the human host.

Microorganisms must rapidly induce and repress various transcriptional networks in order to adapt to the stressful conditions of the infected host. One aspect of this rapid transcriptional response is the ability to remodel chromatin, allowing transcription factors access to the promoters of important stress response genes. Acetylation of specific lysine residues of histone proteins is one mechanism for chromatin remodeling that leads to altered transcription (62). Acetylated histones are preferentially associated with regions of active transcription, and they are thought to recruit the assembly of transcriptional complexes (11, 19, 31). Mutation of histone proteins to prevent acetylation results in defects in transcription and cell growth (32, 39, 58, 68). In eukaryotes, histone proteins are acetylated by the Gcn5 protein as part of larger, multisubunit, chromatin remodeling complexes (11, 19, 31, 55).

These large histone acetyltransferase complexes are recruited to specific gene promoters to modify local chromatin structure and thus regulate transcription. In the budding yeast Saccharomyces cerevisiae, the Spt3-Ada2-Gcn5 (SAGA) complex is involved in global transcriptional regulation. However, further studies of SAGA function, through mapping the localization of the complex to specific loci, have shown that binding is enriched at particular stress-responsive genes. In S. cerevisiae, Gcn5 is involved in regulating transcriptional responses to common environmental stresses, such as high temperature, oxidative damage, high osmolarity, and nutrient deprivation. In the fission yeast Schizosaccharomyces pombe, Gcn5 is involved in a narrower group of stress responses and plays an important role in resistance to salt. Microarray studies of both of these species revealed that acetylation by the SAGA complex is able to remodel the chromatin to transcriptionally regulate genes important for responding to environmental stresses and that these genes tend to be coordinately regulated (33).

Cryptococcus neoformans is an opportunistic human fungal pathogen that causes meningoencephalitis in immunocompromised hosts. Inside the host, C. neoformans lives extracellularly as well as within phagocytic cells. One way in which it responds to these varied stresses is by inducing important virulence factors such as the antioxidant melanin and antiphagocytic polysaccharide capsule (1, 18, 69). It also adapts to grow under conditions of high temperature, oxidative stress, high concentrations of salt, human physiological pH, and high levels of CO₂. Previous work with C. neoformans determined that the Gpa1/cyclic AMP (cAMP) and Hog1/mitogen-activated protein kinase (MAPK) pathways, among others, are important in regulating responses to these common stresses by leading to the transcriptional activation of stress-related genes (35). However, the role of chromatin remodeling in the transcriptional response of C. neoformans to stress is still unknown. Therefore, in this work we examine the function of C. neoformans Gcn5 (CnGcn5) in adaptation to the host environment, paying particular attention to its role in transcriptional regulation of host stress response genes. We demonstrate that histone acetyltransferase activity is necessary for the C. neoformans response to specific conditions associated with the host environment, and it is therefore essential for virulence.

MATERIALS AND METHODS

Strains and media. C. neoformans and S. cerevisiae strains used in this study are listed in Table S2 in the supplemental material. All C. neoformans mutants were created in the H99 background unless otherwise stated. Strains were maintained on rich medium (1% yeast extract, 2% peptone, 2% dextrose [YPD]), and selective media contained either nourseothricin (100 mg/liter; Werner Bio-
Agents, Jena-Cospeda, Germany) or neomycin (G418) (200 mg/liter; Clontech, Takara-Bio Inc.). Niger seed medium was made as previously described (37). Stress media were created by adding 44.1 g calcium chloride, 8.4 g lithium chloride, 111.8 g potassium chloride, 9.71 g caffiene, 0.1 g SDS, or 87.6 g sodium chloride to 1 liter of rich medium before autoclaving.

**Molecular biology techniques.** Standard techniques for Southern hybridization were performed as described previously (56). *C. neoformans* genomic DNA for Southern blot analysis was prepared using cetyltrimethylammonium bromide (CTAB) phenol-chloroform extraction as described previously (50).

**Gene disruption.** The wild-type *GCN5* allele was mutated by precisely replacing the entire open reading frame with a neomycin resistance (Nat) resistance allele (40). The gcn5::nat resistance allele was created by using PCR overlap extension and biolistically transformed into the H99 strain, using previously described methods (23, 40, 60). Several independent gcn5Δ mutant colonies had identical phenotypes in vitro and showed deletion of the gene by PCR. The TOC1 strain was chosen for further study, and Southern blot analysis confirmed deletion of the *GCN5* allele.

To reconstitute the *gcn5*Δ mutant strain with the wild-type allele, the *GCN5* locus was amplified from H99 genomic DNA and biolistically cotransformed into the *gcn5*Δ strain with a separately amplified neomycin resistance allele to create strain TOC3, as previously described (28). The wild-type *GCN5* allele was amplified with primers 5′-TGCAGGTGAACCTTA-5′ and 5′-GCTGAGGATCCA-3′, and then cloned into the pCN50 plasmid to create a GFP::*GCN5* fusion protein under the constitutive histone H3 promoter (46). pTO1 was then biolistically transformed into the TOC1 *gcn5*Δ mutant strain background to create strain TOC9.

