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Convergent Evolution of Calcineurin Pathway Roles in Thermotolerance and Virulence in Candida glabrata

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ABSTRACT Candida glabrata is an emerging human fungal pathogen that is frequently drug tolerant, resulting in difficulties in treatment and a higher mortality in immunocompromised patients. The calcium-activated protein phosphatase calcineurin plays critical roles in controlling drug tolerance, hyphal growth, and virulence in diverse fungal pathogens via distinct mechanisms involving survival in serum or growth at host temperature (37° and higher). Here, we comprehensively studied the calcineurin signaling cascade in C. glabrata and found novel and uncharacterized functions of calcineurin and its downstream target Crz1 in governing thermotolerance, intracellular architecture, and pathogenesis in murine ocular, urinary tract, and systemic infections. This represents a second independent origin of a role for calcineurin in thermostolerant growth of a major human fungal pathogen, distinct from that which arose independently in Cryptococcus neoformans. Calcineurin also promotes survival of C. glabrata in serum via mechanisms distinct from C. albicans and thereby enables establishment of tissue colonization in a murine systemic infection model. To understand calcineurin signaling in detail, we performed global transcript profiling analysis and identified calcineurin- and Crz1-dependent genes in C. glabrata involved in cell wall biosynthesis, heat shock responses, and calcineurin function. Regulators of calcineurin (RCN) are a novel family of calcineurin modifiers, and two members of this family were identified in C. glabrata: Rcn1 and Rcn2. Our studies demonstrate that Rcn2 expression is controlled by calcineurin and Crz1 to function as a feedback inhibitor of calcineurin in a circuit required for calcium tolerance in C. glabrata. In contrast, the calcineurin regulator Rcn1 activates calcineurin signaling. Interestingly, neither Rcn1 nor Rcn2 is required for virulence in a murine systemic infection model. Taken together, our findings show that calcineurin signaling plays critical roles in thermostolerance and virulence, and that Rcn1 and Rcn2 have opposing functions in controlling calcineurin signaling in C. glabrata.

KEYWORDS phosphatase
calcium
calmodulin
Crz1
Rcn1
Rcn2
thermotolerance
cell wall integrity
ER stress
drug tolerance
pH homeostasis
urinary tract
infection
ocular infection
virulence

Candida glabrata is an emerging human fungal pathogen. Most strains have reduced antifungal drug susceptibility, thus making treatment challenging (Lagrotteria et al. 2007; Pfaller et al. 2011a). The poor susceptibility of C. glabrata to antifungal drugs contributes to the high mortality rate (~50%) associated with C. glabrata candidemia (Klevay et al. 2009). Resistance to echinocandins and azoles was most prevalent among nosocomial bloodstream infection (BSI) isolates of C. glabrata compared with other Candida species (Pfaller et al. 2011a). Although C. albicans accounts for 48% of Candida BSIs, C. glabrata ranked second, accounting for 18% of infections across multiple
geographic regions, including Asia-Pacific, Europe, Latin America, and North America (Pfaller et al. 2011b). Besides BSI, Candida species also cause urinary tract infections (UTI). Fungal UTIs are often caused by Candida species, which account for ~10% of all nosocomial UTIs related to indwelling catheters, with C. glabrata accounting for approximately 15% of all Candida isolates (Kaufman et al. 2000; Lundstrom and Sobel 2001). Surprisingly, a study performed by Phillips and Karlowicz (1997) showed that 42% of hospital-acquired urinary tract infections in neonatal intensive care units were caused by Candida species. For Candida ocular infections, C. glabrata was previously rarely seen; however, several recent studies report that C. glabrata infects human corneas, especially in patients undergoing keratoplasty (Caldwell et al. 2009; Djalllian et al. 2001; Kitzmann et al. 2009; Lee et al. 2011; Tappeiner et al. 2009). The pathogenic mechanisms by which C. glabrata causes ocular infections have not been reported.

Ccalcineurin is a calcium/calmodulin-dependent serine/threonine-specific protein phosphatase that comprises a catalytic A (Cna1) and a regulatory B calcium-binding subunit (Cnb1). Upon stimulation with calcium, calmodulin associates with the calcineurin AB heterodimer, stimulating phosphatase activity and converting signals to various outputs by dephosphorylating its downstream targets. Upon dephosphorylation by activated calcineurin, the transcription factor Crz1 in fungi or nuclear factor of activated T cells (NFAT) in mammals migrates to the nucleus to regulate gene expression. Because active calcineurin is an AB heterodimer, the loss of Cnb1 often results in destabilization of Cna1 (Chen et al. 2010a). Calcineurin is essential for growth at elevated temperatures in the human fungal pathogen Cryptococcus neoformans (Odom et al. 1997) and the protozoan parasite Leishmania major (Naderer et al. 2011), but it has not been demonstrated to have a role in controlling thermotolerance of ascomycetes, including Candida species. The intracellular architecture defects of C. neoformans and L. major calcineurin mutants upon thermal stress have not been investigated. In C. glabrata, calcineurin and Crz1 have been demonstrated to play functions in antifungal drug tolerance, cell wall integrity, and virulence in a murine systemic infection model (Miyazaki et al. 2010). However, the mechanism for C. glabrata calcineurin and Crz1 requirement in serum survival and virulence in other murine infection models remains elusive.

The calcineurin- and/or Crz1-regulated transcriptome has been investigated in Saccharomyces cerevisiae, C. albicans, Aspergillus fumigatus, and the rice blast fungal pathogen Magnaporthe oryzae (Karababa et al. 2006; Kim et al. 2010; Malavazi et al. 2009; Yoshimoto et al. 2002). The regulation pattern of the transcriptome is diverged among the four species. Because C. glabrata calcineurin and crz1 mutants exhibited novel phenotypes distinct from these species in terms of hypersensitivity to thermal stress, ER stress, and antifungal drugs, microarray analyses to explore the targets of calcineurin and Crz1 in C. glabrata will extend our understanding of calcineurin signaling.

Regulators of calcineurin (RCN) are a novel family of calcineurin regulators found in eukaryotic cells. In S. cerevisiae, two regulators of calcineurin (Rcn1 and Rcn2) have been demonstrated to control calcineurin signaling. In C. albicans, the function of Rcn1 has been associated with calcineurin signaling (Reedy et al. 2009). In C. glabrata, the functions of Rcn1 and Rcn2 in controlling calcineurin signaling were recently reported (Miyazaki et al. 2011a). C. glabrata Rcn1 displayed both stimulatory and inhibitory effects, whereas Rcn2 showed only inhibitory activities on calcineurin signaling (Miyazaki et al. 2011a). Interestingly, Rcn1 is required for micafungin and fluconazole tolerance in addition to ER stress induced by tunicamycin (Miyazaki et al. 2011a). However, the roles of Rcn1 and Rcn2 in temperature sensitivity, pH response, and virulence remain unclear. In mammals, the homolog of S. cerevisiae Rcn1 is named DSCR1/Rcn1/calcipressin and is overexpressed in the brain of patients with Down’s syndrome (Rothermel et al. 2003). In addition, Baek et al. (2009) demonstrated that DSCR1, a negative regulator of calcineurin located on chromosome 21 and thus present in three copies in patients with Down’s syndrome, has antiangiogenic activity and inhibits tumor formation.

In this study, we comprehensively studied the roles of the calcineurin signaling cascade in stress responses, antifungal drug tolerance, and pathogenesis of C. glabrata. We demonstrated that C. glabrata calcineurin (Cna1 and Cnb1) and/or Crz1 are required for thermotolerance, ER stress, pH homeostasis, and virulence in murine ocular, urinary tract, and systemic infection models. In addition, global transcript profiling analysis revealed 30 calcineurin and Crz1-activated targets, which explain, at least in part, the requirement of calcineurin signaling in cell wall integrity, thermotolerance, and pathogenesis. Furthermore, we demonstrated that a novel regulator of calcineurin 2 (Rcn2) is a downstream target of calcineurin and Crz1 and acts as a feedback inhibitor of calcineurin signaling in response to calcium, whereas Rcn1 functions as a positive regulator of calcineurin signaling.

