**Abstract**

Invasive fungal infections, which kill more than 1.6 million patients each year worldwide, are difficult to treat due to the limited number of antifungal drugs (azoles, echinocandins, and polyenes) and the emergence of antifungal resistance. The transcription factor Crz1, a key regulator of cellular stress responses and virulence, is an attractive therapeutic target because this protein is absent in human cells. Here, we used a CRISPR-Cas9 approach to generate isogenic \( \text{crz1}^- \) strains in two clinical isolates of caspofungin-resistant \( \text{C. glabrata} \) to analyze the role of this transcription factor in susceptibility to echinocandins, stress tolerance, biofilm formation, cell size, and pathogenicity. These results strongly suggest that Crz1 inhibitors may play an important role in the development of novel therapeutic agents against fungal infections considering the emergence of antifungal resistance and the low number of available antifungal drugs.

**Introduction**

In the last years invasive fungal infections (IFI), which are associated with around 1.6 million deaths (similar to tuberculosis and 3-fold higher than malaria infections) have become a public health problem [1, 2]. \( \text{Candida, Aspergillus, Cryptococcus,} \) and \( \text{Pneumocystis} \) are the main fungal pathogens responsible for the majority cases of serious fungal diseases. Among the genus...
the Pontificia Universidad Javeriana in Bogotá, Colombia ID20291. Work in JP’s laboratory is funded by Grant PGC2018-095047-B-100 from Ministerio de Ciencia e Innovación and InGEMICS (B2017/BMD-3691) from Comunidad de Madrid CAM to JP.

**Competing interests:** P.L.P received grants from Astellas, Basilea, MSD and Pfizer and speaker’s fees from Gilead, Basilea, Pfizer and MSD. The other authors declare not have any conflict of interest to disclose.

*Candida, Candida albicans* is the most commonly isolated species, with close to 30% mortality rates in candidemia [3, 4]. However, non-*C. albicans* species such as *Candida glabrata* have emerged as a frequent cause of life threatening IFI becoming the second or third most frequent species. For this species, the associated mortality is about 50% [5, 6].

While current therapeutic options for IFI are limited to only three classes of drugs (polyenes, azoles and echinocandins), the emergence of resistant strains to some of these molecules is even more concerning. In the case of *C. glabrata*, antifungal resistance mechanisms have been relatively well characterized [7, 8]. It is known that this species, commonly resistant to azoles, has high genetic plasticity and can acquire resistance to other drugs easily [8, 9]. *C. glabrata* shows increasing resistance to echinocandins, which is now considered the first-line of therapy for candidemia [10]. The main resistance mechanism is associated with mutations in the FKS1 and/or FKS2 genes that encode the subunits of the 1,3-β-D-glucan synthase protein, the target of echinocandins, leading to multi-drug resistance (MDR) [7, 11].

Although there is an urgent need to develop new antifungal drugs, the close sequence homology of targets between the host and the pathogen makes this task challenging [12, 13]. However, in recent years, some metabolic pathways have become attractive targets due to their role in host adaptation and cellular stress responses [14]. Among these, signaling pathways have been broad studied such as the Ras/cAMP/PKA pathway, calmodulin/calcineurin pathway (CaM/Cal), TOR (target of rapamycin) and mitogen-activated protein kinase (MAPK) signaling pathways [15–18].

The calmodulin/calcineurin (CaM/Cal) is a conserved pathway from fungi to humans [19]. In yeast, this pathway is involved in ion homeostasis, sphingolipid and cell wall biosynthesis, protein trafficking, ubiquitin signaling, autophagy, adaptation to stress and most importantly, in antifungal resistance [20–23]. A key element of this route is the complex formed by the proteins Cnb1, Cna1, Hsp90 and the transcription factors Crz1 (calcineurin-responsive zinc finger 1) in yeast or the NFAT (nuclear factor of activated T cells) in mammals. Upon activation by dephosphorylation (a process mediated by the CaM/Cal-Hsp90 complex), Crz1 translocates to nucleus and through its C2H2 zinc finger motif binds to a specific element in the CDRE gene promoter (calcineurin dependent response element), initiating the activation of about 87 genes, among which is the FKS2 gene [24–27]. *C. albicans* and *C. glabrata* lacking CaM/Cal fail to survive in the presence of membrane stressors, as occurs in *S. cerevisiae* mutants, have a reduced MIC to antifungals and have attenuated virulence in mouse models [28, 29]. In *C. glabrata* and *C. lusitaniae*, calcineurin-Crz1 signaling controls the virulence in a murine model of systemic infection [26, 30]. Growth defects at alkaline pH and at elevated temperature have also been reported in different *crz1Δ* Candida species [31, 32]. Nevertheless, the implication of Crz1 in stress response, biofilm formation and cell morphology in echinocandin-resistant *C. glabrata* clinical isolates has not been evaluated. Furthermore, susceptibility of *Crz1Δ* mutants of these resistant isolates has also to be evaluated for a better understanding of the putative therapeutic impact of this target.

Over the last decade, the innovative discovery of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 has revolutionized genome editing in many organisms, including fungi [33, 34]. However, few studies have been published using CRISPR-Cas9 in *C. glabrata* [35–38]. This approach can be used to transform and accelerate drug discovery and development by enabling a fast and accurate editing of genomic information in biological model systems [39, 40]. This study aimed to assess the role of Crz1 in the resistance to caspofungin in two *C. glabrata* resistant clinical isolates. For this purpose, we used a CRISPR-Cas9 genome editing approach to generate isogenic *crz1Δ* strains in clinical isolates and analyzed the role of this transcription factor in the resistance to echinocandins, tolerance to stress conditions, biofilm formation and cell wall composition. We have also addressed the virulence of
the obtained mutants in a non-vertebrate (Galleria mellonella) and vertebrate (Swiss mouse) models of systemic candidiasis, supporting the role of this factor as a promising antifungal target in this clinically relevant yeast.

Materials and methods

Isolates and media

Two echinocandin-resistant isolates CAGL1875 and CAGL1256 obtained from blood and urine cultures of hospitalized patients in intensive care unit of Centre Hospitalier Universitaire de Nantes, France were described in a previous study by our research group [20, 41]. Isolates were streaked from a glycerol stock onto yeast extract peptone dextrose agar (YPD) and grown for 24 h at 37 °C. Genome sequencing is available in the NCBI BioProject database with the accession number PRJNA692260.

Construction of CRISPR-Cas9 mutants for CgCRZ1

C. glabrata isolates CAGL1875 and CAGL1256 were used as the parent strain to construct the CRISPR-Cas9 mutants for CgCRZ1. sgRNAs were generated by phosphorylation and annealing of complementary single stranded DNA oligonucleotides sgCRZ1-up (GATCGATAACCATTAATTTCTCGACCG) and sgCRZ1-rev (AAAACGGTCGAGAAATTATGGTTATC) and inserted into the pV1083 vector previously digested with BsmBI I and treated with calf intestinal phosphatase CIP (NEB) to generate the recombinant plasmid pV1382-sgRNA. The insertion of the guide was confirmed by sequencing with the o-seq-sgRNA primer (GGCTAGCGGTAAAGGTGCG). Repair templates (Mutagenic template-up: GCATGAAATGGCGATGTATATGAACAA GATAACAAATAACCATAATTCTCGACCTAATGAGGATCCCAGAATAT and Mutagenic template-rev: TGAATAATGTAGACTCACTCAGTATATTCTTGGAATCCCTATTAGGTCGAGAAC) were generated with 53-bp oligonucleotide primers containing 23-bp overlaps at their 3’ ends centred at the mutation point, which consisted of two stop codons. Primers CRZ1-F (GCAATGATCCGTAAAGGTGCG) and CRZ1-R (TGAAAATGTAGACTCACTCAGTATATTCTTGGAATCCCTATTAGGTCGAGAAC) were used to amplify the repair donor DNA fragment. The donor DNA and the recombinant plasmid pV1382-sgRNA were used to transform C. glabrata cells CAGL1875 and CAGL1256 by electroporation [42].