**Microscopy.** Bright-field (transmitted light), differential interference contrast (DIC) microscopy, and fluorescent images were captured with a Zeiss Axio Imager.A1 fluorescent microscope equipped with an AxioCam mrm digital camera. To visualize capsule, cells were grown under inducing conditions and then stained with India ink on glass slides. Images were collected at a magnification of ×63. To visualize GFP and 4′,6-diamidino-2-phenylindole (DAPI), cells were incubated in ice-cold methanol for 1 min, washed three times in phosphate-buffered saline (PBS), and stained with DAPI before images were collected at a magnification of ×63, using a 488-nm wavelength for GFP fluorescence and a 350-nm wavelength for DAPI.

**RNA and cDNA preparation.** Strains were incubated to mid-logarithmic phase in 5 ml of YPD and then washed three times with sterile water before being incubated in DMEM for 3 h. Cells were washed three times before centrifugation, freezing on dry ice, and lysis. RNA preparation was performed as previously described, using the constitutive *GPD1* gene to normalize the samples (46).

**Microarray and data analysis.** The microarray used in this study was developed by the Cryptococcus Community Microarray Consortium with financial support from the National Institutes of Health and the Burroughs Wellcome Fund (http://microarray.wustl.edu/services/microarray/cryptococcus_neoforms). RNA labeling and hybridization were performed by the Duke University Microarray Core Facility according to their standard protocols for custom spotted arrays (16, 46). Data were analyzed using JMP genomics v. 4.1 (SAS Institute, Cary, NC), and initial background subtraction was performed. We used analysis of variance (ANOVA) normalization and false discovery rate (FDR) analysis to calculate differences between treatment effects for pairs of inducing conditions. Genes were considered for further evaluation if they had log2-transformed fold changes greater than 2-fold with a P value of <0.001 (16, 64). The .gal file for the Cryptococcus version 2 microarray contains probes specific for serotype A and D strains of *C. neoformans*. Cryptococcus genome annotation for probes specific for serotype D strains JEC21 and B5351 was obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Cryptococcus genome annotation for probes specific for serotype A strain H99 was obtained from the Broad Institute (http://www.broadinstitute.org/). Chromosome coordinates for individual *C. neoformans* serotype D probes are genome Build 2 Version 1 specific. The .gal file is provided as a .txt file in a tab-delimited format (see Table S3 in the supplemental material).

**Virology and data analysis.** The virulence of the *C. neoformans* strains was assessed using a murine inhalation model of cryptococcosis as described previously (55). Ten female A/et mice were inoculated intranasally with 106 CFU of *C. neoformans* cells of the wild-type (H99), *gcn5*Δ mutant (TOC1), or *gcn5Δ*::G418 complemented strain (TOC3). Mice were monitored daily for signs of infection and were sacrificed at predetermined clinical endpoints predicting mortality. The statistical significance between the survival curves of all animals infected with each strain was evaluated using the log rank test (survival analysis). All studies were performed in compliance with the institutional guidelines for animal experimentation.

**RESULTS**

**Identification of the *C. neoformans* homologue of S. cerevisiae Gcn5.** In a search of the *C. neoformans* genome for transcriptional regulators that may be involved in the response to host stresses, we identified the *C. neoformans* homologue of the *S. cerevisiae* acetyltransferase protein Gcn5 (gene identification [ID] CNAG_00375). Compared with *S. cerevisiae*, the *C. neoformans* Gcn5 protein has a conserved histone acetyltransferase domain (71% identity) and a conserved bromodomain (53% identity) (Fig. 1A). The bromodomain of Gcn5 is thought to be involved in the interactions between the acetyltransferase and the histone, and it is required for stabilizing the
association between other components of the SAGA complex and the promoters of target genes (9, 59). Of the 19 predicted \(C. \) neoformans proteins containing an acetyltransferase domain, only this putative Gcn5 orthologue also contains a bromodomain. Comparison of the \(C. \) neoformans protein with \(C. \) albicans Gcn5 showed similar sequence conservation for these two domains. Further in silico analysis revealed that the \(C. \) neoformans genome also contains predicted orthologues for the majority of the other elements of the SAGA complex, including the major components Spt3 and Ada2p (Table 1). Notably, it is missing a clear orthologue of Spt20, which is essential for the structural integrity of the SAGA complex in \(S. \) cerevisiae. ClustalW alignment showed that the \(S. \) cerevisiae and \(C. \) neoformans orthologues of Gcn5 have the highest degree of sequence similarity among all of the SAGA complex proteins.

**\(C. \) neoformans Gcn5 complements an \(S. \) cerevisiae histone acetyltransferase mutant.** Despite conserved functional domains, the CnGcn5 protein has a predicted extended N-terminal region that is quite divergent from the \(S. \) cerevisiae Gcn5 protein (27% identity). To determine if CnGcn5 is a functional Gcn5 orthologue, we generated a transgenic \(S. \) cerevisiae \(gcn5\Delta \) (\(Scgcn5\Delta \)) strain expressing CnGcn5. The \(CnGcn5 \) cDNA was expressed under the \(S. \) cerevisiae \(GAL7 \) inducible promoter, using the \(p\)YES yeast expression vector. The \(S. \) cerevisiae \(gcn5\Delta \) mutant strain was constructed by deleting the entire gene coding region with the nourseothricin resistance marker. Deletion of the gene was confirmed by both PCR and Southern blot analysis. To ensure that all mutant phenotypes observed were due to deletion of \(Gcn5 \), we complemented the strain by integrating the wild-type allele into the \(gcn5\Delta \) mutant strain, creating \(gcn5\Delta + Gcn5 \) strain TOC3. In this strain, all mutant phenotypes were complemented.

