Calcineurin Controls Hyphal Growth, Virulence, and Drug Tolerance of Candida tropicalis

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Candida tropicalis, a species closely related to Candida albicans, is an emerging fungal pathogen associated with high mortality rates of 40 to 70%. Like C. albicans and Candida dubliniensis, C. tropicalis is able to form germ tubes, pseudohyphae, and hyphae, but the genes involved in hyphal growth machinery and virulence remain unclear in C. tropicalis. Recently, echinocandin- and azole-resistant C. tropicalis isolates have frequently been isolated from various patients around the world, making treatment difficult. However, studies of the C. tropicalis genes involved in drug tolerance are limited. Here, we investigated the roles of calcineurin and its potential target, Crz1, for core stress responses and pathogenesis in C. tropicalis. We demonstrate that calcineurin and Crz1 are required for hyphal growth, micafungin tolerance, and virulence in a murine systemic infection model, while calcineurin but not Crz1 is essential for tolerance of azoles, caspofungin, anidulafungin, and cell wall-perturbing agents, suggesting that calcineurin has both Crz1-dependent and -independent functions in C. tropicalis. In addition, we found that calcineurin and Crz1 have opposite roles in controlling calcium tolerance. Calcineurin serves as a negative regulator, while Crz1 plays a positive role for calcium tolerance in C. tropicalis.

Candida tropicalis is one of the most common Candida species that causes disease in humans, especially in tropical climates. C. tropicalis is responsible for 3 to 66% of cases of candidemia, depending on the geographic region (1–3). Non-albicans Candida species (NACS), including C. tropicalis, C. glabrata, C. krusei, C. dubliniensis, and C. parapsilosis, have increasingly been responsible for nosocomial bloodstream infections (4, 5) and account for almost 50% of nonsuperficial Candida infections (6). Mortality rates of 40 to 70% have been associated with the presence of C. tropicalis in the bloodstream, and these rates can be affected by other factors, such as leukemia, neutropenia, central venous catheters, parental nutrition, and extended time in intensive care units (7–9).

Within the last few years, C. tropicalis drug-tolerant or -resistant isolates have frequently been isolated from patients and environmental samples (10–14). For example, García-Effron et al. showed that 7.5% (3/40) of clinical C. tropicalis isolates were caspofungin resistant owing to amino acid substitutions in beta-1,3-glucan synthase (Fks1p) that resulted in caspofungin-based therapy failures (10). An Asian national antifungal surveillance program found reduced susceptibility of C. tropicalis to fluconazole (12). Recently, Yang et al. reported that C. tropicalis strains isolated from environmental soil also showed reduced susceptibility to medical and agricultural azoles, advocating for the prudent use of azoles in agriculture (11). So far, few studies have focused on C. tropicalis drug resistance mechanisms. For example, Jensen et al. demonstrated that an S80P mutation of Fks1p leads to echinocandin resistance in C. tropicalis (15). Vandeputte et al. found that overexpression of C. tropicalis ERG11 (CtERG11), the gene encoding lanosterol 14α-demethylase, is associated with a missense mutation that might be responsible for the acquired azole resistance of a clinical C. tropicalis isolate (16). Eddouzi et al. showed that CtERG3 and CtERG11 mutations participate in azole resistance (17). Chen et al. demonstrated that the loss of heterozygosity of FCY2, a gene encoding purine-cytosine permease, enables C. tropicalis to develop flucytosine resistance (18). Thus, the mechanisms that C. tropicalis deploys for drug resistance still remain elusive and require further investigation.

The ability to undergo a morphogenic switch between yeast and hyphal growth is a major virulence factor for Candida albicans (19). For example, mutants locked in either the pseudohyphal (tup1/tup1) or yeast (cph1/cph1 egf1/egf1) form exhibit attenuated virulence in murine systemic infection models (20, 21). Although dimorphic transitions have been extensively studied in C. albicans, their studies in C. tropicalis are limited. For example, Porman et al. demonstrated that the overexpression of C. tropicalis WOR1 (CtWOR1), a master regulator of the white-opaque switch (22), promotes filamentous growth and biofilm formation of C. tropicalis (23). Thus, it will be of interest to study the C. tropicalis genes involved in dimorphic transitions and virulence.

Calcineurin, a potential drug target in fungi, is a calcium/calmodulin-dependent serine/threonine-specific protein phosphatase that is comprised of a catalytic subunit A (Cna1) and a regul
C. tropicalis strains used in this study

| Candida tropicalis strain | Genotype | Background |
|---------------------------|----------|------------|
| MYA3404                   | Prototrophic wild type | Clinical isolate |
| YC130                     | cnb1Δ::SAT1-FLP/CNB1   | MYA3404 |
| YC146                     | cnb1Δ::FRT/CNB1        | YC130   |
| YC454                     | cnb1Δ::FRT/cnb1Δ::SAT1-FLP | YC146   |
| YC132                     | cnb1Δ::SAT1-FLP/CNB1   | MYA3404 |
| YC142                     | cnb1Δ::FRT/CNB1        | YC132   |
| YC466                     | crz1Δ::SAT1-FLP/CNZ1   | MYA3404 |
| YC188                     | crz1Δ::FRT/CNZ1       | YC173   |
| YC494a                    | crz1Δ::FRT/crz1Δ::SAT1-FLP | YC188   |
| YC176                     | crz1Δ::SAT1-FLP/CNZ1   | MYA3404 |
| YC190                     | crz1Δ::FRT/CNZ1       | YC176   |
| YC499c                    | crz1Δ::FRT/crz1Δ::SAT1-FLP | YC190   |

a Two independent cnb1/cnb1 mutants.

b Two independent crz1/crz1 mutants.

c The source of all strains except the clinical isolate was this study.

Roles of Calcineurin and Crz1 in *C. tropicalis*

C. tropicalis calcium binding subunit (Cnb1). Upon stimulation with calcium, calmodulin associates with the calcineurin A C-terminal domain, stimulating phosphatase activity by dislodging the auto-inhibitory domain and converting signals to downstream targets, such as the transcription factor Crz1, by dephosphorylation. Dephosphorylated Crz1 migrates into the nucleus and regulates gene expression. Because active calcineurin is an AB heterodimer, the loss of the Cnb1 subunit often results in destabilization of the Cna1 catalytic subunit (24). Although the roles of calcineurin in hyphal growth of *C. albicans*, if any, remain unclear, calcineurin is required for hyphal growth in several fungal pathogens, including *C. dubliniensis*, *Aspergillus fumigatus*, and *Magnaporthe oryzae* (24).