Transformed yeasts were growth in YPD supplemented with nourseothricin selection marker of potentially CRISPR-Cas9 edited mutants. Nourseothricin-resistant clones were evaluated by detection with PCR and BamHI-digestion analysis. PCR was performed on genomic DNA using the primers CRZ1-CF (GGGTTCCTGTCAAAAACTCGTCCCTCAATTTAATTGATCCGTAAAGGTGCG) and CRZ1-CR (CAAATGATCCGTAAAGGTGCG). Finally, CRZ1 mutants were confirmed by sequencing.

Antifungal susceptibility testing

Antifungal susceptibility testing was conducted using the Clinical and Laboratory Standards Institute broth microdilution method (CLSI-BMD), following the M27-A3 guidelines [43]. Briefly, Isolates and mutants were streaked from a glycerol stock onto YPD and grown for 24 h at 37 °C. Colonies were suspended in 1 mL phosphate buffered saline (PBS) and diluted in liquid RPMI 1640 medium to 10^3 cells/mL in a 96-well plate, containing a gradient of two-fold dilutions per step of antifungal (CAS, MCF), with the first well contain no drug. MICs were visually, and densitometry determined as the lowest concentration of drug that caused a significant diminution (MIC/2 or >50%) compared with that of the drug-free growth control after 24 h of incubation. Quality control was ensured by testing the CLSI-recommended strains C. parapsilosis ATCC 22019 and C. krusei ATCC 6258 [44].
Stress-related phenotypic assays

To examine the potential role of CgCRZ1 mutant’s cellular protection to heat and oxidative stresses, associated or not to caspofungin was assessed. For heat-shock stress, drop tests were performed by spotting serial dilutions of C. glabrata (10^6 to 10^3 cells/mL) onto YPD agar plates with CaM/CaL inhibitors fluphenazine (Fph), tacrolimus (Fk506), cyclosporin A (CsA) (15 μg/mL), caspofungin (1 μg/mL) or both compounds. The plates were incubated at 37˚C and 40˚C for 24 h. For oxidative stress, YPD plates were prepared as previously except that the medium was supplemented with the naphthoquinone menadione (0.2 and 0.4 mM), a cytotoxic quinone that generates superoxide. The plates were incubated at 37˚C for 24 h [20].

Confocal microscopy

Yeast cells collected by centrifugation (4000 g, 5 min,) and suspended in 200 μL phosphate buffered saline were stained with 10 μg/mL concanavalin A–Alexa Fluor 594 conjugate (Molecular Probes, Eugene, OR) and 20 μg/mL Fluorescent Brightener 28 (Sigma) for 25 min in the dark. Then, cells were washed twice in PBS and fixed with 4% paraformaldehyde for 30 min. Fluorescence-stained sections were examined under a Nikon A1 RSI microscope with a magnification of ×60 at constant Z-steps of 1 μm. The laser confocal system comprised a 65-mW multi-Ar laser. Three-dimensional (3D) images were processed with NIS elements version 3.21 (Nikon Instruments Inc.) and Volocity 3D image analysis software version 6.01 (PerkinElmer). Cell size measurements and total cell volume were determined using ImageJ software.

Biofilm formation

C. glabrata isolates and mutants were grown in Sabouraud medium (Biomerieux, France) and incubated at 37˚C for 24 h. Two hundred μL of Candida cell suspensions (10^6 cells/mL) in RPMI-1640 (SIGMA®), Saint Quentin Fallavier, France) with MOPS adjusted to pH 7 were seeded in 96-well microdilution wells with GDHK-1325 250mm Gam polyurethane catheter pieces (Hechingen, Germany) and allowed to adhere for 24 h at 37˚C. The non-adherent cells were removed by redispersing catheter pieces in new microplates wells. Follow by incubation for 24 h at 37˚C for biofilm formation phase. Then, catheter pieces were washed twice with PBS and finally 100 μL of RPMI-1640 plus 10 μL of resazurin (700μM) was added to each well and incubated at 37˚C for 2–4 h [45]. Finally, fluorescence was measured at 560 nm with an emission at 590 nm. The results were expressed in arbitrary fluorescence unit (AU). Statistical analysis was performed using Graph Pad Prism version 5.0, Software Inc., La Jolla, CA, USA.

Invertebrate Galleria mellonella model

Killing assays were performed in G. mellonella as described by Fallon, 2012 [46]. Briefly, larvae of late stages (fifth and sixth) between 250 to 330 mg and a length of approximately 2 cm were selected. A group of 10 larvae was used for the following controls: absolute control, disinfection, and inoculation. To compare mortality three biological replicates were performed with 10 larvae for each isolate evaluated. C. glabrata parent and mutant isolates were grown in Sabouraud dextrose agar and incubated for 48 h at 37˚C. Suspensions adjusted to 1x10^9 UFC/mL using Neubauer chamber were used to inoculate 10 larvae per Candida isolate. Larvae receive 10 μL of inoculum and 10 μL of caspofungin (100 mg/L), by injection into the last left and right pro-leg respectively using a 0.5mL (BD®) gauge insulin syringe. After inoculation,
larvae were placed in Petri dishes and incubated in darkness at 37˚C. The larvae were monitored for 10 days, and survival outcome was determined; larvae were considered dead when no response was observed following touch.

**Mice use and care**

Females Swiss mice (Janvier Labs, Le Genest-Saint-Isle, France) with a body weight of ~29 g were obtained and allowed to acclimate for 7 days prior to use. Environmental controls for the animal room were set to maintain a temperature of 16 to 22˚C, a relative humidity of 30 to 70%, and a 12:12 hourly light-dark cycle. All efforts were made to minimize suffering.

According to humane endpoints defined by the Animal Welfare Organization of the animal house, a score is calculated as a function of observations made. Based on this score and on the thresholds reached, corrective measures have been defined to limit the pain and suffering of animals ranging from improvement of well-being to euthanasia.

After inoculation of the Candida suspension, the animals were observed twice a day and scored daily. It consists of evaluating and assigning a score ranging from 0 to 2 for each of the following parameters: temperature, appearance of the hair, general morphology, weight, physical appearance of the face (eyelids, eye, vibrations), behavioural appearance (group behaviour, activity, motor deficit, spasms/tremors) and breathing.

A score between 8 and 14 corresponds to moderate pain and involves the use of opioid analgesic: buprenorphine. It is used at the dosage of 0.05 mg/kg in SC, 2 to 3 times a day, depending on the evolution of the state of the mouse. Experience shows that the duration of administration is short (<24h), the evolution of systemic candidiasis being sudden and rapid. The worsening of the condition is of very poor prognosis. Thus, animals with a score above 15 are classified as seriously ill and euthanized.