To determine if the \(C. \) neoformans Gcn5 protein controls responses to stresses that are relevant for pathogenesis, we examined growth of the mutant strain under various host stress conditions. One of the major stresses that \(C. \) neoformans encounters in the host is high temperature, and strains with defects in high-temperature growth are generally avirulent (49). When incubated on rich medium at 37°C, the \(gcn5\Delta \) strain had a significant delay in growth compared to the wild type (Fig. 2A). In contrast, the \(C. \) neoformans \(gcn5\Delta \) strain had transgenic strain expressing CnGcn5 was able to grow well at 25°C after 2 days, unlike the \(Scgcn5\Delta \) mutant strain. At 30°C, expression of \(CnGcn5 \) also supported growth of this strain after 3 days. The transgenic strain failed to grow at 30°C on glucose-containing medium, in which \(CnGcn5 \) expression from the \(GAL7 \) promoter was repressed (Fig. 1B). This established a conserved function between \(CnGcn5 \) and \(ScGcn5 \), suggesting that CnGcn5 is also a histone acetyltransferase.

**\(Gcn5 \) is involved in stress tolerance.** The \(S. \) cerevisiae and \(S. \) pombe \(gcn5\Delta \) mutants display defects in the cellular response to many stresses, including elevated temperatures, high salt concentrations, and nutrient deprivation. The effect of Gcn5 on the cell response to stress is linked to its activity as a histone acetyltransferase and transcriptional regulator. The SAGA complex is recruited by activator proteins such as Gcn4 to the promoters of stress response genes, which leads to the transcriptional induction of these genes (33, 66). During osmotic stress, Gcn4 has been shown to bind to the promoter of \(HAL1 \), a regulator of the ion transporter \(Ena1 \), and is necessary for the induction of \(HAL1 \) gene expression and subsequent salt/osmotic tolerance (47).

To investigate the role of Gcn5 in \(C. \) neoformans, we created the \(gcn5\Delta \) strain TOC1 in which we replaced the entire gene coding region with the nourseothricin resistance marker. Deletion of the gene was confirmed by both PCR and Southern blot analysis. To ensure that all mutant phenotypes observed were due to deletion of \(Gcn5 \), we complemented the strain by integrating the wild-type allele into the \(gcn5\Delta \) mutant strain, creating \(gcn5\Delta + Gcn5 \) strain TOC3. In this strain, all mutant phenotypes were complemented.

| \(S. \) cerevisiae protein | \(C. \) neoformans gene ID | % Identity |
|--------------------------|--------------------------|-----------|
| Gcn5                     | CNAG_00375               | 44        |
| Spt3                     | CNAG_05290               | 34        |
| Tra1                     | CNAG_07377               | 29        |
| Spt7                     | CNAG_03850               | 16        |
| Ada3                     | CNAG_04674               | 18        |
| Taf5                     | CNAG_05428               | 27        |
| Spt8                     | CNAG_06597               | 30        |
| Spt20                    | No homologue             |           |
| Taf12                    | CNAG_06861               | 19        |
| Taf6                     | CNAG_02536               | 19        |
| Ada1                     | No homologue             |           |
| Ada2                     | CNAG_01626               | 37        |
| Spt29                    | CNAG_06392               | 16        |
| Taf10                    | CNAG_01972               | 21        |
| Taf9                     | CNAG_07565               | 29        |

**FIG. 1.** Identification of \(C. \) neoformans histone acetyltransferase Gcn5. (A) Alignment of the Gcn5 proteins of \(S. \) cerevisiae, \(C. \) albicans, and \(C. \) neoformans shows highest homology in the acetyltransferase domain and the bromodomain. The \(C. \) neoformans protein has a diverged N-terminal region. (B) \(C. \) neoformans Gcn5 complements the \(S. \) cerevisiae \(gcn5\Delta \) mutant. The \(CnGcn5 \) cDNA was cloned into the \(p\)YES yeast expression vector under the \(GAL1 \) promoter and transformed into the \(S. \) cerevisiae \(gcn5\Delta \) mutant strain. Cells containing the \(CnGcn5 \) gene or an empty vector were incubated on inducing (galactose) or noninducing (glucose) medium and incubated at 25°C or 30°C.
in order to determine whether Gcn5 function is regulated by localization, we created a GFP-Gcn5 fusion protein and examined localization under inducing and noninducing conditions. We fused the green fluorescent protein gene to the N terminus of the GCN5 gene and expressed it under the control of a constitutive histone promoter. We introduced this plasmid (pTO1) by biolistic transformation into the gcn5Δ mutant strain to create the gcn5Δ+gfp-GCN5 strain TOC5. We showed that the fusion protein was functional by complementation of temperature sensitivity and other gcn5Δ phenotypes (data not shown). Using epifluorescent microscopy, we observed a nuclear pattern of localization after growth for 24 h in YPD both at 30°C and under high-temperature stress at 37°C (Fig. 2C). We also observed nuclear localization after induction in DMEM (data not shown). Therefore, in contrast to some other pathogens, the CnGcn5 localization appears to be constitutively nuclear.