MATERIALS AND METHODS

Ethics statement

Animals studies conducted in the Division of Laboratory Animal Resources (DLAR) facilities at Duke University Medical Center (DUMC) were handled with good practice as defined by the United States Animal Welfare Act and in full compliance with the guidelines of the DUMC Institutional Animal Care and Use Committee (IACUC). The murine systemic and urinary tract infection models were reviewed and approved by the DUMC IACUC under protocol numbers A238-09-08 and A219-08-08, respectively.

Murine ocular infection studies conducted at the animal house facility of the Department of Microbiology and Parasitology, University of Perpetual Help–Dr. Jose G. Tamayo Medical University (UPH-DJGTMU) were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the United States Animal Welfare Act, and the Republic of the Philippines Animal Welfare Act of 1998 (RA No. 8485), and in full conformity with the guidelines set forth in the UPH-DJGTMU research manual. The protocol was formally approved by the UPH-DJGTMU institutional review board after review by the Regional Institute for Tropical Medicine Institutional Animal Care and Use Committee (RITM-IACUC) under research protocol No. 010. All animal infection experiments were conducted by properly trained personnel, including licensed veterinarians.

Yeast strains, media, and chemicals

Yeast strains used in this study are listed in Table 1. YPD (1% yeast extract, 2% peptone, 2% glucose) liquid medium and agar (2%) and SC (6.7 g yeast nitrogen base without amino acids, 1 x amino acid stock, 20 g glucose, 20 g agar in 1 liter) media were used in this study. YPD medium containing 100 μg/ml nourseothricin was used to select transformants. YPD medium containing 150 mM HEPES buffered at pH 2, 5, 8, or 9 was used to study pH tolerance. FK506 (Astellas Pharma Inc.), cyclosporin A (CsA; LC Laboratories), sodium dodecyl sulfate (SDS; Fisher), fetal bovine serum (Invitrogen), calcifluor white (CFW, fluorescent brightener 28; Sigma), Congo red (Sigma), fluconazole (Bedford Laboratories), posaconazole (Sequoia Research Products Ltd.), ketoconazole (Sigma), voriconazole (Sigma), caspofungin (Merck), micafungin (Astellas Pharma Inc.), anidulafungin (Pfizer Inc.), tunicamycin (Sigma), and dithiothreitol (DTT; Sigma) were added to the media at the concentrations indicated.
Identification of *C. glabrata* gene orthologs

The *C. glabrata* orthologs of the genes encoding *C. albicans* calcineurin subunits Cna1 and Cnb1 and the calcineurin target Crz1 were identified by reciprocal BLAST searches between the two species, with the reciprocal best BLAST hit orthologs in *C. glabrata* being the CNA1 (CAGL0L11110g), CNB1 (CAGL0L00605g), and CRZ1 (CAGL0M06831g) genes. *C. glabrata* YPS5 (CAGL0E01771g, putative aspartyl protease) and RCN2 (CAGL0J04158g, regulator of calcineurin 2) were identified by reciprocal BLAST searches using the *S. cerevisiae* genome and genes. The *C. glabrata* ortholog of *C. albicans* RCN1 (regulator of calcineurin 1) was identified by reciprocal BLAST searches between *C. glabrata* and *C. albicans*, identifying the reciprocal best BLAST hit as *C. glabrata* RCN1 (CAGL0E06248).

Gene disruptions in *C. glabrata*

All deletion strains were generated from the prototrophic CBS138 (ATCC2001) background using the SAT1 flipper (Reuss et al. 2004). All primers used in strain construction are listed in Table S1. For the CNA1 gene disruption, approximately 1 kb of the 5’ (amplified with primers JC49/JC50, Table S1) and 3’ (amplified with primers JC51/JC52) noncoding regions (NCR) of the CNA1 ORF were PCR-amplified from genomic DNA of the wild-type strain CBS138. The 4.2 kb SAT1 flipper sequence was amplified from plasmid pSFS2A (Reuss et al. 2004) with primers JC17/JC18. The three PCR products were treated with ExoSAP-IT (USB Corp.) to remove contaminating primers and dNTPs and then combined in a 1:3:1 molar ratio (5’ CNA1NCR, SAT1 flipper; 3’ CNA1NCR) to generate the disruption allele by overlap PCR using flanking primers JC49/JC52, resulting in an ~6.2 kb 5′CNA1NCR-SAT1 flipper-3′CNA1NCR CNA1 disruption allele. The CNA1 gene was disrupted in the wild-type strain CBS138 by transformation with 0.2–1.0 μg of gel-purified disruption cassette DNA extracted with the Frozen-EZ Yeast Transformation Kit (Zymo Research). Two independent nourseothricin-resistant cna1 mutants (YC67 and YC98, Table 1) were obtained from two separate transformations.

A similar approach was employed to disrupt the CNB1 and CRZ1 genes, with ~1 kb of the 5’ and 3’ noncoding regions used for homologous recombination. To generate the ~6 kb cnb1 disruption allele, the overlap PCR DNA products 5’ CNB1NCR (amplified with primers JC134/JC135), SAT1 flipper (amplified with primers JC17/JC18), and 3’ CNB1NCR (amplified with primers JC136/JC137) were mixed in a 1:3:1 molar ratio and amplified with primers JC138/JC139 (~100 bp closer to the CNB1 ORF compared with JC134/JC137, respectively, preserving primers JC134/JC137 for further integration confirmation). Two independent nourseothricin-resistant cnb1 mutants (YC191 and YC193, Table 1) derived from two separate transformations were obtained. To generate the ~6 kb crz1 disruption allele, 5’ CRZ1NCR (amplified with primers JC142/JC143), SAT1 flipper (amplified with primers JC17/JC18), and 3’ CRZ1NCR (amplified with primers JC144/JC145) were combined and amplified with primers JC146/JC147 (~100 bp closer to the CRZ1 ORF compared with JC142/JC145, respectively). Two independent nourseothricin-resistant crz1 mutants (YC67 and YC182, Table 1) derived from two separate transformations were obtained.

To disrupt the RCN1 gene, 5’ RCN1NCR and 3’ RCN1NCR were amplified with primers JC459/JC460 and JC461/JC462, respectively. JC463/JC464 primers were used to amplify the RCN1 disruption allele via overlap PCR including three fragments: 5’ RCN1NCR, SAT1 flipper, and 3’ RCN1NCR. Two independent rcn1 mutants (YC553 and YC556) were obtained. To disrupt the RCN2 gene, 5’ RCN2NCR and 3’ RCN2NCR were amplified with primers JC441/JC442 and JC443/JC444, respectively. JC445/JC446 primers were used to amplify the RCN2 disruption allele via

| Table 1 | Candida glabrata and Cryptococcus neoforms strains |
|----|---|---|---|
| **Strain** | **Genotype** | **Parent** | **Reference** |
| CBS138 | Prototrophic wild-type | Clinical isolate | Dujon et al. (2004) |
| YC67\a | cna1\a::SAT1-FLP | CBS138 | This study |
| YC98\b | cna1\b::SAT1-FLP | CBS138 | This study |
| YC191\h | cnb1\h::SAT1-FLP | CBS138 | This study |
| YC193\h | cnb1\h::SAT1-FLP | CBS138 | This study |
| YC267\c | crz1\c::SAT1-FLP | CBS138 | This study |
| YC182\c | crz1\c::SAT1-FLP | CBS138 | This study |
| YC531\h | rcn1\h::SAT1-FLP | CBS138 | This study |
| YC535* | rcn1\h::SAT1-FLP | CBS138 | This study |
| YC594* | rcn1\h::FRT | YC581 | This study |
| YC594* | rcn2\h::FRT | YC581 | This study |
| YC111* | cna1\h::FRT | YC98 | This study |
| YC535* | cna1\h::FRT | YC98 | This study |
| YC549* | cna1\h::FRT | YC193 | This study |
| YC504* | cnb1\h::FRT | YC193 | This study |
| YC502* | crz1\h::FRT | YC182 | This study |
| YC532* | crz1\h::FRT | YC502 | This study |
| YC534* | crz1\h::FRT | YC502 | This study |
| CBS138 | Prototrophic wild-type | Clinical isolate | Perfect et al. (1993) |
| H99 | cna1::NAT | H99 | Kojima et al. (2006) |
| KK1 | cna1::NAT | H99 | Kojima et al. (2006) |

\a,b,c,d,e,f,g,h Two independently derived cna1, cnb1, crz1, rcn1, rcn2, cna1 rcn2, cnb1 rcn2, or crz1 rcn2 mutants, respectively.