**Systemic C. glabrata infection model**

Mice were immunosuppressed by subcutaneous injection of 30 mg/kg prednisolone (Hydrocortanycin®) one day before challenge. On day 0, mice were infected intravenously (100 μL) with a blastoconidia suspension (10⁸ yeast/mL) of WT CAGL1875 clinical isolate (in vitro caspofungin-resistant, 19 mice) or the crz1Δ C. glabrata (in vitro caspofungin-susceptible, 20 mice). One hour after infection, mice of each group (WT and crz1Δ) were separated in 2 subgroups that were treated intraperitoneally once daily for 4 days. One received a 2.5 mg/kg body weight of caspofungin (OHRE Pharma, Tours, France) treatment, the other (control subgroup) received 100 μL of distilled water. Survival was monitored for 7 days post inoculation. No mice died during the experiment.

At the end of the assay (day 7), the spleen and kidney of euthanized mice (by cervical dislocation) were excised and weighed. Tissues were homogenized and serially diluted 100 to 10000-fold in sterile saline solution, then plated onto Sabouraud dextrose agar and incubated for 48 h to determine the number of colony-forming units (CFUs). Tissue fungal burden was expressed as the average Log(CFU)/gram of tissue. Mean CFUs in kidneys and spleens were compared between WT and crz1Δ C. glabrata and with vehicle control.

**Ethical approval**

The mice experimental protocol was approved by the Ethic Committee on Animal Testing (committee N°006) and was authorized by the French Ministry of Higher Education, Research and Innovation (APAFIS-#9710-2017042410186554 v4).
Statistical analysis
Experiments were performed on three independent biological replicates; survival curves were constructed using the method of Kaplan and Meier, then the curves were compared using the Log-Rank (Mantel-Cox) test. For the mouse model, statistical comparisons were made by analysis of variance (Two-way ANOVA) followed by a Tukey–Kramer post hoc test. Statistical models were constructed and analyzed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant. Cell size measurements were done using the following formula: \( \frac{4}{3} \pi \left( \frac{r^3}{\text{diameter} + \text{height} / 2} \right) \).

Results
Disruption of CRZ1 in clinical isolates using CRISPR-Cas9
\( crz1 \Delta \) mutants were generated using the new unified solo vectors developed by Vyas et al., that allows efficient genetic engineering in \( C. \ glabrata \) incorporating both Cas9 and sgRNA into a single vector with a dominant marker [38]. In silico analysis of the CRZ1 orf using the Chop-Chop server (https://chopchop.cbu.uib.no) was used to identify sequences likely to be used as CRISPR guides. A guide starting at 215 bp from the starting methionine was selected and cloned into the expressing vector. This plasmid was used to transform the CAGL1875 and CAGL1256 clinical isolates, which have a caspofungin-resistant phenotype with a repair template which incorporate double-stop codon and a \( \text{BamHI} \) restriction site. Nourseothricin-resistant colonies were then checked for the correct CRZ1 disruption by CRZ1 amplification followed by \( \text{BamHI} \) digestion. Finally, the clones that showed a double band in agarose gel were confirmed by sequencing (Fig 1).

\( CRZ1 \) disruption restores caspofungin-susceptibility phenotype in resistant \( C. \ glabrata \) clinical isolates
The \( C. \ glabrata \) isolates CAGL1875 and CAGL1256 which exhibited a deletion in the \( FKS2 \) gene (F659del) were previously reported as resistant to caspofungin (MIC > 16 μg/mL) by our group [20]. Micafungin resistance was confirmed with MIC values (> 0.5 μg/mL). The disruption of CRZ1 allowed the restoration of echinocandin-susceptibility of the two isolates, since the \( crz1 \Delta \) mutants showed low MIC values for caspofungin (MIC 0.25 and 0.5 μg/mL) and micafungin (MIC 0.03 and 0.06 μg/mL), respectively (Fig 2).

\( CRZ1 \) disruption does not affect thermotolerance but leads to increased susceptibility to oxidative stress
To understand the heat and oxidative stress response in \( crz1 \Delta \) mutants, we analyzed growth in the presence of menadione and compared growth at 37 and 40 °C in solid drop assays. Spot test at 37 °C or after heat shock at 40 °C did not show major differences between the growth of mutants and WT isolates. However, at 37 °C the use of CaM/CaL inhibitors (Fph, Fk506 and CsA) slightly affected the growth, and the association of the CaM/CaL inhibitors with caspofungin seriously compromised it, being more pronounced for the mutants. At 40 °C, the presence of CaM/CaL inhibitors alone and in combination with caspofungin strongly affected the growth (Fig 3A). Regarding oxidative stress, the addition of 0.2 and 0.4 mM menadione caused a significant decreased growth of mutants compared with their parental isolates. Moreover, \( crz1 \Delta \) mutants were mostly affected in the presence of CaM/CaL inhibitors and unable to grow in presence of caspofungin. At 0.4 mM menadione, the WT isolates showed a slight reduction of growth in presence of calcineurin inhibitors whereas the \( crz1 \Delta \) mutants were unable to
grow at this concentration. The combination with caspofungin completely inhibited growth of all the strains (Fig 3B).

crz1Δ mutants display changes in cell wall composition and yeast size

Different responses to caspofungin could be caused by differences in the cell wall among strains. We therefore analysed cell wall composition of the indicated strains by staining cells with concanavalin A (ConA), a lectin probe with strong affinity for yeast α-mannans and calcofluor white, which binds chitin. We observed that ConA staining was similar in both the mutants and parental strains and the fluorescent green colour was homogeneously distributed throughout the cell wall of all the strains. By contrast, staining with calcofluor white showed clear differences between mutants compared with their parental isolates, with a few preferences toward bud scars (Fig 4A). Interestingly, we observed that CRZ1 disruption in WT clinical isolates resulted in increased cell sizes, reaching values of 29 ± 4 µm compared to (9 ± 3 µm of the WT strains (p < 0.02) (Fig 4B).

Fig 1. Construction of CRISPR mutants for CgCRZ1. A. The plasmid pV1382 contains CgCAS9, the nourseothricin resistant gene (NatR) and SNR52 promoter that control the expression of the desired sgRNA. B. pV1382 permits a rapid cloning by BsmBI digestion followed by ligation of annealed oligos (boldface type) containing the CgCRZ1 sequence of synthetic guide RNA (in red bracket). C. Scheme of CgCRZ1 mutagenesis mediated by CRISPR/Cas9. sgCRZ1 guides the nuclease Cas9 to the specific genomic site and allows DSB next to protospacer adjacent motif (PAM). Homology directed repair (HDR) with a repair template containing two stop codons (TAA-TGA) and one BsmBI restriction site allows further analysis of crz1 mutants by PCR.

https://doi.org/10.1371/journal.pone.0265777.g001
**Fig 2. Susceptibility determination.** (A) Caspofungin (B) Micafungin, minimal inhibitory concentration (star) by broth microdilution method. The green colour bar represents relative growth. Dotted red line indicates cut-off values (C) Caspofungin (CAS) and micafungin (MCF) Etest determination using RPMI agar supplemented with 2% glucose. The $10^6$ cell/mL yeast suspensions were spread on RPMI agar plates, MIC readings were made following 24 h incubation at 35 ºC. The MIC was read as the drug concentration that leads to 80% of inhibition.

https://doi.org/10.1371/journal.pone.0265777.g002

**Fig 3. Stress responses.** (A) Isolates were heat-shocked at 37 and 40 ºC with 15 μg/mL CaM/CaL inhibitors (fluphenazine (Fph), tacrolimus (Fk506), cyclosporin A (CsA)) and with or without 1 μg/mL caspofungin (Cas) (B) and were tested for oxidative stress with 0.2 and 0.4 mM menadione with or without CaM/CaL inhibitors.

https://doi.org/10.1371/journal.pone.0265777.g003
Δ mutants exhibit a reduced capacity of biofilm formation

To evaluate the role of Crz1 in biofilm formation, we compared the ability to form biofilms using the polyurethane catheter pieces methodology of crz1Δ mutants and their parental isolates. Both clinical isolates were able to form biofilm, but biofilm development was significantly reduced when CRZ1 was disrupted (threefold decrease \( p < 0.001 \)) (Fig 4C).