Gcn5 regulates capsule in *C. neoformans*. Upon entering the infected host, *C. neoformans* induces a large polysaccharide capsule. This structure protects the fungal cells in many ways, including inhibiting phagocytic cell function and activating complement distant from the cell surface (14, 54, 61). To determine whether Gcn5 is involved in regulating this host-specific phenotype, we incubated wild-type and gcn5Δ mutant cells in Dulbecco’s modified Eagle’s medium (DMEM) at 30°C as an inducing condition for polysaccharide capsule. To observe the capsule size, we stained the cell background with India ink and examined the zone of exclusion around the cells. Microscopic analysis of the cells established that the gcn5Δ mutant strain demonstrated a significantly decreased capsule size compared to that of the wild type (Fig. 3A). When the cells were incubated in DMEM at 37°C and 5% CO2 to more accurately mimic human physiological conditions, the gcn5Δ mutant strain did not display visible capsule by India ink exclusion. This capsule defect is not due to the previously demonstrated growth defects at 37°C, as the gcn5Δ strain eventually grows to saturation at this temperature, albeit at a lower rate. The gcn5Δ mutant displayed markedly decreased capsular phenotype even when incubated to saturation. Additionally, it also failed to produce capsule when incubated in either low-iron medium or 10% Sabouraud medium, two distinct growth conditions that also induce capsule in wild-type strains (data not shown).

Recent work has shown that certain mutants that do not demonstrate surface capsule may still secrete wild-type levels of exopolysaccharide, presumably due to defects in capsule attachment instead of capsule secretion or biosynthesis (46). Therefore, the gcn5Δ acapsular phenotype was further explored by testing the culture filtrate for secreted exopolysaccharide. This allowed us to differentiate between defects in capsule production, secretion, and attachment. Strains were incubated to equal cell densities in DMEM for 1 week at 30°C to induce capsule, after which the medium was heated to denature enzymes and filtered to remove cellular debris and to isolate secreted capsular polysaccharides. The filtrate was separated by electrophoresis on a 0.6% agarose gel, transferred to a nylon membrane, and immunoblotted with an anti-GXM capsular antibody (monoclonal antibody [MAb] 18b7) (65). Compared to the wild type, the gcn5Δ mutant strain secreted capsular polysaccharide with a distinct pattern of electro-
systems from yeasts to humans, histone acetyltransferase activity can be inhibited by specific drugs. We examined two drugs, garcinol and anacardic acid, for their ability to mimic the gcn5Δ mutation. We primarily examined the effect of garcinol, a polyisoprenylated benzophenone derivative, with documented histone acetyltransferase activity inhibition (6). Addition of garcinol to wild-type C. neoformans cells phenocopied the temperature sensitivity of the gcn5Δ mutation. After 24 h of incubation, addition of 0.5 μM garcinol to wild-type cells caused a statistically significant growth defect at 37°C and not at 30°C. At 5 μM, growth at 37°C was completely inhibited (Fig. 5A). We further investigated garcinol-induced temperature sensitivity by performing a growth curve with and without garcinol at 37°C for both the wild-type strain and the gcn5Δ mutant strain. Addition of 0.8 μM garcinol caused a severe defect in growth for the wild-type strain. Again, after 24 h, there was no statistically significant difference in growth between the garcinol-treated cells and the gcn5Δ mutant cells (Fig. 5B). However, treatment of the gcn5Δ mutant cells with 0.8 μM garcinol led to death of the strain after 24 h. This suggests that garcinol has off-target effects in addition to Gcn5 inhibition. Therefore, we examined the effect of a second histone acetyltransferase inhibitor, anacardic acid. Similar to the effect of garcinol, treatment with 10 μM anacardic acid induced temperature sensitivity in the wild-type strain (see Materials and Methods; also, data not shown).

We also tested whether compounds with antifungal activity displayed additive effects with Gcn5 inhibition. The gcn5Δ strain was more sensitive than the wild type to the antifungal effects of FK506, even at 30°C (Fig. 5C). FK506 acts by binding to FKBP12 and actively inhibiting the calcineurin pathway (44). FK506 has maximum efficacy at 37°C, as blocking the calcineurin pathway leads to abolishment of growth at elevated temperatures (44, 45). This increased sensitivity to drug was not due to a general defect in drug resistance, as the gcn5Δ mutant strain and the wild type had similar MICs against the antifungal drug fluconazole at 30°C.

Gcn5 regulates the expression of genes involved in host stress response. Phenotypic analysis demonstrated that the C. neoformans gcn5Δ mutant is defective in responding to several host-related stresses, including elevated temperature, oxidative

FIG. 4. Virulence analysis of the gcn5Δ mutant strain. A/Jcr mice were inoculated intranasally with 5 × 10⁵ cryptococcal cells and monitored for survival.