In this study, we comprehensively studied the roles of calcineurin and Crz1 in hyphal growth in vitro, virulence, drug tolerance, and other stress responses in *C. tropicalis*. We demonstrated that *C. tropicalis* calcineurin and Crz1 are required for hyphal growth, micafungin tolerance, and virulence in a murine systemic infection model. Meanwhile, *C. tropicalis* calcineurin but not Crz1 was shown to govern azole tolerance and cell wall integrity. Our data suggest that calcineurin is a potential drug target and calcineurin inhibitors could be combined with current antifungal drugs for therapy.

**MATERIALS AND METHODS**

**Yeasts strains, media, and chemicals.** The *C. tropicalis* strains used in this study are listed in Table 1. The following media were used in this study: yeast extract-peatone-dextrose (YPD; 1% yeast extract, 2% peptone, 2% glucose) liquid medium and agar (2%), yeast extract-malt extract-agar (2% yeast extract, 1% peptone, 2% maltose) (YPD) medium was used to amplify 5′-end ORF terminal primers and resulted in nourseothricin-resistant mutants (YC130 and YC132; Table 1) obtained from two separate transformations. Liquid YPM (1% yeast extract, 2% peptone, 2% maltose) medium was used to drive expression of the FLP recombinase under the control of the *C. albicans* MAL2 promoter. The SAT1 flipper was then excised, which left an FLP recombinase target (FRT) sequence and resulted in nourseothricin-resistant mutants (YC130 and YC132; Table 1). The first allele of the CNB1 gene was disrupted in wild-type strain MYA3404 by transformation with 0.2 to 1 μg of gel-purified disruption DNA using a Frozen-EZ yeast transformation kit (Zymo Research). Two independent heterozygous nourseothricin-resistant mutants (YC130 and YC132; Table 1) were obtained from two separate transformations. Liquid YPM (1% yeast extract, 2% peptone, 2% maltose) medium was used to drive expression of the FLP recombinase under the control of the *C. albicans* MAL2 promoter. The SAT1 flipper was then excised, which left an FLP recombinase target (FRT) sequence and resulted in nourseothricin-sensitive CNB1/cnb1 mutant strains (YC146 and YC142).

**Strain construction.** Both alleles of the *C. tropicalis* CNB1 and CRZ1 genes were disrupted with the SAT1 flipper (25). For CNB1 gene disruption, approximately 1-2 kb 5′ (amplified with primers JC182/1C183; see Table S1 in the supplemental material) and 3′ (amplified with primers JC184/JC185) noncoding regions (NCRs) of the CNB1 open reading frame (ORF) (CNB1NCR) were PCR amplified from genomic DNA of genome-sequenced reference strain MYA3404 (26). The 4-2 kb SAT1 flipper sequence was amplified from plasmid pSFS2A (25) with primers JC17/JC18. The three PCR products were treated with ExoSAP-IT (USB Corp.) to remove contaminating primers and deoxyxynucleoside triphosphates and then combined in a 1:3:1 molar ratio (5′ CNB1NCR, SAT1 flipper, and 3′ CNB1NCR) to generate the disruption allele by overlap PCR using flanking primers JC186/JC187 (which are ~100 bp closer to the CNB1 ORF than JC182/JC185, respectively, with primers JC182/JC185 being reserved for use for further integration confirmation), resulting in an ~6-5 kb CNB1ncr/SAT1 flipper-5′ CNB1ncr CNB1 disruption allele.

The first allele of the CNB1 gene was disrupted in wild-type strain MYA3404 by transformation with 0.2 to 1 μg of gel-purified disruption DNA using a Frozen-EZ yeast transformation kit (Zymo Research). Two independent heterozygous nourseothricin-resistant mutants (YC130 and YC132; Table 1) were obtained from two separate transformations. Liquid YPM (1% yeast extract, 2% peptone, 2% maltose) medium was used to drive expression of the FLP recombinase under the control of the *C. albicans* MAL2 promoter. The SAT1 flipper was then excised, which left an FLP recombinase target (FRT) sequence and resulted in nourseothricin-sensitive CNB1/cnb1 mutant strains (YC146 and YC142).

**Serial dilution growth assays.** Cells were grown overnight at 30°C and washed twice with distilled H2O (dH2O), and the optical density at 600 nm (OD600) was measured. Cells were resuspended in an appropriate volume of dH2O to achieve 1 OD unit/ml. Three microliters of 5-fold serial dilutions of each strain was spotted onto solid medium with a multichannel pipette. The plates were then incubated at the temperatures indicated below for 48 h and photographed.

**Growth curve and doubling time measurement.** To determine whether the loss of calcineurin and Crz1 affects cell growth at 37°C, we measured the growth curves and doubling times of the strains. For growth curves, cells were grown overnight at 30°C, washed twice with dH2O, diluted to 0.1 OD600 unit/ml in fresh YPD medium, and incubated at 37°C with shaking at 200 rpm. The OD600 of the cultures was measured at 0, 3,
6, 9, 12, 24, 48, 72, and 96 h via microplate spectrophotometer readings (Spectra MAX 190; Molecular Devices). The experiments were performed in triplicate, and the data were plotted using Prism (version 5.03) software.

The doubling time was calculated by using the formula T=ln2/(ln(OD/TOD)) where OD and TOD represent the OD_{oo} at time T and the initial time (time zero), respectively. The log-phase time points from 0 to 6 h were chosen.

**Murine systemic infection model.** Five- to 6-week-old male CD1 mice from The Jackson Laboratory (n = 10 for each group, except n = 9 for the wild type) were used in this study. This was a single experiment because 10 mice per group provided sufficient power to obtain statistically significant P-values. *C. tropicalis* strains were grown in 5 ml YPD overnight at 30°C with shaking at 250 rpm. 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 amount of PBS to obtain an inoculum of 2.5 × 10^7 cells/ml. Two hundred microliters (5 × 10^6 cells) were used to infect mice by lateral tail vein injection. The course of infection was monitored for up to 42 days. The survival of mice was monitored twice daily, and moribund mice (mice that were unable to eat or drink, whose body weight was reduced by >30%, or that were hunched) were euthanized with CO_2.