Disruption of CRZ1 results in a decreased virulence in two different animal models

Virulence was first addressed in the non-vertebrate model of Galleria mellonella. C. glabrata crz1Δ mutants exhibited a significantly attenuated virulence compared with the WT isolates.
Indeed, C. glabrata WT CAGL1875 and CAGL1256 caused 100% mortality of larvae by day 4 and 5, respectively, whereas crz1Δ mutants caused 60% mortality after 10 days of follow-up. In contrast to what occurred for WT isolates, treatment with caspofungin at 1 μg/larvae proved to be effective in prolonging survival of larvae infected with crz1Δ mutants, which exhibited 80% of survival at day 10 of experiment (Fig 5).

In a mouse model of disseminated candidiasis, 8 days after intravenously yeast inoculation, a high number of colony-forming units (CFUs) of the C. glabrata WT isolates were found in the kidney (10⁵ CFU/g) and the spleen (10⁴ CFU/g). These values were rather similar for crz1Δ mutants. As expected, the kidney fungal burden after infection with the caspofungin-resistant WT isolate was not affected by treatment with caspofungin. By contrast, caspofungin treatment led to a significantly lower fungal load (p < 0.005) in the group infected with the crz1Δ mutant (Fig 6). Regarding the number of CFUs in the spleen, no statistical differences were found between WT and mutant (p > 0.005) (data not shown).

Fig 5. Time-kill curves. A. C. glabrata 1875 B 1256, wild type (circle) and crz1Δ (square) exposed to caspofungin 100 mg/L. The data are expressed as the percentages of survival. Log-rank (Mantel–Cox) test with p-values of < 0.05 was used to indicate statistical significance. As follows, p < 0.05 * p < 0.02 ** and p < 0.001 ***. Not significant (NS).

https://doi.org/10.1371/journal.pone.0265777.g005

(p < 0.005). Indeed C. glabrata WT CAGL1875 and CAGL1256 caused 100% mortality of larvae by day 4 and 5, respectively, whereas crz1Δ mutants caused 60% mortality after 10 days of follow-up. In contrast to what occurred for WT isolates, treatment with caspofungin at 1 μg/larvae proved to be effective in prolonging survival of larvae infected with crz1Δ mutants, which exhibited 80% of survival at day 10 of experiment (Fig 5).

In a mouse model of disseminated candidiasis, 8 days after intravenously yeast inoculation, a high number of colony-forming units (CFUs) of the C. glabrata WT isolates were found in the kidney (10⁵ CFU/g) and the spleen (10⁴ CFU/g). These values were rather similar for crz1Δ mutants. As expected, the kidney fungal burden after infection with the caspofungin-resistant WT isolate was not affected by treatment with caspofungin. By contrast, caspofungin treatment led to a significantly lower fungal load (p < 0.005) in the group infected with the crz1Δ mutant (Fig 6). Regarding the number of CFUs in the spleen, no statistical differences were found between WT and mutant (p > 0.005) (data not shown).
Discussion

The spread of antifungal resistance and the limited number of available antifungal drugs amplify the need to identify new fungal targets for development of novel therapeutic alternatives. Here, we demonstrate that CRZ1 disruption leads to echinocandin susceptibility in echinocandin clinical resistant C. glabrata isolates. This highly encouraging result could be supported by the well-known regulation of multiple biological processes governed by the CaM/CaL pathway and it is in agreement with previous studies showing the involvement of CaM/CaL and the downstream effector Crz1 in the antifungal response [26, 47, 48]. Indeed, glucan biosynthesis inhibitors exhibited fungicidal activity when combined with CaM/CaL inhibitors or by using calcineurin mutants of different Candida species [26, 49–51].

The ability of yeast cells to adapt to stress conditions and to activate cellular protection mechanisms represents an important survival strategy [52]. The CaM/CaL converges with other stress response pathways, such as the PKC and the PKA signaling pathways [53–55]. Here, C. glabrata crz1Δ mutants were subjected to heat stress and oxidative stress conditions. Our results suggest that although CaM/CaL is required for thermotolerance in C. glabrata, Crz1 is only involved in yeast growth at high temperatures, in concordance to what has been previously described [31, 47]. On the other hands, the antioxidant capacity of C. glabrata, mainly associated with the catalase Cta1, is higher than that of S. cerevisiae and C. albicans. Cta1 is controlled by the transcription factors Yap1, Msn2, and Msn4 and modulated by pathways other than the CaM /Cal [56]. Otherwise, our data show that Crz1 has a significant role in the oxidative stress tolerance in C. glabrata similarly to that reported for Metarhizium acridum [57]. This is an interesting result since it has been reported that the growth capacity in

![Kidney](https://doi.org/10.1371/journal.pone.0265777.g006)

Fig 6. Fungal burden in kidneys from mice inoculated with $10^7$ yeast cells of caspofungin-resistant C. glabrata 1875 wild-type or crz1Δ strain and treated daily with (or without) caspofungin as indicated. Statistical analysis was carried out using the analysis of variance (two-way ANOVA) with post Tukey-Kramer test.

https://doi.org/10.1371/journal.pone.0265777.g006
oxidative stress conditions was not affected by CaM/CaL inhibitors neither in calcineurin mutants [20, 55, 58] and therefore is a CaM/CaL-Crz1-independent phenomenon.

Regarding survival strategy, the fungal cell wall represents the first structure recognized by immune cells and a key defense against all external attacks, including those mediated by the host immune system. Since the cell wall of fungi is not present in human cells, it is an attractive target for antifungal drug development [59]. Yeast cell walls are principally composed by glucans, chitin, and glycoproteins. Yeast remodels their cell wall over time in response to environmental changes, a process controlled mainly by the cell wall integrity (CWI) pathway [60]. The concept that the cell wall is dynamic and that structural rearrangements occur due to the upregulation of pressure-induced signaling pathways is not novel [59]. For example, in response to caspofungin, which induces a weakening of the cell wall, the yeast increases the production of chitin and/or mannan [61, 62]. For instance, we recently published the proteomic changes induced by the exposure to caspofungin in *C. glabrata*. We found that the proteins that were most abundant after caspofungin treatment were involved in DNA binding, i.e., CAGL0M06831g (Cg.Crz1) CAGL0J11440g (Ca.Srp1), CAGL0L10021g (Ca.Dbp5) and CAGL0C01683g (Ca.Isw1). Of interest are the proteins involved in antifungal responses, CAGL0M06831g (Cg.Crz1) (CaM/CaL-pathway), CAGL0J00539g (Cg.Slt2) (PKC-pathway) and CAGL0J11440g (Ca.Srp1) [55]. Moreover, in *C. albicans* caspofungin produced changes in expression of genes encoding cell wall maintenance proteins, including FKS, as well as ECM21, ECM33, FEN12 and PHR1 [63]. In this study, the CRZ1 disruption resulted in an apparent increase in chitin. Since Crz1 is involved in cell wall biogenesis and regulation of FKS genes, this may be a compensatory mechanism in response to cell wall destabilizing [26]. As described in *S. cerevisiae* [64].