FIG. 3. Gcn5 is required for capsule attachment to the cell. (A) gcn5Δ cells have a capsule defect. Cells were incubated in Dulbecco’s modified Eagle’s medium at 30°C for 24 h. Capsule was assessed by staining with India ink and visualizing the zone of exclusion (magnification, ×63; bar, 10 μm). (B) The gcn5Δ mutant strain is able to secrete polysaccharide. Electrophoretic mobilities and quantities of shed polysaccharide from 3 C. neoformans strains were assessed by an immunoblotting technique of culture medium filtrate, using an anti-GXM antibody to probe for capsule as previously described. Cells were incubated in DMEM for 1 week at 30°C before being filtered. The arrow indicates the direction of electrophoresis. The cap59Δ mutant is included as a negative control as this strain secretes no discernible capsular polysaccharide.

A histone acetyltransferase inhibitor phenocopies the gcn5Δ mutant strain for temperature sensitivity. In a wide range of
stress, and capsule-inducing conditions. We hypothesized that this is due to an inability to regulate adaptive transcriptional responses through chromatin remodeling. Without Gcn5, it is likely that the strain is unable to appropriately acetylate histones, and thus, the strain is unable to recruit the transcriptional complexes needed to induce gene expression. Therefore, to determine which processes are transcriptionally regulated by Gcn5, we performed comparative transcriptional profiling between the wild type and the gcn5Δ mutant strain. Both the wild-type and the gcn5Δ mutant strains were incubated for 3 h in DMEM at 37°C to mimic host conditions, so that all differences in gene expression can be attributed to the mutation of Gcn5. The RNA was purified and hybridized against a C. neoformans serotype A/D genome microarray, which uses 7,738 70-mer probes to represent all predicted genes in the C. neoformans genome. Our results indicate that 417 putative genes in this growth condition are significantly differentially regulated in the gcn5Δ mutant strain (Table 2; see also Table S1 in the supplemental material).

We examined the transcriptional profiling data to identify genes that may offer mechanisms for the observed phenotypic defects in the gcn5Δ mutant strain. When examining mechanisms for the capsule defect, we found that Gcn5 regulates the expression of multiple glucosidases, including Kre61, which are involved in cell wall biosynthesis and capsule architecture (24, 25). Reverse transcription-PCR (RT-PCR) results confirmed a 6-fold-decreased expression of Kre61 in the gcn5Δ strain compared to the wild type (data not shown). β-Glucan synthases also had lower expression in the mutant strain, suggesting that alterations in cell wall structure may occur in the gcn5Δ mutant strain, with resulting defective capsule attachment.

Two categories of genes that demonstrated increased transcription in the wild-type strain compared to the gcn5Δ mutant strain (potential Gcn5-dependent induction) were those encoding transcription factors and kinases. Therefore, in addition to directly regulating gene expression by histone acetylation, Gcn5 may also indirectly regulate gene expression and protein activity. In contrast, several mitochondrial and ribosomal genes showed decreased expression in the wild type, suggesting that Gcn5 normally acts to repress these genes.

Two putative catalase A proteins demonstrated decreased expression in the gcn5Δ mutant strain, perhaps leading to the increased sensitivity of the gcn5Δ mutant to hydrogen peroxide. In addition, FKBP12, the binding target of the drug FK506, showed increased expression in the gcn5Δ mutant compared to that in the wild type, perhaps resulting in increased sensitivity to FK506 since FK506-FKBP12 binding is the mechanism of this drug’s toxicity to the cell.

**DISCUSSION**

Our results indicate that Gcn5 plays a critical role in C. neoformans adaptation to the host. In many organisms, Gcn5 orthologues serve as histone acetyltransferases within large, multisubunit chromatin remodeling complexes, such as the SAGA complex. In S. cerevisiae, the SAGA complex is important in global transcriptional activation and in regulating the expression of highly inducible stress response genes (33). This complex is directed to the promoters of target genes by activators such as Gcn4p, Snf1p, and Gal4p. Once at the promoters, the histone acetylation of H3 and H2B allows transcriptional activation of these target genes (29, 34, 41). We have found putative homologues for the majority of the components of the yeast SAGA complex in the C. neoformans genome, with two exceptions: Spt20 and Ada1 were not readily identifiable by sequence homology. Of those genes present, the Gcn5 protein showed the most sequence identity, with the acetyltransferase and bromodomains sharing 71% and 53% identity, respectively. C. neoformans has a unique extended N-terminal domain. This sequence divergence did not prevent the CnGcn5 protein from complementing the S. cerevisiae gcn5Δ mutant defects in high-temperature growth, thus establishing a conserved histone acetyltransferase activity for CnGcn5. Although the N termini of other Gcn5 orthologues determine regulated subcellular localization, we have demonstrated that CnGcn5 appears to be constitutively localized to the nucleus.
\textit{C. neoformans} exists in a niche very different from that of \textit{S. cerevisiae}. As both an environmental fungus and an opportunistic pathogen, \textit{C. neoformans} must be able to respond to a wide variety of stresses. Inside a human host, the pathogen is subjected to high CO\textsubscript{2}, high temperatures, low iron, nutrient starvation, and osmotic stress. As a facultative intracellular pathogen, it also has to survive inside the macrophage, where it is subjected to the acidity of the phagolysosome and the oxidative bursts of the host cells. In response to the host, \textit{C. neoformans} induces the virulence factors melanin and polysaccharide capsule, and it additionally initiates multiple signaling pathways for adapting to host stress. The Hog1/MAPK pathway uses a two-component system to sense extracellular signals and regulate expression of genes involved in osmotic, antifungal, and heat stresses, among others (2, 3, 4, 35). The cAMP pathway uses G-protein-coupled receptors to sense the host environment and to activate several transcription factors, such as Nrg1 and Rim101, to regulate stress gene expression and production of capsule and melanin (1, 16, 46, 51). Additionally, the Ras and calcineurin signaling pathways are required for adaptation to high-temperature growth (7, 36, 43, 45). This demonstrates that this pathogenic microorganism uses numerous pathways to regulate adaptation to the host. However, the role of chromatin remodeling in \textit{C. neoformans} response to the host was previously unknown.