All experimental procedures were carried out according to NIH guidelines and Duke IACUC protocols for the ethical treatment of animals. Appropriate dilutions of the cells were plated onto YPD and incubated at 30°C for 48 h to confirm the numbers of CFU and viability.

To determine fungal burden, both kidneys and the spleen of *C. tropicalis*-infected mice (n = 5 for each strain) were dissected at day 10 postinfection. Half-organ portions were weighed, transferred to a 15-ml Falcon tube filled with 5 ml PBS, and homogenized for 10 s at 13,600 rpm/min (Power Gen 500; Fisher Scientific). Tissue homogenates were serially diluted, and 100 μl was plated onto a YPD plate. The plates were incubated at 30°C for 48 h to determine the number of CFU per gram of kidney or spleen. The identity of the colonies recovered from the organs was confirmed by PCR and by growth or no growth on YPD medium containing 0.01% SDS (for cnb1/cnb1 mutants) or 0.4 M CaCl_2 (for ccr1/ccr1 mutants). The significance of differences in fungal burden was determined using one-way analysis of variance (ANOVA) and Dunnett’s multiple-comparison test. For histopathological analysis, half-organ samples of kidney and spleen were fixed in 10% phosphate-buffered formalin (Fisher), and Gomori methenamine silver (GMS) and hematoxylin-eosin (H&E) stainings were performed by the Department of Pathology at Duke University. After slide preparation, each sample was examined by microscopy for analysis of Candida colonization (GMS) and tissue necrosis (H&E). Images were captured using an Olympus Vanox microscope (PhotoPath; Duke University Medical Center).

**Scanning electron microscopy.** The cultures were excised from the agar and fixed in 3% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 6.8) for 2 days at 4°C. They were then rinsed in three 30-min changes of 0.1 M Na cacodylate buffer (pH 6.8), followed by a graded dehydration series of 2-h changes in cold 30% and 50% ethanol (EtOH), and held overnight in 70% EtOH. Dehydration was completed with 1-h changes of cold 95% and 100% EtOH at 4°C and then warmed to room temperature in 100% EtOH. Two additional 1-h changes of room temperature 100% EtOH completed the dehydration series. The samples were then critical point dried in liquid CO_2 (Samdri-795; Tousimis Research Corp., Rockville, MD) for 15 min at the critical point. The agar pieces were mounted on stubs with double-stick tape, pressed down completely around the edge, and then sealed with silver paint to ensure good conductivity. The samples were then sputter coated with 50-Å Au/Pd (Hummer, version 6.2; Anatech USA, Hayward, CA). Samples were stored in a vacuum desiccator until they were viewed under a JEOL JSM 5900LV scanning electron microscope at 15 kV.

**Murine ocular infection model.** The *C. tropicalis* wild type (MYA4404), cnb1/cnb1 mutants (YCA454, YCA466), and ccr1/ccr1 mutants (YC494, YC499) and *C. albicans* strain SC5314 were grown overnight in YPD broth at 25°C. Ten milliliters of broth culture was pelleted by centrifugation at 3,000 rpm for 10 min and then washed three times with PBS (pH 7.4). Cells were resuspended in PBS and then diluted to a concentration equal to 10^6 CFU/μl. The concentration of *Candida* cells was determined by using the spectrophotometer optical density reading at a wavelength of 600 nm and multiplying it by a conversion factor in which 1 OD_{oo} unit is equivalent to 3 × 10^7 cells/ml. The numbers of CFU and cell viability were verified by plating cells onto YPD agar plates with incubation for 48 h at 25°C.

Six- to 8-week-old outbred ICR mice (22 to 30 g) were purchased from the Research Institute for Tropical Medicine (RITM), Alabang, Philippines. Animals were handled in accordance with the ARVO statement for the use of animals in ophthalmic and vision research. The murine keratomycosis induction protocol described previously for *C. dubliniensis* (27) was performed with minor modification and was approved by the University of Perpetual Help Institutional Review Board. Briefly, mice were immunocompromised by intraperitoneal administration of cyclophosphamide (180 to 200 mg/kg of body weight) on days 5, 3, and 1 prior to inoculation of the test strains. Before applying the inoculum, the mice were placed under general anesthesia by intramuscular injection of tiletamine hydrochloride-zolazepam hydrochloride (10 to 15 mg/kg of body weight; Zoletil 50; Virac, Australia), followed by topical application of proparacaine hydrochloride ophthalmic solution (Alcaine; Alcon-Couvreur, Belgium) in the right eyes until the blink sensation was lost. Excess solution in the eye was removed with a sterile cotton swab. Eyes were superficially scarified before applying the inoculum. An inoculum with 10^6 CFU/μl was distributed uniformly by rubbing the eye for a few seconds with the eyelid. Sterile PBS was applied in negative controls. Clinical scoring of disease severity of fungal keratitis was assessed for 8 days as described previously (27). The visual scoring system (28) evaluates three physical features of the eyes, namely, (i) the area of opacity, (ii) the density of opacity, and (iii) surface irregularity. A grade of 0 to 4 was assigned for each of these parameters to yield a maximum score of 12. At 4 and 8 days postinfection (p.i.), three mice were sacrificed by cervical dislocation. For mouse groups showing low infection rates (<6 mice), only 1 or 2 eyes were evaluated for histological evaluation after 8 days. Eyes were removed and fixed in neutral formalin solution (10% formaldehyde in PBS) before being submitted for histological staining and examination. Two-group comparisons were analyzed using Student’s t test. A P value of <0.05 was considered statistically significant.