Table 1. Candida glabrata and Cryptococcus neoforms strains

<table>
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<tr>
<th><strong>Strain</strong></th>
<th><strong>Genotype</strong></th>
<th><strong>Parent</strong></th>
<th><strong>Reference</strong></th>
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overlap PCR including three fragments: 5’ RCN2^NCR, SAT1 flipper, and 3’ RCN2^NCR. Two independent rcn2 mutants (YC525 and YC531) were obtained.

To obtain the rcn1 rcn2 double mutant, we disrupted the RCN1 gene in the rcn2 mutant background (YC581, the SAT1 flipper was removed via culturing YC531 in YPD medium and replicating onto nourseothricin-containing medium to confirm the loss of SAT1 flipper). To obtain the cna1 rcn2 (YC535 and YC549), cnb1 rcn2 (YC539 and YC601), and crz1 rcn2 (YC532 and YC534) double mutants, we disrupted the RCN2 gene in SAT1 flipper-free (methods described above) cna1 (YC111), cnb1 (YC504), and crz1 (YC502) mutants, respectively.

**Transmission electron microscopy**

Transmission electron microscopy (TEM) of C. glabrata was accomplished as follows. Cells were grown overnight in YPD at 24°C and washed twice with dH2O. Then 0.1 OD600 of cells (in 100 μl) was spread on YPD agar plates and incubated for 24 hr at 24°C, 37°C, and 40°C. Cells were collected from agar plates by washing in 0.2 M sodium cacodylate buffer (pH 6.8), collected by centrifugation (~4,000 rpm, 3 min in a table-top centrifuge), resuspended in 2% glutaraldehyde plus 0.05% malachite green oxalate in 0.1 M sodium cacodylate buffer, and incubated at 4°C for 2 days. Fixed cells were collected by centrifugation, resuspended, washed with 0.1 M sodium cacodylate buffer, centrifuged, supernatant removed, and post-fixed with 0.8% K2Fe (CN)6, 1% OsO4, 0.1 M sodium cacodylate for 2 hr at room temperature. The cells were washed twice with 0.1 M sodium cacodylate buffer and stained with 1% tannic acid for 1 hr at room temperature. Then cells were washed with 0.1% sodium cacodylate buffer for 5 min followed by two washes in dH2O for 5–10 min each and stained with 1% uranyl acetate in water overnight at 4°C. Samples were washed in molecular-grade distilled water, embedded in an agarose pellet, and prepared for embedding. The cured peg was trimmed, sectioned, and mounted on copper grids. Grids were post-stained prior to viewing. Sections were viewed and imaged with a Philips/FEI CM 12 Transmission EM (FEI Company, Hillsboro, OR) with Advanced Microscopy Techniques Corp. (AMT) 2K × 2K digital camera (Danvers, MA) at the Duke University Department of Pathology.

**Fluorescence microscopy**

Cells were grown overnight in YPD at 24°C and washed twice with dH2O. Then 0.1 OD600 of cells (in 100 μl) was spread on YPD agar plates and incubated for 24 hr at 24°C and 37°C. The Alexa Fluor 488 phalloidin (Cat #A12379; Life Technologies) staining of actin was performed based on the protocol described by Adams and Pringle (1991) with minor modifications. In brief, cells were spun and resuspended in 3.7% (vol/vol) formaldehyde in PBS for 1 hr, washed, and resuspended in PBS (100 μl). Cells were then stained with 20 μl of 6.6 μM (or 0.2 U/μl) phalloidin stock for 1 hr in the dark. Stained cells were washed five times with PBS, resuspended in 100 μl PBS in the absence of mounting medium, and observed using the standard green fluorescence filter set.

**Time-kill curve for strains exposed to fluconazole, micafungin, or serum**

Cells were grown overnight at 24°C, washed twice with dH2O, counted with a hemocytometer, and then 5 × 10^6 cells were added to 5 ml of fresh YPD medium ± fluconazole/micafungin or 5 ml of 100% fetal bovine serum to achieve 10^6 cells/ml. Cells were cultured at 24°C with shaking at 250 rpm. The cells surviving after 0, 3, 6, 9, and 24 hr were serially diluted onto YPD medium, and CFUs were counted after 48 hr of incubation at 24°C. The experiments were performed in triplicate, and data were plotted using Prism 5.03.

**Culture growth, harvesting, and total RNA extractions for microarray experiments**

Strains were grown overnight at 24°C, washed twice with dH2O, diluted to 0.2 OD600/ml in YPD, and incubated for 3 hr at 24°C. For wild-type strains, cells in log-phase were diluted to 0.2 OD600/ml (10 ml) in YPD in the presence or absence of FK506 (1 μg/ml) while cna1 (YC98) and crz1 (YC182) mutants were diluted to 0.2 OD600/ml (10 ml) in YPD. Following 3 hr of incubation at 37°C with shaking at 250 rpm, the 10 ml cultures were immediately added to 15 ml methanol (60%) chilled in a dry ice-ethanol bath to stop cellular processes and RNase activity. Cells were pelleted at 3000 rpm at −4°C, flash frozen with liquid N2, and stored at −80°C prior to total RNA extraction. The total RNAs were extracted using the RNeasy Mini Kit (Qiagen). RNA quality was assessed with the RNA 6000 Nano Kit of the Agilent Bioanalyzer 2100 to ensure RNA Integrity Number (RIN) scores ≥ 7.

**Probe preparation, microarray hybridization, and data analysis**

Total RNA samples were reverse-transcribed to cDNA and labeled with either Cy3 or Cy5, according to a modification of the protocol described by Wapinski et al. (2010). We employed a wheel hybridization design to compare all samples to each other with dye-swaps, for four comparisons per sample on 10 microarrays per biological replicate. The entire hybridization scheme was repeated for two biological replicates. After hybridization and washing per the manufacturer’s instructions, arrays were scanned using an Agilent scanner and analyzed with Agilent’s Feature Extraction software V10.5.1. The median intensities of probes corresponding to a specific ORF were used to estimate the expression values for each gene. We then fit linear models to these gene expression data using the limma library of the R Bioconductor Package (Gentleman et al. 2005), which computes fold change, indicates the direction and quantity of the differential gene expression between the samples, and provides summary statistics, including T- and B-statistics and an adjusted P value that takes into account the false discovery rate. For each comparison in each study, Q-values were computed using the R package. Microarray results have been deposited at NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31167).

**Quantitative determination of expression by real-time RT-PCR**

DNase I (Turbo DNA-free; Ambion) was used to eliminate genomic DNA contamination. One microgram of DNA-free total RNAs was reverse-transcribed to cDNA by the Affinity Script qPCR cDNA Synthesis Kit (Agilent). PCR reactions of 25 μl included 10 ng cDNA (in 10 μl), 12.5 μl of 2× qPCR master mix (Brilliant SYBR Green Kit; Agilent), 0.5 μl of 5 μM forward primer, 0.5 μl of 5 μM reverse primer, 1.125 μl of nuclease-free H2O, and 0.375 μl of ROX dye. Quantitative PCR conditions were the following: 95°C/10 min (denaturation); 95°C/15 sec, 60°C/1 min (40×, cycling stage); 95°C/15 sec, 60°C/1 min, 95°C/15 sec (melting curve). Primers for probes were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) and are listed in Table S1. The ABI PRISM 7900HT machine and StepOne v2.1 (Applied Biosystems) were used to determine ΔΔCt and relative quantity (RQ). The bar graphs of ACTI normalized RQ compared with the wild-type (CBS138) were created with Prism 5.03.
Mouse studies

Urinary tract infection model: Six- to eight-week-old female C3H/HeJ mice (stock #000659, n = 5 for each group) and C3H/HeOuJ (stock #000635, n = 8 for the group) from the Jackson Laboratory were used in this study. C. glabrata strains were grown in 5 ml SD + CAA medium (6.7 g yeast nitrogen base minus amino acids, 6 g casamino acids, 20 g glucose in 1 L dH2O) overnight at 24°C. Cultures were washed twice with 10 ml of phosphate buffered saline (PBS) and diluted to yield an infection inocula of 10⁶ cells/ml after hemocytometer counting. Prior to infection, mice were anesthetized with i.p. injection of 100 µl of 5-fold diluted pentobarbitral (final concentration of 10 mg/ml in PBS). Thirty microliters (3 × 10⁶ cells) were used to infect mice via the urinary tract for 15 sec aided by polyethylene catheter tubing (BD catalog #427400) and syringe (BD catalog #309659) with 30 G × 1/2 needle (BD catalog #305106). Bladder and kidney tissue samples were harvested on day 7 and homogenized with 5 ml PBS for 5 s at 17,500 rpm (for kidneys) or at 24,000 rpm (for bladder) (Power Gen 500; Fisher Scientific). Tissue homogenates were serially diluted and 100 µl was plated onto YPD solid medium. The plates were incubated at 24°C for 72 hr to determine CFUs per organ. Appropriate dilutions of the inocula were plated onto YPD at 24°C for 48 hr to confirm cell viability. All experimental procedures were carried out according to NIH guidelines and Duke IACUC protocols for the ethical treatment of animals.