In other pathogens, the elements of the SAGA complex have recently been implicated in regulating pathways that are essential for virulence. Sellam et al. demonstrated that defects in the Ada2 protein in \textit{Candida albicans} lead to poor protein folding, hypersensitivity to oxidative stress and antifungals, and defects in virulence (57). In addition, although the Spt3 protein is required for pseudohyphal and invasive growth in \textit{S. cerevisiae}, \textit{spt3}/H9004 mutants of \textit{C. albicans} lead to poor protein synthesis in animal models of candidiasis, confirming the importance of reversible yeast-hyphal transitions in this fungal pathogen and showing the involvement of the SAGA complex regulation of gene expression in \textit{C. albicans} virulence (38). These data demonstrate that the gene targets of conserved histone acetyltransferase complexes may differ according to the environmental niche, but the overall importance of gene regulation by histone acetylation is maintained in very divergent species. Other pathogens, such as the malaria parasite \textit{Plasmodium falciparum}, also maintain homologues of the SAGA complex. Moreover, treatment with histone acetyltransferase inhibitors causes changes in global gene expression and parasite development.

### Table 2. Representative genes regulated by Gcn5 that may be involved in host response

| Category and gene ID | Annotation | Fold change (wild-type strain\textasciitilde\textit{gcn5}\Delta strain) |
|----------------------|------------|---------------------------------------------------------------|
| **Capsule**          |            |                                                               |
| CND06160             | Glucosidase, β-glucan synthesis-associated protein Kre61 | 8.27 |
| CND00120             | Mannosyltransferase, putative                           | 4.21 |
| CN03890              | Glycogen debranching enzyme, putative                    | 2.43 |
| CNE2490              | Glycoside hydrolase, cytoplasm protein                    | 2.38 |
| CNE2630              | Glucan 1,4-α-glucosidase, putative                       | 2.21 |
| CNA07240             | CAP64 gene product, related                              | 2.18 |
| CND04970             | Glycosyltransferase family 31                           | 2.07 |
| CNH03390             | α-α-Trehalose-phosphate synthase (UDP forming)           | 2.05 |
| CNC01640             | Glucosidase, putative                                    | 2.03 |
| CNAG\_01341          | Mannose-6-phosphate isomerase                            | 2.05 |
| CND04560             | Mannose 6-phosphate isomerase                            | 2.16 |
| CNAG\_04574          | Dolichol phosphate-mannose biosynthesis                   | –4.44 |
| **Oxidative stress and drug sensitivity** |            |                                                               |
| CNL06340             | Catalase A                                              | 2.58 |
| CNK00780             | Oxidoreductase                                          | 2.22 |
| CNA05600             | Catalase A, putative                                    | 2.19 |
| CNB01800             | Macrolide-binding protein FKBP12                        | –2.92 |
| CNF03740             | Protein kinase TOR1                                     | 2.73 |
| **Transcription factors and signaling** |            |                                                               |
| CNC03220             | Transcription factor                                     | 7.58 |
| CNAG\_01611          | Regulator of G-protein signaling                        | 6.98 |
| CNAG\_03346          | Prb protein, putative transcription factor               | 3.3  |
| **Other**            |            |                                                               |
| CNAG\_00456          | Na-K-Cl transporter, putative                            | 7.12 |
| CNA05130             | Transporter EA1                                         | 2.12 |
| CNAG\_01455          | Ribosomal L39 protein                                   | –5.04 |
| CNA05600             | Ribosomal S30 superfamily                               | –3.63 |
| CNE01150             | NADH-ubiquinone oxidoreductase 12-kDa subunit, mitochondrial precursor, putative | –4.59 |
| CNAG02760            | Mitochondrial import inner membrane translocase subunit Tim13, putative | –4.29 |

*This list contains representative functionally annotated genes whose expression was at least 2-fold different between wild-type and \textit{gcn5}\Δ mutant strains after induction in DMEM for 3 h, with a \textit{P} value of <0.05. Annotations were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov), the Broad Institute (http://www.broadinstitute.org), and BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST), with additional hand editing.*
indicating that chromatin remodeling as a mechanism for responding to the host environment is not limited to fungal pathogens (17, 20).