**RESULTS**

**Identification of calcineurin and Crz1 orthologs in *C. tropicalis*.** The *C. tropicalis* orthologs of the *C. albicans* and Saccharomyces cerevisiae calcineurin regulatory subunit (CNB1) and the calcineurin target CRZ1 genes were identified by reciprocal BLAST searches between the two species and in all cases identified a reciprocal best BLAST hit ortholog as the *C. tropicalis* CNB1 (CTRGL_02450) and CRZ1 (CTRGL_02450) genes (26). *C. tropicalis* Cnb1 shares 91% and 61% identity over the full-length protein with its corresponding *C. albicans* and *S. cerevisiae* orthologs, respectively (see Fig. S1A in the supplemental material), while Crz1 shares 54% and 21% identity (see Fig. S2A in the supplemental material) over the full-length protein with its corresponding *C. albicans* and *S. cerevisiae* orthologs, respectively. *C. tropicalis* Cnb1 has four helix E-loop-helix F (EF) hand Ca^{2+} binding motifs (see Fig. S1B in the supplemental material), while Crz1 shares two C2H2 zinc finger domains with the respective orthologs in *C. albicans* and *S. cerevisiae* (see Fig. S2B in the supplemental material).

**Calcineurin is required for hyphal growth.** The *C. tropicalis* genes involved in the dimorphic transition, an important virulence factor, have not been identified. Based on previous studies on the roles of calcineurin in plant and human fungal pathogens...
We hypothesized that calcineurin signaling might be required for the dimorphic transition. To test this hypothesis, we disrupted the calcineurin (CNB1) and CRZ1 genes in the genome-sequenced C. tropicalis MYA3404 isolate. Here, we investigated the roles of calcineurin and Crz1 in hyphal growth. We demonstrated that calcineurin and Crz1 are required for hyphal growth in filament-inducing spider medium (carbon source starvation), while calcineurin but not Crz1 controls hyphal growth in another filament-inducing medium (50% serum or SLAD [nitrogen source starvation]) or nutrient-rich YPD medium (Fig. 1A). In liquid 100% serum, calcineurin mutants also exhibited attenuated hyphal growth, while crz1/crz1 mutants showed wild-type hyphal growth (Fig. 1B). Under a high-resolution scanning electron microscope, we found that calcineurin mutants exhibited mainly yeast and a few pseudohyphal forms, while crz1/crz1 mutants exhibited wild-type hyphae and invasive growth in solid 50% serum agar medium (Fig. 2).

Deletion of calcineurin and Crz1 attenuates virulence in mice. It has been demonstrated that C. tropicalis exhibits greater or reduced virulence in animal infection models than several C. albicans strains. However, in general, C. tropicalis is considered to be the second most virulent Candida species in mice, after C. albicans. Previous studies showed that C. tropicalis is able to colonize and form hyphae in murine kidneys. Here, we found that C. tropicalis calcineurin mutants (YC454 and YC466) exhibited significantly attenuated virulence, based on survival curves, compared with the wild type (P < 0.0001) (Fig. 3A). C. tropicalis wild-type strain MYA3404 caused 100% mortality of mice by day 10, while independent calcineurin mutants resulted in only 10% mortality, even after 42 days (Fig. 3A). Interestingly, the crz1/crz1 mutants (YC494 and YC499) exhibited intermediate virulence between the wild-type and calcineurin mutants (P < 0.001 compared to the wild type; P < 0.005 compared to calcineurin mutants). This suggests that calcineurin control of pathogenesis in mice is in part mediated by Crz1 in C. tropicalis (Fig. 3A).

To determine colonization ability, we performed fungal burden analyses in the kidneys and spleens of mice infected with the wild-type and mutant strains. In contrast to C. glabrata, but similar to C. albicans and C. dubliniensis, the C. tropicalis wild type preferentially colonized the kidneys rather than the spleen (Fig. 3B). The calcineurin mutants exhibited a 736-fold and 13-fold reduced fungal burden in the kidneys (P < 0.01) and spleens (P < 0.05), respectively, compared with the wild type (Fig. 3B). Meanwhile, the crz1/crz1 mutants (YC494 and YC499) exhibited a 5.2- and 4.8-fold reduced fungal burden in the kidneys (P = 0.04) and spleens (P = 0.12), respectively, compared with the wild type (Fig. 3B). Taken together, mice infected with calcineurin and crz1/crz1

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**FIG 1** Calcineurin is required for hyphal growth in C. tropicalis. (A) Hyphal growth of C. tropicalis wild-type (WT) and mutant strains on filament-inducing agar plates. Cells were grown overnight, washed twice with dH2O, and serially diluted to 10⁷ cells/ml (based on an OD₆₀₀ of 1 being equal to 4 × 10⁷ cells/ml). One hundred microliters containing ~100 cells was spread on a variety of filament-inducing media and incubated at 37°C for the number of days indicated. The experiments were repeated at least three times, and one representative image is shown. Bar = 0.1 mm. (B) Hyphal growth of C. tropicalis wild-type and mutant strains in liquid bovine calf serum (100%). Cell preparations were as described above with minor modifications. Two microliters of cells at an OD₆₀₀ of 1/ml were added to microtiter wells prefilled with 98 µl of 100% bovine calf serum, resulting in an OD₆₀₀ of 0.00004 (~1.6 × 10⁷ cells) in each well. Cultures in the 96-well polystyrene plates were incubated at 37°C without shaking for 24 h. Bar = 40 µm.
mutants exhibited a reduced fungal burden in the kidneys and a marginally reduced fungal burden in the spleen (Fig. 3B).

In histopathological analyses, similar to published studies for *C. albicans* and *C. dubliniensis* (27, 32), GMS-stained kidney tissues revealed that the *C. tropicalis* wild-type strain readily forms hyphae and proliferates extensively (Fig. 4, left). Here, we demonstrated that *C. tropicalis* calcineurin mutants had an impaired ability to colonize kidney tissues, while *crz1/crz1* mutants continued to form hyphae in the kidneys (Fig. 4, left). Colonization by the *C. tropicalis* wild type and mutants was not observed in the spleen (data not shown). In the H&E staining, tissue damage or necrosis...
was observed only in mice infected with the wild type or \textit{crz1/crz1} mutants and not in those infected with the calcineurin mutants (Fig. 4, right).