Murine systemic infection model: Five- to six-week-old male CD1 mice from the Jackson Laboratory (n = 10 for each group) were utilized in this study. C. glabrata strains were grown in 5 ml liquid YPD medium overnight at 24°C. Cultures were washed twice with 10 ml of phosphate buffered saline (PBS), and the cells were then resuspended in 2 ml of PBS. Cells were counted with a hemocytometer and resuspended in an appropriate volume of PBS to obtain an infection inocula concentration of 2 × 10⁶ cells/ml. Two hundred microliters (4 × 10⁶ cells) were used to infect mice by lateral tail vein injection. Appropriate dilutions of the cells were plated onto YPD solid medium and incubated at 24°C for 48 hr to confirm cell viability. C. glabrata–infected mice were sacrificed and dissected on day 7 post-infection. The kidney and spleen tissues were removed, weighed, transferred to a 15 ml Falcon tube filled with 5 ml PBS, and homogenized for 5 s at 19,000 rpm (IKA T25; Cole-Parmer). Tissue homogenates were serially diluted, and 100 µl was plated onto YPD solid medium. The plates were incubated at 24°C for 48 hr to determine CFUs per gram of organ. The identity of organ-recovered colonies was confirmed by PCR. All experimental procedures were carried out according to NIH guidelines and Duke IACUC protocols for the ethical treatment of animals.

Murine ocular infection model: Candida strains were grown in YPD overnight at 25°C. Cultures were washed three times with sterile PBS (pH 7.4). Cells were diluted to a concentration of 10⁶ CFU/5 µl. The concentration was determined by using the spectrophotometer optical density reading at a wavelength of 600 nm and multiplying it by a conversion factor of 1 OD₆₀₀ equivalent to 3 × 10⁷ cells/ml. Inoculum concentration was verified by plating on YPD for 48 hr at 25°C.

For murine ocular infection, six- to eight-week-old outbred ICR mice (20–28 g; Research Institute for Tropical Medicine, Alabang, Philippines) were used in the experiment in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The previously described keratomycolysis protocol (Chen et al. 2011) was used for Candida infection with minor modifications and was approved by the University of Perpetual Help Institutional Review Board. Before the infection procedure, mice were anesthetized by intravenous injection of Zoletil 50 (10–15 mg/kg body weight; Virac, Australia) followed by topical application of proparacaine hydrochloride ophthalmic solution (Alcaine; Alcon-Couvreur, Belgium) to the eyes. Once animals were anesthetized, the right eye was superficially scarified in a grid-pattern by a sterile 25-gauge hypodermic needle, and 5 µl of Candida solution (10⁶ CFU) was placed into each eye. The inoculum was distributed uniformly by rubbing the eye for few seconds with the eyelid. A mock-infection experiment was performed using sterile PBS as a control. Disease severity of fungal keratitis was assessed for 8 days with the aid of a dissecting microscope. In this procedure, corneal involvement was assessed and scored according to three parameters: (i) area of opacity, (ii) density of opacity, and (iii) surface regularity. A grade of 0 to 4 was assigned based on each of these criteria to yield a maximum score of 12.

Statistical analysis

Statistical analysis was conducted using Prism 5.03 software (GraphPad, La Jolla, CA). The significance of differences in fungal burden was determined using one-way ANOVA and Dunnett’s multiple comparison tests. For optimal serum growth and real-time RT-PCR, the significance of differences was determined by one-way ANOVA and Bonferroni’s multiple comparison tests. For shrunk cell percentage and murine ocular infection, the Student unpaired t-test was used to determine significance. P < 0.05 was considered significant.

RESULTS AND DISCUSSION

The calcineurin pathway plays a critical role in thermotolerance in C. glabrata

Fungal pathogens must adapt to elevated temperatures and other environmental stresses to successfully infect their mammalian hosts. How these environmental cues are sensed is not clear. In the human fungal pathogen C. neofor mans, calcineurin mutants are unable to grow at 37°C and as a result are avirulent in an animal model of cryptococcal meningitis (Odom et al. 1997). In the protozoan parasite L. major, a calcineurin mutant (cnb) loses viability at 34°C and thus fails to survive in mice (Nader et al. 2011). To date, a requirement of calcineurin for high-temperature growth has not been reported in ascomycetes, including species such as S. cerevisiae (Cayet et al. 1991), C. albicans (Bader et al. 2006), A. fumigatus (Steinbach et al. 2006), and M. oryzae (Choi et al. 2009). Here, we demonstrate that high-temperature growth in C. glabrata is controlled by calcineurin and Crz1. C. glabrata calcineurin mutants (cnal and cnbl) exhibited a temperature-sensitive (ts) phenotype at 40°C, whereas crz1 mutants exhibited intermediate growth between the wild-type strain and calcineurin mutants (Figure 1A), suggesting the calcineurin pathway governs growth at elevated temperature. The growth of the wild-type strain in medium containing the calcineurin inhibitors FK506 or cyclosporin A (CsA) at 40°C phenocopies the ts growth of calcineurin mutants in the absence of calcineurin inhibitors (Figure 1A). We observed that cnal and cnbl mutants, and the wild-type strain in the presence of FK506 or CsA, exhibited slight growth defects at 37°C but not at 24°C (Figure 1A). The growth defects of calcineurin mutants were not simply due to a general reduced rate of growth because wild-type strain and calcineurin pathway mutants had similar growth kinetics at 24°C (Figure 1B). Calcineurin mutants were not viable after 48 hr growth at 40°C and could not be rescued after transfer to 24°C for 48 hr (Figure 1C). When cells were growing in log phase at 40°C, cnal and cnbl mutants exhibited a significantly shrunk cell
morphology [65.6 ± 15.3% and 75.0 ± 9.4% of the cellular population, respectively, compared with the wild-type (1.7 ± 1.5%), P < 0.002; Figure 1D]. The shrunk cell morphology was also observed in wild-type cells at 40°C in the presence of FK506 or cyclosporin A (Figure S1). The crz1 mutants showed an intermediate proportion of shrunk cells (29.3 ± 9.4%) compared with the wild-type and calcineurin mutants (Figure 1D). Interestingly, the growth defects of the calcineurin mutants at 40°C were rescued by the presence of an osmotic stabilizer (1 M sorbitol; Figure 1E, left panel), which is in contrast to C. neoformans calcineurin mutant for which the osmotic stabilizer did not rescue the growth of the mutant at 37°C (Figure 1E, right panel). Our data suggest a novel role for the calcineurin pathway in controlling thermal stress response of C. glabrata and a convergent role of calcineurin in promoting high-temperature growth of C. glabrata and C. neoformans.