*C. glabrata* normally produces blastoconidia measuring 2 to 8 microns in diameter. Interestingly, in crz1Δ mutants, markedly enlarged “giant” (approximately 29 microns) blastoconidia were observed. Similar features related to cell size have been described in calcineurin mutants of *Mucor circinelloides* and for *Aspergillus flavus* conidia after calcineurin-Crz1 signaling pathway modulation [65, 66]. However, this giant cell production does not enhance *C. glabrata* virulence. Although we do not know the molecular mechanism for this phenomenon, we postulate that Crz1 disruption could lead to compensatory hyperactivation of the CWI and cAMP/PKA signaling pathways involved in cell size. Since, overexpression of PKA pathway members such as Pka1 caused an increase in cell size, moreover, the relation between Ca2+/CaM-PKA is well documented [67, 68]. In the dimorphic *M. circinelloides*, an emerging opportunistic pathogen, PKA activity is elevated during yeast growth in the presence of FK506, and in CaM/CaL mutants [65].

Biofilm formation is another important factor in the understanding of cellular adaptation. Infections resulting from the formation of biofilms, if unsuccessfully managed, can have devastating consequences, progressing to IFI with a high risk of mortality [69, 70]. Then, the biofilm-forming capacity is one of the most important *Candida* virulence factors. Although CaM/CaL, HSP90 and mitogen-activated pathways have been associated with biofilm antifungal resistance acquisition, their role in biofilm development has been little studied [55, 70, 71]. In *C. glabrata*, adherence step of biofilm formation is mediated by epithelial Epa- and Awp-adhesins (Awp1–13). The EPA family is composed of 23 genes, among which *EPA1, EPA6*, and *EPA7* are the most relevant [59, 71]. In a previous study, we reported that fluphenazine/caspofungin combination reduced the ability of biofilm formation [20]. Here, we demonstrated by an *in vitro* model using polyurethane catheter pieces, that CRZ1 disruption significantly affected biofilm development, confirming CaM/CaL-Crz1 pathway implication or CaM/CaL-Crz1 related pathways.
Deletion of CRZ1 reduces virulence in several human and plant pathogens such as *Aspergillus fumigatus* [66], *Cryptococcus neoformans* [72], *Magnaporthe oryzae* [73], and *Botrytis cinerea* [74]. In *M. acridum*, deletion of CRZ1 leads to down-regulation of hydrophobins, proteins involved in cell surface hydrophobicity, adhesion and virulence [57]. Our data also showed that Crz1 had an important role in the pathogenicity of *C. glabrata* as was observed in the *Galleria mellonella* model.

Regarding the susceptibility pattern of *crz1* mutants, *in vivo* experiments confirm the *in vitro* susceptibility results. Indeed, caspofungin treatment reduces the larvae mortality and kidney fungal burden in the candidiasis disseminated models. However, as previously described caspofungin did not reduce the number of colonies of *crz1Δ* mutants in the spleen [75]. A significant change in the spleen fungal load was only observed with high dose of caspofungin and lower *C. glabrata* inoculum [76]. These observations could be due to the pharmacokinetic of caspofungin in the spleen which has already informed is superior in the liver and the kidneys, and in fact, is inferior to fluconazole and amphotericin B concentrations detected in the spleen [77, 78]. Moreover, in a murine model, infection with *cnb* mutants resulted in a greater reduction in fungal burden compared to *crz1* mutants. Interestingly, a decreased burden was observed in the kidney; unfortunately, *in vivo* antifungal activity was not evaluated [47].

Currently, drugs against CaM and Cal proteins exist and are used in humans as psychotropic and for immunosuppressive therapy, respectively. Nevertheless their potential use as antifungal therapy is not possible, due to the similarity of CaM/Cal targets between humans and fungi [24, 79]. Identification of novel selective drugs without side effects (e.g., immunosuppression) remains required. To achieve this objective, transcription factors are attractive as novel antifungal targets since they are evolutionarily divergent between fungi and humans and hence can be exploited as selective approach [80, 81]. Recently Malik et al, in an *in-silico* study described the potential antifungal properties of natural compounds against *Rhizoctonia solani Crz1* [82], which confirms that the design of Crz1 inhibitors could be a successful therapeutic strategy for fighting life-threatening fungal diseases and increase of echinocandin resistance.

**Conclusions**

In summary, the use of CRISPR-Cas9 allowed us to generate and evaluate phenotypically *crz1Δ* mutants from echinocandins-resistant *C. glabrata* isolates and to demonstrate their crucial role in *in vitro* and *in vivo* susceptibility to echinocandin, stress tolerance, biofilm formation, and virulence. In this sense, our results strongly suggest that inhibitors of Crz1 could have an important role in the development of novel therapeutic drugs to combat fungal infections, considering the increase of resistance phenomenon and the low number of antifungal drugs available.

**Acknowledgments**

We thank the UTE-IRS2 (University of Nantes) for housing the mice and the MicroPICell facility, SFR-Santé, INSERM, CNRS, UNIV Nantes, CHU Nantes, Nantes, France, member of the national infrastructure France-BioImaging supported by the French National Research Agency (ANR-10-INBS-04).

**Author Contributions**

**Conceptualization:** Andres Ceballos-Garzon, Elvira Roman, Jesús Pla, Patrice Le Pape, Claudia M. Parra-Giraldo.

**Data curation:** Andres Ceballos-Garzon, Elvira Roman, Patrice Le Pape.
Formal analysis: Andres Ceballos-Garzon, Elvira Roman, Jesús Pla, Patrice Le Pape, Claudia M. Parra-Giraldo.

Funding acquisition: Patrice Le Pape, Claudia M. Parra-Giraldo.

Investigation: Andres Ceballos-Garzon, Elvira Roman, Fabrice Pagniez, Daniela Amado, Carlos J. Alméciga-Díaz, Patrice Le Pape.

Methodology: Andres Ceballos-Garzon, Fabrice Pagniez, Daniela Amado, Carlos J. Alméciga-Díaz.

Project administration: Claudia M. Parra-Giraldo.

Resources: Patrice Le Pape.

Supervision: Patrice Le Pape, Claudia M. Parra-Giraldo.

Validation: Jesús Pla.

Writing – original draft: Andres Ceballos-Garzon.

Writing – review & editing: Andres Ceballos-Garzon, Elvira Roman, Jesús Pla, Patrice Le Pape, Claudia M. Parra-Giraldo.