\textbf{Calcineurin controls ocular infection in a murine keratitis model.} \textit{C. tropicalis} and other \textit{Candida} species are frequently isolated worldwide in ocular regions of patients with candidemia or endophthalmitis (33–37). However, the mechanisms and genes that operate during \textit{C. tropicalis} ocular infections are largely unknown. Here, we investigated the roles of calcineurin for \textit{C. tropicalis} in a murine keratitis model. The corneal virulence of the \textit{C. tropicalis} wild type, \textit{crz1/crz1} mutants, and \textit{C. albicans} (Ca) type strain SC5314 and not in animals infected with \textit{C. tropicalis} calcineurin mutants. (B) Each cornea of an immunosuppressed mouse was inoculated with $10^8$ yeast cells of each strain, and the disease severity was scored for 8 days. \textit{C. albicans} type strain SC5314 served as a reference control. Mice infected with \textit{C. tropicalis} calcineurin mutants or the PBS control exhibited normal corneas. The disease scores of mice infected with the \textit{C. tropicalis} wild type or \textit{crz1/crz1} mutants and \textit{C. albicans} strain SC5314 exhibiting visible signs of keratitis were plotted.

\begin{figure}[h]
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\caption{\textit{C. tropicalis} calcineurin mutants are attenuated in a murine ocular infection model. (A) Clinical photographs of corneas of immunosuppressed (cyclophosphamide-treated) ICR mice 8 days after inoculation with $10^8$ yeast cells. Fungal keratitis (red arrows) was observed only in animals infected with the \textit{C. tropicalis} (Ct) wild type, \textit{crz1/crz1} mutants, and \textit{C. albicans} (Ca) type strain SC5314 and not in animals infected with \textit{C. tropicalis} calcineurin mutants. (B) Each cornea of an immunosuppressed mouse was inoculated with $10^8$ yeast cells of each strain, and the disease severity was scored for 8 days. \textit{C. albicans} type strain SC5314 served as a reference control. Mice infected with \textit{C. tropicalis} calcineurin mutants or the PBS control exhibited normal corneas. The disease scores of mice infected with the \textit{C. tropicalis} wild type or \textit{crz1/crz1} mutants and \textit{C. albicans} strain SC5314 exhibiting visible signs of keratitis were plotted.}
\end{figure}
scores observed for either of the calcineurin mutants were significantly lower than those for the wild type \( (P < 0.001, \text{Student’s } t \text{ test}) \), indicating an important role of calcineurin in the corneal virulence of \textit{C. tropicalis}. However, the two independent \textit{C. tropicalis} \textit{crz1/crz1} mutants caused an average of 83% of the wild-type level of keratitis, with a mean keratitis score of 8.3, and thus exhibited corneal virulence similar to that of the wild type (Fig. 5).

**Calcineurin is required for cell wall integrity and drug tolerance in \textit{C. tropicalis}**. A straightforward explanation for the attenuated virulence of \textit{C. tropicalis} calcineurin mutants is their hyphal growth defects (Fig. 1 and 2). However, it is possible that other mechanisms are required for calcineurin to establish and maintain infections. The maintenance of cell wall integrity is important for virulence in multiple fungal pathogens (24, 39–41). Nevertheless, the roles of the \textit{C. tropicalis} genes involved in cell wall integrity remain unclear. Here, we demonstrated that calcineurin is essential for cell wall integrity based on the sensitivity of calcineurin mutants to SDS (which compromises cell wall integrity), Congo red (which intercalates between glucan polymers), or tunicamycin (which blocks the synthesis of N-linked glycoproteins) (Fig. 6A). However, the \textit{crz1/crz1} mutants did not exhibit sensitivity to hyphal growth defects (Fig. 6A). In contrast, the \textit{crz1/crz1} mutants did exhibit sensitivity to triazoles (Fig. 6B), suggesting that either calcineurin control of cell wall integrity is Crz1 independent or Crz1 is redundant with other factors or pathways (Fig. 7).

Antifungal drug-resistant \textit{C. tropicalis} isolates are frequently isolated from patients with candidemia or leukemia and can pose treatment challenges (10, 12, 15, 42, 43). Calcineurin is a potential drug target on the basis of its requirement for drug tolerance and virulence in multiple fungal pathogens (24, 44–46). Information on the roles of the \textit{C. tropicalis} genes involved in drug tolerance is

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**FIG 6** Calcineurin is required for cell wall integrity, drug tolerance, and cation homeostasis in \textit{C. tropicalis}. (A) Calcineurin mutants are sensitive to cell wall integrity-damaging agents (SDS and Congo red) and an endoplasmic reticulum stress-inducing chemical (tunicamycin). Cells were grown overnight in YPD at 30°C, 5-fold serially diluted, spotted onto YPD medium containing SDS, Congo red, or tunicamycin at the concentrations indicated, and then incubated at 30°C for 48 h and photographed. (B) Calcineurin mutants are sensitive to echinocandins. Cells were grown overnight in YPD at 30°C, 5-fold serially diluted, and spotted onto YPD medium containing caspofungin, micafungin, or anidulafungin at the concentrations indicated, and then incubated at 30°C for 48 h and photographed. (C) Calcineurin mutants did not exhibit sensitivity to triazoles. Cells were grown overnight in YPD at 30°C, 5-fold serially diluted, and spotted onto YPD medium containing fluconazole (FLC), posaconazole (PSC), or voriconazole (VRC) at the concentrations indicated, and then incubated at 30°C for 48 h and photographed. (D) Roles of calcineurin and Crz1 in controlling cation homeostasis in \textit{C. tropicalis}. Cells were grown overnight in YPD at 30°C, 5-fold serially diluted, and spotted onto YPD medium containing CaCl\textsubscript{2} (with or without FK506), MnSO\textsubscript{4}, LiCl, or NaCl at the concentrations indicated. The plates were incubated at 30°C for 36 h.

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in different fungal pathogens. Here, we investigated the roles of C. tropicalis calcineurin and Crz1 in controlling cation homeostasis and found that C. tropicalis calcineurin and Crz1 play opposite functions in controlling Ca\(^{2+}\) homeostasis. Calcineurin mutants exhibited tolerance to Ca\(^{2+}\) stress, while crz1/crz1 mutants showed sensitivity but the wild type did not (Fig. 6D). In response to other cations, we demonstrated that calcineurin, but not Crz1, is required for Mn\(^{2+}\) homeostasis, while neither calcineurin nor Crz1 appeared to be involved in Na\(^{+}\) or Li\(^{+}\) homeostasis (Fig. 6D and data not shown).