The intracellular architecture of calcineurin mutants upon thermal stress in C. neoformans and L. major remains elusive. Here, we examined cell structures of C. glabrata wild-type and calcineurin pathway mutants upon growth at 24°C, 37°C, and 40°C via transmission electron microscopy. C. glabrata calcineurin and crz1 mutants exhibit abnormal cell structures compared with the wild-type at 37°C or above (Figure 2 and Figure S2), but not at 24°C (data not shown). Calcineurin and crz1 mutants exhibit irregular plasma membrane structures (red arrowheads in Figure 2, E, H, and K) at 37°C, whereas the wild-type strain shows an intact and regular plasma membrane structure (Figure 2B). A similar disruption of the plasma membrane has not been reported for S. cerevisiae calcineurin mutants. Because actin forms patches associated with the plasma membrane in S. cerevisiae (Doyle and Botstein 1996; Waddle et al. 1996), we examined whether C. glabrata calcineurin mutants might exhibit defective cortical actin patch structures. However, we found no defects in forming cortical actin patches based on phalloidin staining of calcineurin mutant cells at 37°C (Figure S3). Calcineurin and crz1 mutants show fewer budding cells at 37°C compared with the wild-type (data not shown), suggesting a reduced growth rate or cell-cycle arrest of calcineurin pathway mutants. When grown at 40°C for 24 hr, calcineurin mutants could not be recovered due to cell death, whereas crz1 mutants exhibit misshapened organelles (Figure S2C) and irregular plasma membrane structures (Figure S2D) compared with the wild-type. Altogether, our data suggest that the calcineurin pathway is required for maintaining intact plasma membrane structure in C. glabrata.

To determine whether calcineurin was required for thermotolerance in all C. glabrata strains, we tested the growth of 19 clinical C. glabrata isolates at elevated temperatures in the presence of FK506 or CsA. We found that 16% (3/19) of C. glabrata clinical isolates exhibit temperature sensitivity in the presence of a calcineurin inhibitor (Figure S4), suggesting that the requirement of calcineurin at elevated temperatures has been evolutionarily diverged in distinct isolates of the species. One of the 3 clinical isolates that exhibited temperature sensitivity in the presence of FK506 or CsA was the type strain and genome-sequenced reference strain CBS138, used as the parental wild-type strain in this study. It is possible that a specific mutation occurred in the genome of the C. glabrata ancestor that resulted in reduced fitness, such as a temperature-sensitive response to calcineurin inhibitors secreted by the bacterium Streptomyces tsukubaensis in the soil (Goto et al. 1987). Because most C. glabrata strains did not exhibit the ts phenotype in response to calcineurin inhibitors, it suggests that additional modifier mutations might be present that suppress the ts phenotype. Further studies that isolate high-temperature-resistant mutants from C. glabrata CBS138 in the presence of a calcineurin inhibitor and screening suppressors or sequencing the whole genome will shed light on the roles of C. glabrata calcineurin in thermotolerance.

**Calcineurin contributes to tissue colonization in a murine urinary tract infection model**

*Candida* species account for 10–15% of all nosocomial urinary tract infections related to indwelling catheters (Lundstrom and Sobel 2001). *C. albicans* (52%), *C. glabrata* (16%), *C. tropicalis* (8%), and *C. parapsilosis* (4%) were the major *Candida* species found in the urine of 861 patients with funguria (Kauffman et al. 2000). Recently, Silva et al. (2010) reported that C. glabrata strains exhibited a better ability to colonize silicone surfaces in the presence of urine than *C. tropicalis* or *C. parapsilosis*, which may explain why *C. glabrata* is an emerging pathogen in hospitalized and catheterized patients. *C. glabrata* epithelial adhesin triple knockout mutant (epa1,6,7) has been demonstrated to have a decreased ability to colonize the bladder in the murine urinary tract infection model (Cormack et al. 1999). However, other factors that might contribute to urinary tract infections due to *C. glabrata* are unclear. We demonstrated that calcineurin contributed to murine urinary tract infections. C. glabrata cna1 and cnb1 mutants exhibited a 7.5-fold (P < 0.001) and 3.7-fold (P < 0.01) reduced fungal burden in the kidneys, respectively, whereas crz1 mutants exhibited no difference compared with the wild-type strain (Figure 3A). Nevertheless, we were unable to determine the fungal burden in bladders when we used the C3H/HeJ inbred mouse strain, which serves as a vesico-ureteric reflux (VUR) model (Murawski et al. 2010) and is the only mouse model that has worked for this purpose in our laboratory. VUR is defined by the retrograde passage of pathogens from the bladder into the ureters and pelvis of the kidneys. The percentage of VUR with the C. glabrata infection in C3H/HeJ mice was 91.2% (31/34), indicating that most C. glabrata cells migrated from the bladder to the kidneys; therefore, the fungal burden in the bladders remained very low due to the nature of this mouse model (Figure S5A). The mouse strains CD1 and CBA/J have been used to determine the fungal burden of C. glabrata in bladders and kidneys, but the model was unsuccessful due to poor colonization of the bladder and kidneys with percentages of VUR of 34% and 50%, respectively (data not shown).

**Mouse TLR4 is not required for protection from C. glabrata urinary tract infection**

C3H/HeJ mice have a Toll-like receptor 4 (TLR4) mutation. TLR4 is a type of pattern recognition receptor (PRR) that recognizes lipopolysaccharides from Gram-negative bacteria and initiates innate immunity. Recently, Leal et al. (2010) showed that killing of *A. fumigatus* was reduced in a TLR4(−/−) murine keratitis model, suggesting that TLR4 is required for *A. fumigatus* infection. In *C. albicans*, the role of mammalian TLR4 for *Candida* recognition or infection varies in different studies (Gil and Gozalbo 2006; Netea et al. 2006) and appears to be dependent upon the *C. albicans* isolates tested (Netea et al. 2010). However, the role of TLR4 against *C. glabrata* infection is unclear. Our data demonstrated that C. glabrata can establish infection in kidneys of TLR4(−/−) C3H/HeJ mice following urinary tract inoculation but not in other TLR4(+/+) CD1 or CBA/J mice. To determine whether TLR4 has role in *C. glabrata* infection in kidneys, we tested the growth of TLR4(+/−) C3H/HeOuJ mice, which have a similar genetic background as C3H/HeJ mice. Our experiments showed that the C. glabrata wild-type strain CBS138 exhibited a similar fungal burden in the kidneys and bladders of TLR4(+/−) C3H/HeOuJ and TLR4(−/−) C3H/HeJ mice in a urinary tract infection model (Figure 3B), suggesting that
We tested whether fungal calcineurin uses different pathogenesis mechanisms to cause infections in the host depending on the infection location (Chen et al. 2010a). The requirement of C. albicans calcineurin for mammalian virulence depends on the specific niche (Bader et al. 2006). For example, calcineurin controls virulence in murine systemic infections, but not in vaginal or pulmonary infection models (Bader et al. 2006). In addition, fungal calcineurin uses different pathogenesis mechanisms to cause infections in the host depending on the infection location (Chen et al. 2010a). We tested whether C. glabrata calcineurin is required for murine systemic infections. Unlike C. albicans and C. dublinensis, C. glabrata preferentially colonizes the spleen rather than kidney tissues. In splenic tissues, both cna1 and cnb1 mutants exhibited a 3.7-fold reduced fungal burden (P < 0.0001), whereas crz1 mutants had a 2.1-fold reduced fungal burden (P < 0.01) (Figure 3C) compared with the wild-type strain. The results of the calcineurin mutants in spleen tissues were similar to those reported by Miyazaki et al. (2010). However, we found that crz1 mutants exhibited reduced fungal burden in the spleen, while Miyazaki and colleagues reported no difference. The differential role of calcineurin mutants in the spleen between the two groups might be due to differences in mouse strains or experimental procedures. In kidney tissues, cna1 and cnb1 mutants exhibited 2.5-fold (P = 0.05) and 3.9-fold (P < 0.01) reduced fungal burdens compared with the wild-type strain, whereas crz1 mutants showed a 2.3-fold reduced fungal burden (P < 0.05) compared with the wild-type strain. Our results of fungal burden in the kidneys were consistent with those reported by Miyazaki et al. (2010). Taken together, C. glabrata calcineurin and Crz1 contribute to virulence in a murine systemic infection model.
Calcineurin pathway mutants are unable to establish murine ocular infection

C. glabrata is an uncommon yeast pathogen in ocular infections, but it has recently been reported with increasing frequency in post-keratoplasty patients, often resulting in ocular morbidity, including loss of vision (Al-Assiri et al. 2006; Caldwell et al. 2009; Grueb et al. 2006; Muzaliha et al. 2010). However, the mechanisms of ocular infection due to C. glabrata have not been studied. We used a murine ocular infection model to investigate the roles of the C. glabrata calcineurin pathway in ocular pathogenesis. The C. glabrata wild-type strain CBS138 established an ocular infection in 43% (6/14) of the immunocompetent CD1 mice, whereas the C. albicans wild-type strain SC5314 established infection in 100% (12/12) of the mice (Figure 4). Ocular infections due to C. albicans were significantly more severe than those caused by C. glabrata at all time points (P < 0.05; Figure S5B), representing the first comparison between C. albicans and C. glabrata in a murine ocular infection model. C. glabrata cna1, cnb1, and crz1 mutants were unable to establish infection, suggesting that the calcineurin pathway controls ocular pathogenesis.