Given these prior results, we hypothesized that chromatin remodeling and transcriptional regulation via Gcn5-mediated histone acetyltransferase activity would be an important way for C. neoformans to rapidly adapt to host conditions. In this study, we show that C. neoformans Gen5 regulates processes required for survival within a human host, such as growth at 37°C, resistance to ROS, and capsule attachment to the cell wall (10, 21, 26, 43, 44, 45). In light of the capsule and host stress response defects, the gen5Δ mutant strain had an expected attenuation in virulence in the mouse inhalation model of cryptococcosis. We also demonstrated that histone acetyltransferase inhibitors are able to result in temperature sensitivity of C. neoformans, similar to mutation of the GCN5 gene.

To better understand the mechanism underlying gen5Δ mutant phenotypes, we examined the genes that were differentially transcribed in the mutant strain compared to the wild type after incubation under in vitro conditions that are similar to those encountered within the infected host. We found that two putative catalase A genes had decreased expression in the gen5Δ mutant strain. Previous studies indicated that overexpression of C. neoformans catalase genes increases resistance to hydrogen peroxide, confirming their role in protection from oxidative stress. However, none of the catalase gene mutations, either singly or in combination, had significant effects on virulence (27). Giles et al. (27) concluded that this was most likely due to redundancy in oxidative stress resistance from the presence of other oxidative stress response pathways. Other studies have also shown that capsule is also important in regulating resistance to oxidative stress, presumably by acting as a buffer against the reactive oxygen species (67). In liquid culture, shed wild-type capsule acts to protect cells from killing by hydrogen peroxide. Therefore, a combination of alterations in capsule attachment, capsule structure, and catalase gene expression may explain the increased sensitivity to oxidative stress in the gen5Δ mutant strain.

Our phenotypic analysis also revealed gen5Δ mutant defects in polysaccharide attachment to the cell wall. When we examined shed polysaccharide content, we determined that the gen5Δ mutant strain produces and secretes capsule, but India ink staining demonstrated that the secreted polysaccharide is not being maintained at the cell wall. This is similar to defects previously documented in the rim101Δ mutant strain, where the cell is unable to anchor the capsule (46). Previous studies on the capsule have implicated cell wall biosynthesis as important in capsule attachment to the cell. A monoclonal antibody against β-glucan inhibits capsule formation around the normally encapsulated wild-type C. neoformans (52). This effect is presumably caused by preventing capsule attachment to the cell wall, although interference with capsular export could not be excluded. The α-glucan synthase enzyme activity, which is important in developing cell wall structure, is also required for C. neoformans capsule attachment to the cell surface (53). Therefore, we examined the transcriptional profiling data for cell wall biosynthesis genes that may be involved in capsule attachment. Our microarray data indicated that several β-glucosidases were differentially expressed in the gen5Δ mutant compared to the wild type. Kre61, a member of the Skn1/Kre6 family, had 8.2-fold-higher expression in the wild type than in the mutant strain, and it is one of the most highly differentially regulated genes. β-Glucosidases have previously been shown to be important in cell wall biosynthesis, capsular architecture, and virulence (24, 25), although Kre61 had no major defect as a single mutant. We also observed Gen5-dependent expression of other putative glucosidases and β-glucan synthases that may also be involved in cell wall biosynthesis and capsule attachment.

When examining our microarray data for other genes involved in host response, we determined that Tor1 and a putative calcineurin A subunit had differential expression in the wild type compared to the gen5Δ mutant strain. We observed a 2.2-fold decrease in a putative calcineurin A gene expression in the gen5Δ strain, which may explain some of the phenotypes that we observed. The calcineurin pathway activates the transcription of genes necessary for growth at high temperatures, high concentrations of carbon dioxide or salt, and growth in alkaline pH (22, 36, 45). We also observed a 2.9-fold increase in the expression of FKBP12 in the gen5Δ mutant strain compared to the wild type. The drug FK506 targets FKBP12, and the accumulation of this complex inhibits the activity of calcineurin (45). An increase in FKBP12 expression is likely to cause the increased sensitivity to FK506 that we observed in the gen5Δ mutant strain.

We also demonstrated that Gcn5 is important in negatively regulating a large number of genes involved in mitochondrial and ribosome processes. This is likely due to acetylation and transcriptional activation of repressors. During host stresses, such as high temperature, reactive oxygen species, or nutrient deprivation, a common cell response is to repress metabolism and protein biosynthesis. Previous work on C. neoformans gene expression during macrophage infection showed that this condition represses 38 known components of the translation machinery (21). In the absence of Gcn5, C. neoformans is unable to repress the expression of many mitochondrial and ribosomal genes. Multiple ribosomal subunits, NADH-ubiquinone oxidase proteins, and cytochrome c oxidase proteins, among others, showed increased expression in the mutant strain relative to the wild-type response to inducing conditions.