**DISCUSSION**

**Roles of calcineurin and Crz1 in hyphal growth of C. tropicalis.** Whether C. tropicalis forms pseudohyphae, hyphae, or both is thought to be isolate and medium dependent. Our data suggest that C. tropicalis is able to form hyphae (see Fig. S3 in the supplemental material) and pseudohyphae (data not shown) on cornmeal solid agar medium. However, so far, the genes involved in hyphal growth, a potential phenotype linked to the virulence of C. tropicalis, are unclear. Calcineurin is required for hyphal growth in C. dubliniensis (27), but any role in C. albicans hyphal growth is unclear because two groups, including our own, were unable to find a role for calcineurin in hyphal growth (45, 56), while another group reported that calcineurin mutants exhibited hyphal growth defects on filament-inducing solid medium (46). Our data suggest that calcineurin is critical for hyphal growth of C. tropicalis (Fig. 1 and 2) and, hence, plays a role similar to its role in hyphal growth of the related species C. dubliniensis. However, the mechanisms via which calcineurin controls the dimorphic transition of C. tropicalis remain to be clarified. It is possible that calcineurin regulates downstream targets important for hyphal growth. One target is the transcription factor Crz1, which serves as a calcineurin target in both S. cerevisiae and C. albicans (57, 58). The roles of Crz1 in hyphal growth of C. albicans remain elusive because Karababa et al. (57) reported that Crz1 is required for hyphal growth, but Noble et al. (59) demonstrated that Crz1 is not critical for hyphal growth in a systematic screen. However, similar to C. dubliniensis (27, 57), we demonstrated that C. tropicalis Crz1 is critical for hyphal growth on spider medium (carbon source starvation), suggesting that the hyphal growth machinery involves Crz1-dependent calcineurin signaling (Fig. 7) and is conserved in the two related species C. dubliniensis and C. tropicalis.

**Roles of calcineurin and Crz1 in virulence of C. tropicalis.** Previous studies of the C. tropicalis genes involved in virulence are limited (2). Our data provide evidence that calcineurin control of C. tropicalis virulence in a murine systemic infection model is Crz1 dependent because crz1/crz1 mutants exhibit virulence interme-

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**TABLE 2** Calcineurin is required for drug resistance in C. tropicalis

| Strain               | MIC or MIC range (µg/ml) |
|----------------------|--------------------------|
|                      | Caspofungin | Ketoconazole | Voriconazole | Fluconazole | Amphotericin B |
| MYA3404 (wild type)  | 0.032       | 0.064        | 0.125        | 2.0         | 0.38–0.5       |
| YC454 cnb1/cnb1      | 0.016       | 0.016        | 0.047        | 1.0         | 0.38–0.5       |
| YC466 cnb1/cnb1      | 0.016       | 0.016        | 0.047        | 1.0         | 0.38–0.5       |
| YC494 crz1/crz1      | 0.064       | 0.094        | 0.19–0.25    | 3.0         | 0.25           |
| YC499 crz1/crz1      | 0.064       | 0.094        | 0.19–0.25    | 2.0–3.0     | 0.25           |

*Cells were grown overnight at 30°C and washed twice with ddH\(_2\)O. Then, cells at an OD of 0.5 (in 500 µl) were spread on RPMI 1640 medium (R04067; Remel). After 20 min, the Etest strips (bioMérieux Corp.) were transferred to the surface of the medium. The MIC was read after 24 h of incubation at 35°C according to the manufacturer’s instructions.*
Whether calcineurin is critical for the growth of *C. tropicalis* in a murine urinary tract infection model (27), the basis of the similar growth curves and doubling times at 37°C (see Fig. S5 in the supplemental material). Our findings on the roles of calcineurin and Crz1 in the virulence of *C. tropicalis* are similar to those on the roles of calcineurin and Crz1 in the virulence of *C. dubliniensis* (27, 57), suggesting conserved functions for calcineurin and Crz1 in the virulence of the closely related species *C. tropicalis* and *C. dubliniensis* in a murine systemic infection model. In contrast, in the murine ocular infection model, *C. tropicalis* calcineurin but not Crz1 is critical for virulence, similar to findings in *C. dubliniensis* (27). Thus, calcineurin is, in general, required for the virulence of *C. tropicalis* and *C. dubliniensis* in both murine systemic and ocular infection models, while Crz1 is required for the virulence of *C. tropicalis* and *C. dubliniensis* only in a murine systemic infection model and not in an ocular infection model, suggesting a specific niche requirement (bloodstream versus ocular surface) of Crz1 in both *C. tropicalis* and *C. dubliniensis* (27).

In addition to being frequently isolated from patients (11, 17, 60–62), *C. tropicalis* has also been isolated from the mouse intestine (where it constitutes up to 65% of the overall fungal component) (63) and environmental compost and soil (11, 64). Previous studies suggest that *C. tropicalis* can be transferred by hand-to-hand contact (65), indicating a potential route for human-human transmission. Further studies to assess if *C. tropicalis* strains isolated from patients originate from an environmental source and whether calcineurin is critical for the growth of *C. tropicalis* isolated from patients and the environment will be important.

Mouse Toll-like receptor 4 (TLR4) is a pattern recognition receptor that recognizes lipopolysaccharides from Gram-negative bacteria and initiates innate immunity. TLR4 has been demonstrated to play a role against *Aspergillus fumigatus* infection in a murine keratitis model (66), while it may play a role in defending *C. albicans*, depending upon the strains used (67). In previous studies, we demonstrated that mouse TLR4 is not critical for defense against *C. glabrata* infection in a murine urinary tract infection model (52). Using C3H/HeJ mice with a TLR4 mutation, which we compared to C3H/HeOuJ mice with wild-type TLR4, we found that mouse TLR4 is not required for defense against *C. tropicalis* in a murine systemic infection model (see Fig. S4 in the supplemental material).