C. glabrata wild-type strain CBS138 can establish murine ocular infection in immunocompetent mice, which is in contrast to the inability of C. dubliniensis to cause ocular infection in an immunocompetent mouse model (Chen et al. 2011). Because C. albicans successfully infected the cornea of immunocompetent mice (100%), we conclude that the relative ability of the three Candida species to cause murine ocular infection is C. albicans > C. glabrata > C. dubliniensis.

Calcineurin is essential for ER stress tolerance and cell wall integrity in C. glabrata

A straightforward explanation for the attenuated virulence of calcineurin mutants is their growth defects (plasma membrane disruption and slower growth) at body temperature (~37°C). However, it is possible that calcineurin is involved in other mechanisms necessary for the establishment and maintenance of infections. The maintenance of the ER stress response is required for successful infection by fungal...
pathogens. For example, Hac1, a transcription factor that controls the ER stress response and unfolded protein response (UPR), is required in A. fumigatus for virulence in a murine invasive aspergillosis model (Richie et al. 2009). The UPR is activated to induce molecular chaperones involved in protein folding upon the accumulation of unfolded or misfolded proteins in the lumen of the ER. Recently, Cheon et al. (2011) demonstrated that the C. neoformans UPR pathway (IRE1 and HXL1) is essential for ER stress tolerance, thermotolerance, and virulence in a murine intranasal infection model. Because C. glabrata calcineurin mutants exhibit similar stress responses to those of C. neoformans UPR pathway mutants, it is possible that calcineurin might function together with the UPR pathway to orchestrate multiple phenotypes in C. glabrata. Thus far, it is unclear whether the ER stress response is required for virulence of C. glabrata.

A link between ER stress response and calcineurin is known in S. cerevisiae (Bonilla et al. 2002), in the plant fungal pathogen Ustilago hordei that causes covered smut of barley (Cervantes-Chavez et al. 2011), and in the protozoan parasite L. major, which causes leishmaniasis in humans (Naderer et al. 2011). For example, U. hordei cna1 and cnb1 mutants were hypersensitive to the chemical ER stressors tunicamycin (TM; blocks the synthesis of N-linked glycoproteins in the ER) and dithiothreitol (DTT; disrupts the formation of disulfide bonds and leads to retention of proteins in the ER) (Cervantes-Chavez et al. 2011). Because Crz1, a downstream target of calcineurin, has not been identified in U. hordei or other basidiomycetes, it will be interesting to investigate whether this ER stress response is mediated by Crz1. We demonstrated that the ER stress response is associated with calcineurin and Crz1 function in C. glabrata. The cna1 and cnb1 mutants exhibited hypersensitive growth on media containing TM or DTT, whereas crz1 mutants showed intermediate phenotypes between the wild-type strain and calcineurin mutants (Figure 5A).

There is limited information linking ER stress and cell wall integrity in fungi. In S. cerevisiae, ER stress has been demonstrated to activate cell wall integrity (CWI) signaling via mitogen-activated protein kinase Shk2/Mpk1 (Krysan 2009; Scrimale et al. 2009). In Candida, Cryptococcus, and Aspergillus species, calcineurin is required for cell wall integrity (Chen et al. 2010a). Here, we showed that calcineurin but not crz1 mutants are hypersensitive to agents that damage the cell wall or membrane, including calcifluor white, Congo red, and SDS at 24°C (Figure 5, A and B). In accord with TEM images (Figure 2), calcineurin and crz1 mutants are hypersensitive to the membrane-damaging agent SDS at 37°C (Figure 5B). The cell wall integrity of Candida species has been demonstrated to be required for virulence in murine systemic infection models (Chen et al. 2010b). Thus, the requirement of calcineurin in cell wall/membrane integrity may affect the pathogenicity of C. glabrata.
Calcineurin controls pH homeostasis in C. glabrata

How fungal pathogens sense environmental pH changes is central to virulence. In _C. albicans_, calcineurin appears to be required for pH homeostasis (Kullas et al. 2007). However, the requirement of calcineurin for pH homeostasis had not been studied in _C. glabrata_. We found that _cna1_ and _cnb1_ mutants were hypersensitive to both acidic (pH 2) and alkaline (pH 8) conditions (Figure 5C), whereas _crz1_ mutants exhibited sensitivity similar to the wild-type, suggesting pH homeostasis is governed solely by calcineurin and not Crz1 in _C. glabrata_.

The _C. glabrata_ GPI-anchored aspartyl protease Yps1 has been demonstrated to be required for pH homeostasis (Bairwa and Kaur 2011) and virulence in a murine systemic infection model (Kaur et al. 2007). Miyazaki et al. (2011b) showed that transcription of _CgYPS1_ was regulated by calcineurin in _C. glabrata_. On the basis of previous studies and ours, we suggest that calcineurin may control pH homeostasis and virulence via Yps1 in _C. glabrata_. The pH of mouse urine is about 5.3, and the pH of blood is nearly 7.2. Because calcineurin is essential for growth at pH 2 and pH 8 but not pH 5 (Figure 5), we suggest that the requirement of calcineurin in pH homeostasis may play a limited role in causing infections.

Optimal growth in serum is controlled by calcineurin but not Crz1 in _C. glabrata_

The survival and proliferation of fungal pathogens in host serum are essential for a successful bloodstream infection. The growth defects of calcineurin mutants in serum have been linked to attenuated virulence of _C. albicans_ and _A. fumigatus_ (Blankenship and Heitman 2005; Steinbach et al. 2007). For example, _C. albicans_ calcineurin is required for survival in calcium stress, and _A. fumigatus_ calcineurin is required for survival in the low-phosphate stress of serum (Chen et al. 2010a). Because _C. glabrata_ calcineurin mutants exhibited attenuated virulence in a murine systemic infection, we investigated whether serum inhibits the growth of _C. glabrata_ calcineurin mutants. Calcineurin, but not _crz1_ mutants, are hypersensitive to serum-containing medium (Figure 6A). We also performed a time-kill assay to determine whether serum is lethal to calcineurin mutants. _C. glabrata_ calcineurin mutants were not killed in serum, but their growth was arrested, whereas the growth of the _crz1_ mutant in serum is similar to that of the wild-type strain (Figure 6B), suggesting the growth arrest in serum was linked to calcineurin but not to Crz1. The addition of the calcium chelators bis(o-aminophenoxy)ethane tetraacetic acid (BAPTA) or ethylene glycol tetraacetic acid (EGTA) to serum did not rescue the growth of calcineurin mutants (data not shown), indicating _C. glabrata_ employs calcineurin in a mechanism distinct from _C. albicans_ to proliferate in serum. In a liquid growth assay in a polystyrene plate, calcineurin mutants exhibited reduced growth in serum compared with the wild-type strain or _crz1_ mutants, whereas in control YPD medium alone there was no difference in growth between
the calcineurin mutants and the wild-type strain (Figure 6C). Our data also support the roles of calcineurin in a murine systemic infection model because attenuated virulence of the calcineurin mutants may be in part due to growth arrest in serum.