References

1. Bongomin F, Gago S, Oladele R, Denning D. Global and Multi-National Prevalence of Fungal Diseases—Estimate Precision. J Fungi. 2017; 3: 57. https://doi.org/10.3390/jof3040057 PMID: 29371573

2. Vallabhaneni S, Mody RK, Walker T, Chiller T. The Global Burden of Fungal Diseases. Infect Dis Clin North Am. 2016; 30: 1–11. https://doi.org/10.1016/j.idc.2015.10.004 PMID: 26739604

3. Hahn-Ast C, Glasmacher A, Mückter S, Schmitz A, Kraemer A, Marklein G, et al. Overall survival and fungal infection-related mortality in patients with invasive fungal infection and neutropenia after myelo-suppressive chemotherapy in a tertiary care centre from 1995 to 2006. J antimicrob chemother. 2010; 65. https://doi.org/10.1093/jac/dkp507 PMID: 20106864

4. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. Nat Rev Dis Prim. 2018; 4: 1–20. https://doi.org/10.1038/s41572-018-0001-z PMID: 29930242

5. Gupta A, Gupta A, Varma A. Candida glabrata candidemia: An emerging threat in critically ill patients. Indian J Crit Care Med. 2015; 19: 151–154. https://doi.org/10.4103/0972-5229.152757 PMID: 25810610

6. Lamoth F, Lockhart SR, Berkow EL, Calandra T. Changes in the epidemiologic landscape of invasive candidiasis. J Antimicrob Chemother. 2018; 73: i4–i13. https://doi.org/10.1093/jac/dkx444 PMID: 29304207

7. Perlin DS. Echinocandin Resistance in Candida. Clin Infect Dis. 2015; 61 Suppl 6: S612–7. https://doi.org/10.1093/cid/civ931 PMID: 26567278

8. Moro F, Jensen RH, Le Pape P, Arendrup MC. Molecular basis of antifungal drug resistance in yeasts. Int J Antimicrob Agents. 2017; 50: 599–606. https://doi.org/10.1016/j.ijantimicag.2017.05.012 PMID: 28669835

9. Healey KR, Jimenez Ortizgoza C, Shor E, Perlin DS. Genetic Drivers of Multidrug Resistance in Candida glabrata. Front Microbiol. 2016; 0: 1995. https://doi.org/10.3389/fmicb.2016.01995 PMID: 28018323

10. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, et al. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. Clin Infect Dis. 2015; 62: civ933. https://doi.org/10.1093/cid/civ933 PMID: 26679628

11. Zimbeck AJ, Iqbal N, Ahlquist AM, Farley MM, Harrison LH, Chiller T, et al. FKS Mutations and Elevated Echinocandin MIC Values among Candida glabrata Isolates from U.S. Population-Based Surveillance. Antimicrob Agents Chemother. 2010; 54: 5042–5047. https://doi.org/10.1128/AAC.00836-10 PMID: 20837754

12. Roemer T, Krysan DJ. Antifungal drug development: challenges, unmet clinical needs, and new approaches. Cold Spring Harbor perspectives in medicine. Cold Spring Harbor Laboratory Press; 2014.
13. Kuchaiev O, Przulj N. Integrative network alignment reveals large regions of global network similarity in yeast and human. Bioinformatics. 2011; 27: 1390–6. https://doi.org/10.1093/bioinformatics/btr127 PMID: 21414992

14. Parente-Rocha JA, Bailão AM, Amaral AC, Taborda CP, Piaccez JD, Borges CL, et al. Antifungal Resistance, Metabolic Routes as Drug Targets, and New Antifungal Agents: An Overview about Endemic Dimorphic Fungi. Mediators Inflamm. 2017; 2017: 1–16. https://doi.org/10.1155/2017/9870679 PMID: 28694566

15. Shapiro RS, Robbins N, Cowen LE. Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiol Mol Biol Rev. 2011; 75: 213–267. https://doi.org/10.1128/MMBR.00045-10 PMID: 21646428

16. LaFayette SL, Collins C, Zaas AK, Schell WA, Betancourt-Quiroz M, Gunatilaka AAL, et al. PKC signaling regulates drug resistance of the fungal pathogen Candida albicans via circuitry comprised of Mkc1, calcineurin, and Hsp90. PLoS Pathog. 2010; 6: e1000169. https://doi.org/10.1371/journal.ppat.1000169 PMID: 20865172

17. Reinoso-Martín C, Schüller C, Schuetzer-Muehlbauer M, Kuchler K. The yeast protein kinase C cell integrity pathway mediates tolerance to the antifungal drug caspofungin through activation of Sti2p mitogen-activated protein kinase signalling. Eukaryot Cell. 2003; 2: 1200–10. https://doi.org/10.1128/EC.2.6.1200-1210.2003 PMID: 14685455

18. Román E, Alonso-Monge R, Miranda A, Pla J. The Mkk2 MAPKK Regulates Cell Wall Biogenesis in Cooperation with the Cek1-Pathway in Candida albicans. Lenardon MD, editor. PLoS One. 2015; 10: e0133476. https://doi.org/10.1371/journal.pone.0133476 PMID: 26197240

19. Chin D, Means AR. Calmodulin: A prototypical calcium sensor. Trends in Cell Biology. Elsevier; 2000. pp. 322–328. https://doi.org/10.1016/s0962-8924(00)01800-6 PMID: 10846684

20. Ceballos Garzon A, Armando D, Robert E, Parra Giraldo CM, Le Pape P. Impact of calmodulin inhibition by fluphenazine on susceptibility, biofilm formation and pathogenicity of caspofungin-resistant Candida glabrata. J Antimicrob Chemother. 2020; 75: 1187–1193. https://doi.org/10.1093/jac/dkz565 PMID: 32011702

21. Chen Y-L, Brand A, Morrison EL, Silao FGS, Bigol UG, Malbas FF, et al. Calcineurin Controls Drug Tolerance, Hyphal Growth, and Virulence in Candida dubliniensis. Eukaryot Cell. 2011; 10: 803–819. https://doi.org/10.1128/EC.00310-10 PMID: 21531874

22. Juvvadi PR, Lee SC, Heitman J, Steinbach WJ. Calcineurin in fungal virulence and drug resistance: Prospects for harnessing targeted inhibition of calcineurin for an antifungal therapeutic approach. Virulence. 2017; 8: 186–197. https://doi.org/10.1080/21505594.2016.1201250 PMID: 27325145

23. Xu H, Fang T, Omran RP, Whiteway M, Jiang L. RNA sequencing reveals an additional Crz1-binding motif in promoters of its target genes in the human fungal pathogen Candida albicans. Cell Commun Signal. 2020; 18: 1. https://doi.org/10.1186/s12964-019-0473-9 PMID: 31900175

24. Thewes S. Calcineurin-Crz1 signaling in lower eukaryotes. Eukaryot Cell. 2014; 13: 694–705. https://doi.org/10.1128/EC.00038-14 PMID: 24681686

25. Miyazaki T, Izumikawa K, Nagayoshi Y, Saijo T, Yamauchi S, Morinaga Y, et al. Functional characterization of the regulators of calcineurin in Candida dubliniensis. FEBS Yeast Res. 2011; 11: 621–630. https://doi.org/10.1111/j.1567-1364.2011.00751.x PMID: 22093746

26. Singh-Babak SD, Babak T, Diezmann S, Hill JA, Xie JL, Chen Y-L, et al. Global Analysis of the Evolution and Mechanism of Echinocandin Resistance in Candida glabrata. Mitchell AP, editor. PLOS Pathog. 2012; 8: e1002718. https://doi.org/10.1371/journal.ppat.1002718 PMID: 22655774