Roles of calcineurin and Crz1 in drug tolerance of *C. tropicalis*. *C. tropicalis* calcineurin mutants are sensitive to cell wall-perturbing agents, such as SDS, Congo red, and tunicamycin (Fig. 6A), indicating that these mutants might be susceptible to anti-fungal drugs that target the cell wall. Indeed, these calcineurin mutants are susceptible to echinocandins, such as caspofungin, micafungin, and anidulafungin (Fig. 6B and Table 2). Meanwhile, *C. tropicalis* calcineurin mutants were found to exhibit susceptibility to azoles in Etest strip analyses and spot dilution assays on solid medium (Table 2 and Fig. 6C). Interestingly, we found that the difference between the wild type and calcineurin mutants based on the results of the spot dilution assays could be seen only at extremely high concentrations of azoles (i.e., 150 μg/ml of fluconazole), which is in contrast to the 1 μg/ml of fluconazole that allowed us to observe a difference between the wild type and calcineurin mutants in *C. albicans* and *C. dubliniensis* (27). The difference might be due to the fact that (i) *C. tropicalis* is intrinsically more tolerant to azoles than *C. albicans* and *C. dubliniensis* and/or (ii) *C. tropicalis* exhibited a higher growth/metabolism rate than *C. albicans* and *C. dubliniensis*. In summary, we demonstrate that calcineurin controls hyphal growth (in response to carbon source starvation), virulence (in a murine systemic infection model), and drug tolerance (micafungin) and that these functions are in part dependent upon Crz1 in *C. tropicalis* (Fig. 7). Meanwhile, *C. tropicalis* calcineurin has Crz1-independent functions (or the Crz1 function is redundant with other factors) for hyphal growth induced by serum or SLAD medium (nitrogen source starvation), cell wall integrity, and tolerance to caspofungin, anidulafungin, or Mn2+-tolerance functions, while calcineurin serves as a negative regulator of Ca2+-tolerance functions (Fig. 7). The requirement for calcineurin in virulence and drug tolerance supports calcineurin as a potential drug target in *C. tropicalis*.

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**REFERENCES**

1. Arendrup MC, Bruun B, Christensen JJ, Fuursted K, Johansen HK, Kjaeldgaard P, Knudsen JD, Kristensen L, Moller J, Nielsen L, Rosenvinge FS, Roder B, Schonheyder HC, Thomsen MK, Truberg K. 2011. National surveillance of fungemia in Denmark (2004 to 2009). J. Clin. Microbiol. 49:325–334. http://dx.doi.org/10.1128/JCM.01811-10.

2. Chai LY, Denning DW, Warn P. 2010. *Candida tropicalis* in human disease. Crit. Rev. Microbiol. 36:282–298. http://dx.doi.org/10.3109/1040841X.2010.489506.

3. Arendrup MC, Fuursted K, Gahrn-Hansen B, Schonheyder HC, Knudsen JD, Jensen IM, Bruun B, Christensen JJ, Johansen HK. 2008. Semi-national surveillance of fungemia in Denmark 2004-2006: increasing incidence of fungaemia and numbers of isolates with reduced azole-susceptibility. Clin. Microbiol. Infect. 14:487–494. http://dx.doi.org/10.1111/j.1469-0691.2008.01954.x.

4. Pfaffer MA, Diekema DJ. 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. 20:133–163. http://dx.doi.org/10.1128/CMR.00029-06.

5. Andes DR, Safdar N, Baddley JW, Playford G, Reboli AC, Rex JH, Sobel
Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink 16.
Yang YL, Lin CC, Chang TP, Lauderdale TL, Chen HT, Lee CF, Hsieh 12.
Yang YL, Wang AH, Wang CW, Cheng WT, Li SY, Lo HJ. 13.
Chen YN, Lo HJ, Wu CC, Ko HC, Chang TP, Yang YL. 14.
Porman AM, Alby K, Kohler JR, DiDomenico B, Naglik JR, Hube B. 15.
Yang YL, Wang AH, Wang CW, Cheng WT, Lo HJ. 16.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 17.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 18.
Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 19.
Gottfredsson M, Vredenburgh JJ, Xu J, Schell WA, Perfect JR. 20.
Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 21.
Gottfredsson M, Vredenburgh JJ, Xu J, Schell WA, Perfect JR. 22.
Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 23.
Gottfredsson M, Vredenburgh JJ, Xu J, Schell WA, Perfect JR. 24.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 25.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 26.
Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 27.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 28.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 29.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 30.
Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 31.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 32.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 33.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 34.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 35.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 36.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 37.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 38.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 39.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 40.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 41.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 42.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 43.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 44.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 45.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 46.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 47.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 48.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 49.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 50.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 51.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 52.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 53.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 54.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 55.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 56.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 57.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 58.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 59.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 60.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 61.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 62.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 63.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 64.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 65.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 66.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 67.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 68.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 69.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 70.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 71.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 72.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 73.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 74.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 75.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 76.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 77.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 78.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 79.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 80.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 81.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 82.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 83.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 84.

Leung AY, Chim CS, Ho PL, Cheng VC, Yuen KY, Lie AK, Au WY, 85.
Gottfredsson M, Alby K, Hirakawa MP, Bennett RJ. 86.
Aspergillus fumigatus. Mol. Microbiol. 77:891–911. http://dx.doi.org/10.1111/j.1365-2958.2010.07254.x.

42. Jiang C, Dong D, Yu B, Cai G, Wang X, Ji Y, Peng Y. 2013. Mechanisms of azole resistance in 52 clinical isolates of Candida tropicalis in China. J. Antimicrob. Chemother. 68:778–785. http://dx.doi.org/10.1093/jac/dks481.

43. Pasquale T, Tomada JR, Ghannoun M, Dipersio J, Bonilla H. 2008. Emergence of Candida tropicalis resistant to caspofungin. J. Antimicrob. Chemother. 61:219. http://dx.doi.org/10.1093/jac/dkm453.

44. Steinbach WJ, Reedy JL, Cramer RA, Jr, Perfect JR, Heitman J. 2007. Harnessing calcineurin as a novel anti-infective agent against invasive fungal infections. Nat. Rev. Microbiol. 5:418–430. http://dx.doi.org/10.1038/nrmicro1680.

45. Cruz MC, Goldstein AL, Blankschhip JR, Del Poeta M, Davis D, Cardenas ME, Perfect JR, McCusker JH, Heitman J. 2002. Calcineurin is essential for survival during membrane stress in Candida albicans. EMBO J. 21:546–559. http://dx.doi.org/10.1093/emboj/21.4.546.

46. Sanglard D, Ischer F, Marchetti O, Entenza J, Bille J. 2003. Calcineurin A of Candida albicans: involvement in antifungal tolerance, cell morphogenesis and virulence. Mol. Microbiol. 48:959–976. http://dx.doi.org/10.1046/j.1365-2958.2003.03495.x.