**Antifungal drug susceptibility is controlled by calcineurin and Crz1 in *C. glabrata***

*C. glabrata* is notorious for its antifungal drug tolerance, which contributes to difficulty in treating infections associated with this species. Our studies demonstrated that calcineurin contributes to virulence in murine urinary tract, systemic, and ocular infections due to *C. glabrata*. Thus, *C. glabrata* calcineurin is a drug target worthy of investigation. Calcineurin mutants (*cna1* and *cnb1*) are hypersensitive to the azoles fluconazole, posaconazole, and ketoconazole (Figure 7A), and to the echinocandins micafungin, caspofungin, and anidulafungin (Figure 7B). In disk diffusion assays, calcineurin mutants exhibit larger and clearer zones of inhibition in the presence of fluconazole or micafungin than the wild-type strain (Figure 7C). Our data suggest that combination therapy with a calcineurin inhibitor and fluconazole or micafungin may improve the treatment outcome of *C. glabrata* infections.

Interestingly, *crz1* mutants showed differential responses to azoles and echinocandins. Unlike the intermediate hypersensitivity phenotypes of *crz1/crz1* mutants in *C. albicans* and *C. dubliniensis* (Chen et al. 2011), *C. glabrata* *crz1* mutants exhibit hyperresistance to azoles (Figure 7A), suggesting that Crz1 might repress genes involved inazole tolerance in *C. glabrata*. In response to echinocandins, *crz1* mutants showed an intermediate phenotype between the wild-type and the calcineurin mutants (Figure 7B), suggesting that echinocandin tolerance is mediated by a calcineurin-dependent Crz1 pathway.

**Genome-wide analysis of calcineurin- and Crz1-dependent targets in *C. glabrata***

To better understand the multiple phenotypes of calcineurin pathway mutants and to identify calcineurin pathway–regulated genes in *C. glabrata*, we compared the genome-wide transcriptome profile of a wild-type strain with an FK506-treated wild-type strain, a *cna1* mutant, and a *crz1* mutant (Figure 8A). We expected that the FK506-treated wild-type strain would have a similar expression profile to that of the *cna1* mutant. The genes regulated by FK506 largely overlapped (90%, 180/200) with differentially expressed genes in a *cna1* mutant (Figure 8B). By looking at calcineurin-upregulated and calcineurin-downregulated genes, the FK506 group overlapped 96% (107/112) and 83% (73/88), respectively, with the *cna1* mutant (Figure 8B). Interestingly, 38% (33/87) of *crz1*-dependent genes are Cna1-independent (Figure 8B), indicating that proteins other than calcineurin might regulate Crz1 and result in differential expression profiles compared with the calcineurin-dependent Crz1 pathway. The genes regulated by FK506 exhibited 29% (32/112, upregulated) and 3% (3/88, downregulated) overlap, respectively, with differentially expressed genes in a *crz1* mutant (Figure 8B), suggesting a strong correlation between FK506-treated wild-type and *cna1* mutant, but not with the *crz1* mutant. In addition, FK506 treatment of wild-type cells resulted in differential expression of 20 genes (Table S2) distinct from those in the *cna1* mutant, including genes encoding plasma membrane ATP-binding cassette (ABC) multidrug transporters (*PDR5, PDR15*, and *YOR1*), the bile acid transporter YBT1, and the transcription factor INO2, possibly due to FK506 inhibition of FKBP12 or other FKBP5s or to direct action on multidrug resistance (MDR) pumps.

Because the requirement of calcineurin in *C. glabrata* is different from *C. albicans* in terms of thermal stress tolerance and optimal serum growth, it is of interest to explore the calcineurin and Crz1 targets in *C. glabrata*. We identified 34 genes that are regulated by...
both calcineurin and Crz1 (Figure 9A), some of which encode proteins involved in cell wall biosynthesis (YPS5, YPS1, YSR3, YPC1, PIR1, HOR7, and NTE1), heat shock protein responses (SBA1 and GRE2), and calcineurin regulation (RCN2). Among 34 genes, 30 genes are upregulated and 3 genes are downregulated by the calcineurin pathway, whereas 1 gene (LIP5) is upregulated in the FK506-treated wild-type and cna1 mutant and is downregulated in the crz1 mutant (Figure 9A). Although the transcription levels of another calcineurin regulator (RCN1) and calcineurin binding protein 1 (CBP1) have been demonstrated to be upregulated by calcineurin and Crz1 in S. cerevisiae and by Crz1 in M. oryzae (Kim et al. 2010; Yoshimoto et al. 2002), it was not regulated by C. glabrata calcineurin and Crz1. Our transcript profiling data suggest that the heat shock response genes (SBA1 and GRE2) regulated by calcineurin and Crz1 may be involved in controlling thermal stress tolerance in C. glabrata. Consistent with a recent study that CgYPS1 was regulated by Cnb1 and Crz1 in C. glabrata (Miyazaki et al. 2011b), our microarray data support this finding and provide a comprehensive view of the transcriptome regulated by the calcineurin pathway. Real-time RT-PCR analysis confirmed that the transcription of CNA1 and CRZ1 was abolished in the cna1 and crz1 mutants, respectively (Figure 9B). The transcription of two control genes (YPS5 and RCN2) determined by real-time RT-PCR was well correlated with the microarray data (Figure 9C).

**Rcn2 is a feedback inhibitor of calcineurin signaling**

Regulator of calcineurin 2 (RCN2/YOR220w) was first identified a decade ago in S. cerevisiae with a genetic screen for endogenous inhibitors of calcineurin (Kingsbury and Cunningham 2000). The transcription of ScRCN2 is activated by calcineurin and Crz1 (Mehta et al. 2009). In C. albicans, microarray data showed that the transcription of CaRCN2 (IPF12084/ORF19.6554, function uncharacterized)
was activated in a calcineurin- and Crz1-dependent manner (Karababa et al. 2006). Miyazaki et al. (2011a) recently showed that transcription of CgRCN2 was regulated by C. glabrata Cnb1 and Crz1. According to our microarray analysis and real-time RT-PCR, we independently demonstrated that the transcription of CgRCN2 was strongly activated by Cna1 and Crz1 in C. glabrata. To test whether Rcn2 is a downstream target of calcineurin and Crz1, we constructed rcn2 mutants and found that calcineurin-controlled cellular functions, such as thermostolerance, drug tolerance, ER stress, and pH homeostasis, were not affected by deletion of RCN2 (Figure 10, A and B), indicating that Rcn2 is not a functional target of calcineurin in C. glabrata. Instead, we found that rcn2 mutants were hypersensitive to Ca2+ (Figure 10, A and C), in contrast to the modestly increased Ca2+ tolerance of calcineurin mutants (Figure 10D, left panel). In S. cerevisiae, cells lacking calcineurin grow better in the presence of high concentrations of Ca2+, suggesting that calcineurin represses Ca2+ tolerance in C. glabrata (Figure 11). The mechanisms by which genes control calcium tolerance are unclear in C. glabrata. Vacuolar Ca2+ ATPase (Pmc1) and vacuolar Ca2+/H+ exchanger (Vcx1) are required for calcium tolerance in S. cerevisiae (Cunningham and Fink 1996); therefore, we hypothesize that the homologs of Pmc1 and Vcx1 also control calcium tolerance in C. glabrata, and it is possible that calcineurin inhibits Pmc1 or Vcx1 via dephosphorylation in C. glabrata. Interestingly, Ca2+ can rescue the temperature-sensitive growth of C. glabrata calcineurin mutants at 40°C (Figure 10D, right panel), indicating that Ca2+ may activate a specific signaling cascade that supports growth at an elevated temperature.

In S. cerevisiae, Rcn2 can inhibit calcineurin activity, and truncation mutations that removed the PxxIxxT-like motif [P/G][V/I][F/V/L] [E/D/N/H/T] of Rcn2 abolished its inhibition of calcineurin signaling (Mehta et al. 2009). We found that C. glabrata and C. albicans Rcn2 also have a PxxIxxT-like motif (Figure S6B), indicating calcineurin inhibition mediated by Rcn2 might be conserved in these three species. Interestingly, the TxxP motif required for calcineurin stimulation found in ScRcn1 and ScRcn1 is not found in CaRcn1 (Figure S6A) or in Rcn2 of S. cerevisiae, C. glabrata, and C. albicans (Figure S6). The synteny analysis of Rcn1 and Rcn2 in S. cerevisiae, C. glabrata, and C. albicans suggests that C. albicans calcineurin regulators are diverged from S. cerevisiae and C. glabrata (Figure S7).