27. O’Meara TR, O’Meara MJ, Polvi EJ, Pourhaghighi MR, Liston SD, Lin Z-Y, et al. Global proteomic analyses define an environmentally contingent Hsp90 interactome and reveal chaperone-dependent regulation of stress granule proteins and the R2TP complex in a fungal pathogen. Leu J-Y, editor. PLOS Biol. 2019; 17: 1–38. https://doi.org/10.1371/journal.pbio.3000338 PMID: 31283755

28. Zakrzewska A, Boorza A, Brul S, Hellingwerf KJ, Klis FM. Transcriptional Response of Saccharomyces cerevisiae to the Plasma Membrane-Perturbing Compound Chitosan. Eukaryot Cell. 2005; 4: 703. https://doi.org/10.1128/EC.4.4.703-715.2005 PMID: 15821130

29. Yu SJ, Chang YL, Chen YL. Calcineurin signaling: Lessons from Candida species. FEMS Yeast Res. 2015; 15: 1–7. https://doi.org/10.1093/femsye/fov016 PMID: 25878052

30. Zhang J, Silao FGS, Bigol UG, Bungay AAC, Nicolas MG, Heitman J, et al. Calcineurin Is Required for Pseudohyphal Growth, Virulence, and Drug Resistance in Candida lusitaniae. Bahn Y-S, editor. PLoS One. 2012; 7: e44192. https://doi.org/10.1371/journal.pone.0044192 PMID: 22952924

31. Chen Y-L, Konieczka JH, Springer DJ, Bowen SE, Zhang J, Silao FGS, et al. Convergent Evolution of Calcineurin Pathway Roles in Thermotolerance and Virulence in Candida glabrata. G3 Genes, Genomes, Genet. 2012; 2: 675–681. https://doi.org/10.1534/g3.112.002279 PMID: 22690377
32. Ruiz A, Serrano R, Aníñor J. Direct regulation of genes involved in glucose utilization by the calcium/calci-neurin pathway. J Biol Chem. 2008; 283: 13923–13933. https://doi.org/10.1074/jbc.M708683200 PMID: 18362157

33. Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. Elife. 2013; 2. https://doi.org/10.7554/eLife.00471 PMID: 23386978

34. Román E, Prieto D, Alonso-Monge R, Pia J. New insights of CRISPR technology in human pathogenic fungi. Future Microbiology. Future Medicine Ltd.; 2019. pp. 1243–1255. https://doi.org/10.2217/fmb-2019-0183 PMID: 31625446

35. Enkler L, Richer D, Marchand AL, Ferrandon D, Jossinet F. Genome engineering in the yeast pathogen Candida glabrata using the CRISPR-Cas9 system. Sci Rep. 2016; 6: 35766. https://doi.org/10.1038/srep35766 PMID: 27767081

36. Cen Y, Timmermans B, Souffriau B, Thevelein JM, Van Dijck P. Comparison of genome engineering using the CRISPR-Cas9 system in C. glabrata wild-type and lig4 strains. Fungal Genet Biol. 2017; 107: 44–50. https://doi.org/10.1016/j.fgb.2017.08.004 PMID: 28822858

37. Moroc L, Fairhead C. A new inducible CRISPR-Cas9 system useful for genome editing and study of double-strand break repair in Candida glabrata. Yeast. 2019; 36: 723–731. https://doi.org/10.1002/yea.3440 PMID: 31945142

41. Vargas-Casanova Y, Carlos Villamil Poveda J, Jenny Rivera-Monroy Z, Ceballos Garzón A, Fierro-Medina R, Le Pape P, et al. Palindromic Peptide LfcinB (21–25)Pal Exhibited Antifungal Activity against Multidrug-Resistant Candida. ChemistrySelect. 2020; 5: 7236–7242. https://doi.org/10.1002/slct.202001329

42. Kohler GA, Kohler K, White TC, Agabian N. Overexpression of a Cloned IMP Dehydrogenase Gene of Candida albicans Confers Resistance to the Specific Inhibitor Mycophenolic Acid. J Bacteriol. 1997.

44. CLSI. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Fourth Informa-tional Supplement M27-S4. 2012.

45. Onyewu C, Blankenship JR, Del Poeta M, Heitman J. Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors against Candida albicans, Candida glabrata, and Candida krusei. Antimicrob Agents Chemother. 2003; 47: 956–964. https://doi.org/10.1128/AAC.47.3.956-964.2003 PMID: 12604527