47. Chen YL, Lehman VN, Lewis Y, Averette AF, Heitman J. 2013. Calcineurin governs thermotolerance and virulence of Cryptococcus gattii. G3 (Bethesda) 3:527–539. http://dx.doi.org/10.1534/g3.112.004222.

48. Choi JH, Kim Y, Lee YH. 2009. Functional analysis of MCNA, a gene encoding a catalytic subunit of calcineurin, in the rice blast fungus Magnaporthe oryzae. J. Microbiol. Biotechnol. 19:11–16.

49. Odom A, Muir S, Lim E, Toffaletti DL, Perfect J, Heitman J. 1997. Calcineurin is required for virulence of Cryptococcus neoformans. EMBO J. 16:2576–2589. http://dx.doi.org/10.1093/emboj/16.10.2576.

50. Steinbach WJ, Cramer RA, Jr, Perfect BZ, Asfaw YG, Sauer TC, Najvar LK, Kirkpatrick WR, Patterson TF, Benjamin DK, Jr, Heitman J, Perfect JR. 2006. Calcineurin controls growth, morphology, and pathogenicity in Aspergillus fumigatus. Eukaryot. Cell 5:1091–1103. http://dx.doi.org/10.1128/EC.00139-06.

51. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402. http://dx.doi.org/10.1093/nar/25.17.3389.

52. Chen YL, Konieczka JH, Springer DJ, Bowen SE, Zhang J, Sião FG, Bungay AA, Bigol UG, Nicolas MG, Abraham SN, Thompson DA, Regev A, Heitman J. 2012. Convergent evolution of calcineurin pathway roles in thermotolerance and virulence in Candida glabrata. G3 (Bethesda) 2:675–691. http://dx.doi.org/10.1534/g3.112.002279.

53. Cunningham KW, Fink GR. 1994. Calcineurin-dependent growth control in Saccharomyces cerevisiae mutants lacking PMC1, a homolog of plasma membrane Ca2+/ATPases. J. Cell Biol. 124:351–363. http://dx.doi.org/10.1083/jcb.124.3.351.

54. Withee JL, Sen R, Cyert MS. 1998. Ion tolerance of Saccharomyces cerevisiae lacking the Ca2+/CaM-dependent phosphatase (calcineurin) is improved by mutations in URE2 or PMA1. Genetics 149:865–878.

55. Zhang J, Sião FG, Bigol UG, Bungay AA, Nicolas MG, Heitman J, Chen YL. 2012. Calcineurin is required for pseudohyphal growth, virulence, and drug resistance in Candida lusitaniae. PLoS One 7:e44192. http://dx.doi.org/10.1371/journal.pone.0044192.

56. Bader T, Schroppel K, Bentink S, Agabian N, Kohler G, Morschhäuser J. 2006. Role of calcineurin in stress resistance, morphogenesis, and virulence of a Candida albicans wild-type strain. Infect. Immun. 74:4366–4369. http://dx.doi.org/10.1128/IAI.00142-06.

57. Karavella M, Valentine E, Pardini G, Coste AT, Bille J, Sanglard D. 2006. CRZ1, a target of the calcineurin pathway in Candida albicans. Mol. Microbiol. 59:1429–1451. http://dx.doi.org/10.1111/j.1365-2958.2005.05037.x.

58. Stathopoulos AM, Cyert MS. 1997. Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast. Genes Dev. 11:3432–3444. http://dx.doi.org/10.1101/gad.11.24.3432.

59. Noble SM, French S, Kohn LA, Chen V, Johnson AD. 2010. Systematic screens of a Candida albicans homozygous deletion library decouple morphogenetic switching and pathogenicity. Nat. Genet. 42:590–598. http://dx.doi.org/10.1038/ng.605.

60. Galan-Ladero MA, Blanco-Blanco MT, Hurtado C, Perez-Giraldo C, Blanco MT, Gomez-Garcia AC. 2013. Determination of biofilm production by Candida tropicalis isolated from hospitalized patients and its relation with cellular surface hydrophobicity, plastic adherence and filamentation ability. Yeast 30:331–339. http://dx.doi.org/10.1002/yea.2965.

61. Chang TP, Ho MW, Yang YL, Lo PC, Lin PS, Wang AH, Lo HJ. 2013. Distribution and drug susceptibilities of Candida species causing candidemia from a medical center in central Taiwan. J. Infect. Chemother. 19:1065–1071. http://dx.doi.org/10.1007/s10156-013-0623-8.

62. Yang YL, Chen HT, Lin CC, Chu WL, Lo HJ, TSARY Hospitals. 2013. Species distribution and drug susceptibilities of Candida isolates in Tsarai. 2013. Diagn. Microbiol. Infect. Dis. 76:182–186. http://dx.doi.org/10.1016/j.diagmicrobio.2013.03.003.

63. Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, Brown J, Becker CA, Fleshner PR, Dubinsky M, Rotter JI, Wang HL, McGovern DP, Brown GD, Underhill DM. 2012. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. Science 336:1314–1317. http://dx.doi.org/10.1126/science.1221789.

64. Cheon SA, Jung KW, Chen YL, Heitman J, Bahn YS, Kang HA. 2011. Unique evolution of the UPR pathway with a novel bZIP transcription factor, Hxl1, for controlling pathogenicity of Cryptococcus neoformans. PLoS Pathog. 7:e1002177. http://dx.doi.org/10.1371/journal.ppat.1002177.

65. Rangel-Frausto MS, Houston AK, Bale MJ, Fu C, Wenzel RP. 1994. An experimental model for study of Candida survival and transmission in human volunteers. Eur. J. Clin. Microbiol. Infect. Dis. 13:590–595. http://dx.doi.org/10.1007/BF01971311.

66. Leal SM, Jr, Cowden S, Hsia YG, Ghannoun MA, Momany M, Pearlman E. 2010. Distinct roles for Dectin-1 and TLR4 in the pathogenesis of Aspergillus fumigatus keratitis. PLoS Pathog. 6:e1000976. http://dx.doi.org/10.1371/journal.ppat.1000976.

67. Netea MG, Gow NA, Joosten LA, Verschueren I, van der Meer JW, Kullberg BJ. 2010. Variable recognition of Candida albicans strains by TLR4 and lectin recognition receptors. Med. Mycol. 48:897–903. http://dx.doi.org/10.3109/13693781003621575.