Figure 9 Genes regulated by the calcineurin pathway in C. glabrata. (A) List of 34 genes regulated by calcineurin and Crz1. There are 30 genes that were upregulated by calcineurin and Crz1, 3 genes downregulated by calcineurin and Crz1, and 1 gene (LIP5) upregulated by calcineurin but downregulated by Crz1. The criteria used to classify genes as upregulated or downregulated in the microarray experiments is a cutoff Q value < 0.001. (B) The transcription of CNA1 and CRZ1 was abolished in the cna1 (YC98) and crz1 (YC182) mutants, respectively. Real-time RT-PCR was used to measure the expression level of each gene. (C) Real-time RT-PCR was used to independently assess the expression levels detected in microarray analyses for YPS5 and RCN2 in wild-type, FK506-treated wild-type, cna1 mutant (YC98), and crz1 mutant (YC182). Statistically significant differences (P < 0.05 based on ANOVA, Bonferroni’s multiple comparison tests) compared with the wild-type are indicated by the asterisks.
a negative regulator of calcineurin in *C. glabrata*. To test this hypothesis, we generated a set of calcineurin pathway and *rcn2* double mutants. The *cna1* *rcn2* and *cnb1* *rcn2* (but not *crz1* *rcn2*) double mutants exhibited a reversal of the Ca\(^{2+}\) hypersensitivity of the *rcn2* mutant, and the addition of FK506 to Ca\(^{2+}\)-containing media also rescued the growth of *rcn2* mutants (Figure 10C). Taken together, our data suggest that Rcn2 serves as a negative regulator of calcineurin and that calcineurin suppresses calcium tolerance in *C. glabrata* (Figure 10D). Interestingly, Rcn2 is not required for virulence in a murine systemic infection model (Figure 10E).

**Rcn1 controls cellular functions similar to calcineurin in *C. glabrata***

Regulator of calcineurin 1 (Rcn1) was first identified in fungi (Gorlach et al. 2000; Kingsbury and Cunningham 2000) and mammals (Fuentes et al. 2000; Rothermel et al. 2000) on the basis of its ability to interact with and inhibit calcineurin when overexpressed. In *S. cerevisiae*, Rcn1 can also stimulate calcineurin signaling (Kingsbury and Cunningham 2000). Rcn1 was independently identified as Cbp1 in a screen for calcineurin-binding proteins in *C. neoformans* and shown to be required for full virulence in a murine systemic infection model (Gorlach et al. 2000). In *C. albicans*, Rcn1 (Ipf13909/Orf19.7770) controls cell membrane integrity and azole tolerance, but not pathogenicity, in murine systemic infection and oropharyngeal candidiasis models (Reedy et al. 2009). In contrast to *S. cerevisiae* (Yoshimoto et al. 2002), we found that calcineurin and Crz1 do not transcriptionally regulate Rcn1 in *C. glabrata*. It is possible that Rcn1 physically interacts with calcineurin to regulate calcineurin signaling. Thus, Rcn1 and calcineurin may act on shared downstream targets. Miyazaki et al. (2011a) recently showed that Rcn1 controls ER stress response and drug tolerance in *C. glabrata*. Our *rcn1* mutants exhibited similar phenotypes and have additional phenotypes, such as hypersensitive growth to altered pH (acidic and alkaline) and elevated temperature (41°C) (Figure 10, A and B), and modest resistance to Ca\(^{2+}\) (Figure 10A). Unlike *C. neoformans* *cbp1* mutants, *C. glabrata* *rcn1* mutants did not exhibit attenuated virulence compared with the wild-type strain in a murine systemic infection model (Figure 10E). Overall, calcineurin mutants exhibit more severe phenotypes than *rcn1* mutants, suggesting that Rcn1 is only partially required for calcineurin function. The *rcn1* *rcn2* double mutant had a virulence level similar to the wild-type strain (\(P = 0.1\) in spleen; \(P = 0.47\) in kidney) in a murine systemic infection model (Figure 10E), and exhibited phenotypes similar to the *rcn1* mutant. This indicates that *rcn1* mutation is epistatic to the *rcn2* mutation in *C. glabrata*. Rcn1 may play a larger role in maintaining calcineurin signaling than the role of Rcn2 in calcineurin inhibition.
stress responses, drug tolerance, and virulence in C. glabrata. Calcineurin and Crz1 are required for various stress responses, including thermotolerance, ER stress, echinocandin tolerance, cell membrane integrity, and virulence. Calcineurin also exhibits Crz1-independent functions, including pH homeostasis and optimal growth in serum. Calcineurin (Cna1 or Cnb1) suppresses calcium tolerance, whereas Crz1 inhibits azole tolerance. Regulator of calcineurin 1 (Rcn1) is an endogenous positive regulator of calcineurin that participates in the control of multiple cellular processes, including thermotolerance, ER stress response, and echinocandin tolerance. Rcn2 acts as a feedback inhibitor of calcineurin function in calcium tolerance. Solid arrows represent promoting functions, and bar-headed arrows represent inhibitory functions within the calcineurin signaling cascade. The dotted arrow represents function as seen in S. cerevisiae, but undetermined in C. glabrata.

In mammalian cells, regulator of calcineurin 1 (DSCR1/RCAN1/calciopressin) is preferentially expressed in the brain of patients with Down’s syndrome and contributes to tumor suppression, suggesting DSCR1 could be explored as a potential therapeutic target (Back et al. 2009). The difference in virulence of C. neoformans ebp1 and C. glabrata rcn1 mutants suggests a divergent role of Cbp1/Rcn1 in controlling pathogenicity between C. neoformans and C. glabrata. Our data also showed that Rcn1 controls azole and echinocandin tolerance (Figure 10A), making drug combination therapy with yet-to-be-developed Rcn1 inhibitors and azoles or echinocandins possible approaches to target emerging C. glabrata infections.

CONCLUSIONS
We have demonstrated in this study that calcineurin and Crz1 contribute to the control of thermotolerance, ER stress response, echinocandin tolerance, cell membrane integrity, and pathogenesis in C. glabrata (Figure 11). In addition, calcineurin, but not Crz1, contributes to pH homeostasis, serum growth, and azole tolerance (Figure 11). In contrast to C. albicans, C. glabrata calcineurin modestly inhibits calcium tolerance, suggesting a divergent role of calcineurin in governing calcium tolerance between C. glabrata and C. albicans. Unlike the role of Crz1 as the positive regulator that controls azole tolerance in C. albicans, C. glabrata Crz1 acts as a negative regulator of azole tolerance (Figure 11), indicating rewiring of this transcription factor among Candida species. The calcineurin regulator Rcn1 positively regulates calcineurin functions, including thermotolerance, ER stress response, and echinocandin tolerance, but not cell membrane integrity and virulence. The other calcineurin regulator, Rcn2, acts as a feedback inhibitor of calcineurin signaling (Figure 11). In the future, determining how calcineurin interacts with other signaling pathways to control core stress responses and how Rcn1 and Rcn2 interact with calcineurin will contribute to our understanding of calcineurin biology and signal transduction.

ACKNOWLEDGMENTS
We thank Cecelia Shertz and Joanne Kingsbury for edits and comments on the manuscript. We acknowledge members of the Heitman and Cardenas laboratories for many helpful discussions. We also thank Sheena Singh-Babak and Leah Cowen for discussion and communication of unpublished results; Mitchell Mutz for providing posaconazole; William Steinbach for anidulafungin; Astellas Pharma for micafungin; Merck & Co., Inc. for caspofungin; Wenhun Li for synten analysis; Cheryl Chan and Anna Averette for assistance with murine urinary and systemic infection models; and Fedelino Malbas, Jr., and the Histopathology Department of the Regional Institute for Tropical Medicine, Philippines, for murine ocular infection models.

This work was supported in part by the Duke University Center for AIDS Research (CFAR), assistance with National Institutes of Health (NIH)-funded program (2P30 AI064518-06 to Y.-L. C.) and NIH/NIADA R01 grant AI50438 (J.H.). This work was also supported by Pilot funds from Astellas Pharma, Inc. and Merck & Co., Inc. (J.H. and Y.-L.C.). The funders had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript.

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Communicating editor: D. J. Gresham

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