51. Longo VD, Fabrizio P. Regulation of longevity and stress resistance: a molecular strategy conserved from yeast to humans? Cell Mol Life Sci. 2002; 59: 593–8. https://doi.org/10.1007/s00018-002-8477-8 PMID: 12169020
53. Levin DE. Cell wall integrity signaling in Saccharomyces cerevisiae. Microbiol Mol Biol Rev. 2005; 69: 262–91. https://doi.org/10.1128/MMBR.69.2.262-291.2005 PMID: 15944456
54. Kafadar KA, Cyert MS. Integration of stress responses: Modulation of calcineurin signaling in Saccharomyces cerevisiae by protein kinase A. Eukaryot Cell. 2004; 3: 1147–1153. https://doi.org/10.1128/EC.3.5.1147-1153.2004 PMID: 15470242
55. Ceballos-Garzon A, Monteoliva L, Gil C, Alvarez-Moreno C, Vega-Vela NE, Englthaler DM, et al. Genotypic, proteomic, and phenotypic approaches to decipher the response to caspofungin and calcineurin inhibitors in clinical isolates of echinocandin-resistant Candida glabrata. J Antimicrob Chemother. 2021; 1: 1–13. https://doi.org/10.1093/JAC/DKAB454 PMID: 34893830
56. Cañellas-Cruz M, Briones-Martín-Del-Campo M, Cañas-Villamar I, Montalvo-Arredondo J, Riego-Ruiz L, Castaño I, et al. High Resistance to Oxidative Stress in the Fungal Pathogen Candida glabrata Is Mediated by a Single Catalase, Cat1p, and Is Controlled by the Transcription Factors Yap1p, Skn7p, Msn2p, and Msn4p. Eukaryot Cell. 2008; 7: 814–825. https://doi.org/10.1128/EC.00011-08 PMID: 18375620
57. Chen X, Liu Y, Keyhani NO, Xia Y, Cao Y. The regulatory role of the transcription factor Crz1 in stress tolerance, pathogenicity, and its target gene expression in Metarhizium acridum. Appl Microbiol Biotechnol. 2017; 101: 5033–5043. https://doi.org/10.1007/s00253-017-8299-9 PMID: 28424845
58. Ghosh MC, Wang X, Li S, Klee C. Regulation of Calcineurin by Oxidative Stress. Methods Enzymol. 2003; 366: 289–304. https://doi.org/10.1016/s0076-6879(03)66022-2 PMID: 14674256
59. De Groot PWJ, Kraneveld EA, Qing YY, Dekker HL, Groß U, Crielaard W, et al. The cell wall of the human pathogenic Zygomycete Mucor circinelloides. PLoS Pathog. 2013; 9. https://doi.org/10.1128/MMBR.69.2.262-291.2005 PMID: 15944456
60. Soriani FM, Malavazi I, Da Silva Ferreira ME, Savoldi M, Von Zeska Kress MR, De Souza Goldman MH, et al. Functional characterization of the Aspergillus fumigatus CRZ1 homologue, CrzA. Mol Microbiol. 2008; 67: 1274–1291. https://doi.org/10.1111/j.1365-2958.2008.06122.x PMID: 18298443
61. Mazáň M, Mazáňová K, Farkaš V. Phenotype analysis of Saccharomyces cerevisiae mutants with deletions in Pir cell wall glycoproteins. Antonie van Leeuwenhoek, Int J Mol Microbiol. 2008; 94: 335–42. https://doi.org/10.1007/s10482-008-9228-0 PMID: 18278564
62. Walker LA, Gow NAR, Munro CA. Elevated Chitin Content Reduces the Susceptibility of Candida species to Caspofungin. Antimicrob Agents Chemother. 2013; 57: 146–154. https://doi.org/10.1128/AAC.01486-12 PMID: 23089748
63. Liu TT, Lee REB, Barker KS, Lee RE, Wei L, Homayouni R, et al. Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in Candida albicans. Antimicrob Agents Chemother. 2020; 49: 2226–2236. https://doi.org/10.1128/AAC.49.6.2226–2236.2005
64. Lagorce A, Hauser N, Labourdette D, Rodríguez C, Martín-Yken H, Arroyo J, et al. Genome-wide analysis of the response to cell wall mutations in the yeast Saccharomyces cerevisiae. J Biol Chem. 2003; 278: 20345–20357. https://doi.org/10.1074/jbc.M211604200 PMID: 12644457
65. Lee SC, Li A, Calo S, Heitman J. Calcineurin Plays Key Roles in the Dimorphic Transition and Virulence of the Human Pathogenic Zygomycete Mucor circinelloides. PLoS Pathog. 2013; 9. https://doi.org/10.1371/journal.ppat.1003625 PMID: 24039585
66. Soriani FM, Malavazi I, Da Silva Ferreira ME, Savoldi M, Von Zeska Kress MR, De Souza Goldman MH, et al. Functional characterization of the Aspergillus fumigatus CRZ1 homologue, CrzA. Mol Microbiol. 2008; 67: 1274–1291. https://doi.org/10.1111/j.1365-2958.2008.06122.x PMID: 18298443
67. Huang G, Huang Q, Wei Y, Wang Y, Du H. Multiple roles and diverse regulation of the Ras/cAMP/protein kinase A pathway in Candida albicans. Mol Microbiol. 2019; 111: 6–16. https://doi.org/10.1111/mmi.14148 PMID: 30299574
68. S O, T K, H F. Regulation of Ca(2+)/calmodulin-dependent protein kinase kinase alpha by cAMP-dependent protein kinase. I. Biochemical analysis. J Biol Chem. 2001; 130: 503–513. https://doi.org/10.1093/oxfordjournals.jbchem.a003013 PMID: 11574079
69. Rajendran R, Sherry L, Nile CJ, Sherriff A, Johnson EM, Hanson MF, et al. Biofilm formation is a risk factor for mortality in patients with Candida albicans bloodstream infection-Scotland, 2012–2013. Clin Microbiol Infect. 2016; 22: 87–93. https://doi.org/10.1016/j.cmi.2015.09.018 PMID: 26432192
70. Taff HT, Mitchell KF, Edward JA, Andes DR. Mechanisms of Candida biofilm drug resistance. Future Microbiology. Future Medicine Ltd London, UK; 2013. pp. 1325–1337. https://doi.org/10.2217/fmb.13.101 PMID: 24059922
71. Rodrigues CF, Rodrigues ME, Silva S, Henriques M. Candida glabrata biofilms: How far have we come? Journal of Fungi. MDPI AG; 2017. https://doi.org/10.3390/jof3010011 PMID: 29371530
72. Moranova Z, Virtudazo E, Hricova K, Ohkusmu M, Kawamoto S, Husicova v, et al. The CRZ1/SP1-like gene links survival under limited aeration, cell integrity and biofilm formation in the pathogenic yeast
cryptococcus neoformans. Biomed Pap. 2014; 158: 212–220. https://doi.org/10.5507/bp.2013.024
PMID: 23640031
73. Zhang H, Zhao Q, Liu K, Zhang Z, Wang Y, Zheng X. MgCRZ1, a transcription factor of Magnaporthe
grisea, controls growth, development and is involved in full virulence. FEMS Microbiol Lett. 2009; 293:
160–169. https://doi.org/10.1111/j.1574-6968.2009.01524.x PMID: 19260966
74. Schumacher J, De Larrinoa IF, Tudzynski B. Calcineurin-responsive zinc finger transcription factor
CRZ1 of Botrytis cinerea is required for growth, development, and full virulence on bean plants. Eukar-
yot Cell. 2008; 7: 584–601. https://doi.org/10.1128/EC.00426-07 PMID: 18263765
75. Graybill JR, Bocanegra R, Luther M, Fothergill A, Rinaldi MJ. Treatment of Murine Candida krusei or
Candida glabrata Infection with L-743,872. Antimicrob Agents Chemother. 1997; 41: 1937–1939.
https://doi.org/10.1128/AAC.41.9.1937 PMID: 9303388
76. Ju JY, Polhamus C, Marr KA, Holland SM, Bennett JE. Efficacies of fluconazole, caspofungin, and
amphotericin B in Candida glabrata-infected p47phox-/- knockout mice. Antimicrob Agents Chemother.
2002. https://doi.org/10.1128/AAC.46.5.1240–1245.2002
77. Felton T, Troke PF, Hope WW. Tissue penetration of antifungal agents. Clin Microbiol Rev. 2014; 27:
68–88. https://doi.org/10.1128/CMR.00046-13 PMID: 24396137
78. Bellmann R, Smuszkiwicz P. Pharmacokinetics of antifungal drugs: practical implications for optimized
treatment of patients. Infection. 2017; 45: 737–779. https://doi.org/10.1007/s15010-017-1042-z PMID:
28702763
79. Juvvadi PR, Lamoth F, Steinbach WJ. Calcineurin as a Multifunctional Regulator: Unraveling Novel
Functions in Fungal Stress Responses, Hyphal Growth, Drug Resistance, and Pathogenesis. Fungal
Biol Rev. 2014; 28: 56–69. https://doi.org/10.1016/j.fbr.2014.02.004 PMID: 25383089
80. Karababa M, Valentino E, Pardini G, Coste AT, Bille J, Sanglard D. CRZ1, a target of the calcineurin
pathway in Candida albicans. Mol Microbiol. 2006; 59: 1429–1451. https://doi.org/10.1111/j.1365-2958.
2005.05037.x PMID: 16468987
81. Bahn Y-S. Exploiting Fungal Virulence-Regulating Transcription Factors As Novel Antifungal Drug Tar-
gets. Hogan DA, editor. PLOS Pathog. 2015; 11: e1004936. https://doi.org/10.1371/journal.ppat.
1004936 PMID: 26181382
82. Malik A, Afag S, Gamal B El, Ellatif MA, Hassan WN, Dera A, et al. Molecular docking and pharmaco-
kINETIC evaluation of natural compounds as targeted inhibitors against Crz1 protein in Rhizoctonia solani.
Bioinformation. 2019; 15: 277–286. https://doi.org/10.6026/97320630015277 PMID: 31285645