In summary, CnGcn5 is able to transcriptionally regulate many genes through chromatin remodeling by acting as a histone acetyltransferase. This highly conserved protein function has been coopted by numerous organisms to regulate the expression of distinct gene sets required for survival in unique microenvironments. Among microbial pathogens, divergent species use histone modification to control the expression of those phenotypes required for survival within their particular host environment. Therefore, histone acetyltransferases may be useful antimicrobial drug targets.

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51. Pukkila-Worley, R., Q. D. Gerrald, P. R. Kraus, M.-J. Boily, M. J. Davis, S. S. Giles, G. M. Cox, J. Heitman, and J. A. Alspaugh. 2005. Transcriptional network of multiple capsule and melanin genes governed by the Cryptococcus neoformans cyclic AMP cascade. Eukaryot. Cell 4:190–201.

52. Rachini, A., D. Pietrella, P. Luco, A. Torosantucci, P. Chiani, C. Bromuro, C. Proietti, F. Bistoni, A. Cassone, and A. Vecchiarelli. 2007. An anti-β-glucan monoclonal antibody inhibits growth and capsule formation of Cryptococcus neoformans in vitro and exerts therapeutic, anticytotoxic activity in vivo. Infect. Immun. 75:5085–5094.

53. Reese, A. J., A. Yoneda, J. A. Breger, A. B. H. Liu, C. L. Griffith, I. Bose, M.-J. Kim, C. Skau, S. Yang, J. A. Sefko, M. Osumi, J.-P. Latge, E. Mylonakis, and T. L. Doering. 2007. Loss of cell wall alpha(1-3) glucan affects Cryptococcus neoformans from ultrastructure to virulence. Mol. Microbiol. 63:1385–1398.

54. Retini, C., A. Vecchiarelli, C. Monari, F. Bistoni, and T. R. Kozel. 1998. Encapsulation of Cryptococcus neoformans with glucuronoxylomannan inhibits the antigen-presenting capacity of monocytes. Infect. Immun. 66:664–669.

55. Roberts, S. M., and F. Winston. 1997. Essential functional interactions of SAGA, a Saccharomyces cerevisiae complex of Spt, Ada, and Gcn5 proteins, with the Sfn/Swi and Srb/Mediator complexes. Genetics 147:451–465.

56. Sambrook J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

57. Sellam, A., C. Askew, E. Epp, H. Lavoie, M. Whiteway, and A. Nantel. 2009. Genome-wide mapping of the coactivator Ada2p yields insight into the functional roles of SAGA/ADA complex in Candida albicans. Mol. Biol. Cell 20:2389–2400.

58. Smith, M. M. 1991. Histone structure and function. Curr. Opin. Cell Biol. 3:429–437.

59. Syntichaki, P., I. Topalidou, and G. Thireos. 2000. The Gcn5 bromodomain co-ordinates nucleosome remodelling. Nature 404:414–417.

60. Toffaletti, D. L., T. H. Rude, S. A. Johnston, D. T. Durack, and J. R. Perfect. 1993. Gene transfer in Cryptococcus neoformans by use of biolistic delivery of DNA. J. Bacteriol. 175:1405–1411.

61. Vecchiarelli, A., C. Retini, D. Pietrella, C. Monari, T. Beccari, and T. Kozel. 1995. Downregulation by cryptococcal polysaccharide of tumor necrosis factor alpha and interleukin-1 beta secretion from human monocytes. Infect. Immun. 63:2919–2923.

62. Wang, L., L. Liu, and S. L. Berger. 1998. Critical residues for histone acetylation by Gcn5, functioning in Ada and SAGA complexes, are also required for transcriptional function in vivo. Genes Dev. 12:640–653.

63. Reference deleted.

64. Wolfinger, R. D., G. Gibson, E. D. Wolfinger, L. Bennett, H. Hamadeh, P. Bushel, C. Afsahi, and R. S. Paules. 2001. Assessing gene significance from cDNA microarray expression data via mixed models. J. Comput. Biol. 8:625–637.

65. Yoneda, A., and T. L. Doering. 2008. Regulation of Cryptococcus neoformans capsule size is mediated at the polymer level. Eukaryot. Cell 7:546–549.

66. Zanton, S. J., and B. F. Pugh. 2004. Changes in genomewide occupancy of core transcriptional regulators during heat stress. Proc. Natl. Acad. Sci. U. S. A. 101:16843–16848.

67. Zaragoza, O., C. J. Chrisman, M. V. Castelli, S. Frases, M. Cuenca-Estrella, J. L. Rodriguez-Tudela, and A. Casadevall. 2008. Capsule enlargement in Cryptococcus neoformans confers resistance to oxidative stress suggesting a mechanism for intracellular survival. Cell. Microbiol. 10:2043–2057.

68. Zhang, W., J. R. Bone, D. G. Edmondson, B. M. Turner, and S. Y. Roth. 1998. Essential and redundant functions of histone acetylation revealed by mutation of target lysines and loss of the Gcn5p acetyltransferase. EMBO J. 17:3155–3167.

69. Zhu, X., J. Gibbons, J. Garcia-Rivera, A. Casadevall, and P. R. Williamson. 2001. Laccase of Cryptococcus neoformans is a cell wall-associated virulence factor. Infect. Immun. 69:5589–5596.