Dissecting the Roles of the Calcineurin Pathway in Unisexual Reproduction, Stress Responses, and Virulence in *Cryptococcus deneoformans*

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ABSTRACT The Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin orchestrates sexual reproduction, stress responses, and virulence via branched downstream pathways in the opportunistic human fungal pathogen *Cryptococcus neoformans*. The calcineurin-binding protein Cbp1, the calcineurin temperature suppressor Cts1, the calcineurin-responsive zinc finger transcription factor Crz1, and the calcineurin targets Pbp1, Tif3, and Puf4, all function downstream of calcineurin to orchestrate distinct cellular processes. To elucidate how the calcineurin pathway regulatory network governs unisexual reproduction, stress responses, and virulence, we have analyzed the self-filamentous *C. deneoformans* strain, XL280α, and generated double mutants of these calcineurin downstream genes. We demonstrated that calcineurin governs unisexual reproduction at different sexual developmental stages, in which the initiation of the yeast–hyphal morphological transition is independent of Crz1, whereas the sporulation process is dependent on Crz1. Calcineurin-dependent unisexual reproduction is independent of the pheromone response pathway. Crz1 synergistically interacts with different calcineurin downstream targets in responding to ER, high-calcium, and cell wall stresses. We observed a widespread synergy suggesting that these proteins function in complex branched pathways downstream of calcineurin with some functional redundancy, which may allow efficient signaling network rewiring within the pathway for prompt adaptation to changing environments. Finally, we showed that deletion of *PBP1* or *TIF3* in the *cna1*Δ mutant background conferred a modest level of growth tolerance at 37°, but that the *cna1*Δ *pbp1*Δ and *cna1*Δ *tif3*Δ double mutants were both avirulent, suggesting that calcineurin may control virulence via mechanisms beyond thermotolerance.

KEYWORDS calcineurin; *Cryptococcus deneoformans*; genetic interactions; unisexual reproduction; stress response; virulence

CALCINEURIN, the Ca^{2+}/calmodulin-dependent protein phosphatase, is conserved from fungi to humans, and it plays central functions in governing key cellular processes (Cyert *et al.* 1991; Liu *et al.* 1991b; Sugiura *et al.* 2002; Aramburu *et al.* 2004). The phosphatase is composed of two subunits: a catalytic subunit, Cna1, and a regulatory subunit, Cnb1 (Klee *et al.* 1979). Calcineurin dephosphorylates and activates transcription factors, NF-AT in mammalian systems, and Crz1, the calcineurin-responsive zinc finger transcription factor 1, in fungi, which results in the translocation of NF-AT and Crz1 into the nucleus (Flanagan *et al.* 1991; Northrop *et al.* 1994; Beals *et al.* 1997; Stathopoulos-Gerontides *et al.* 1999; Park *et al.* 2016; Chow *et al.* 2017). Activation of NF-AT is crucial for skeletal muscle development, immune response, cardiac function, and many other important developmental processes, and dysregulation of the calcineurin pathway has been implicated in many human developmental and neurodegenerative diseases (Clipstone and Crabtree 1992; Steinbach *et al.* 2007; Al-Shanti and Stewart 2009; Furman and Norris 2014; Kipanyula *et al.* 2016; Dewenter *et al.* 2017). Calcineurin phosphatase activity is inhibited by two immunosuppressive drugs, cyclosporin A (CsA) and FK506, which target cyclophilin A and FKBP12, respectively (Stewart *et al.* 1982; Liu *et al.* 1991a; Blankenship *et al.* 2003a).

In many human fungal pathogens, calcineurin orchestrates fungal morphogenesis, stress responses, and virulence (Fox and Heitman 2002; Steinbach *et al.* 2007). In ascomycetes, calcineurin controls hyphal growth, drug tolerance, and virulence in

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multiple *Candida* species, including *Candida albicans*, *Ca. glabrata*, *Ca. lusitaniae*, *Ca. tropicalis*, and *Ca. dubliniensis* (Bader *et al.* 2003; Blankenship *et al.* 2003b; Sanglard *et al.* 2003; Chen *et al.* 2011, 2012, 2014; Zhang *et al.* 2012). In *Ca. albicans*, calcineurin is essential for survival during membrane stress and in serum, which promotes virulence (Cruz *et al.* 2002; Blankenship *et al.* 2003b; Blankenship and Heitman 2005). In another ascomycete, *Aspergillus fumigatus*, calcineurin is required for hyphal maturation and pathogenicity (Steinbach *et al.* 2006; Juvvadi *et al.* 2011, 2014). In the zygomycete, *Mucor circinelloides*, calcineurin is crucial for its dimorphic transition and virulence (Lee *et al.* 2013, 2015). The involvement of calcineurin in fungal virulence renders it a prominent drug target for antifungal treatments (Steinbach *et al.* 2007; Coelho and Casadevall 2016). Several studies have shown synergy between calcineurin inhibitors and other antifungal drugs, which provided new frontiers for novel antifungal therapies (Marchetti *et al.* 2000; Steinbach *et al.* 2004; Cowen and Lindquist 2005).

The basidiomycetous *Cryptococcus* species can cause life-threatening meningoencephalitis in immunocompromised patients, which contributes to almost 200,000 annual mortalities around the world (Park *et al.* 2009; Rajasingham *et al.* 2017). Calcineurin regulates *Cryptococcus* sexual reproduction, stress responses, and pathogenicity (Kozubowski *et al.* 2009). In the *Cryptococcus* species complex, the sister species *Cryptococcus neoformans* and *C. deneoformans* can undergo bisexual reproduction between *MAT***a** and *MAT*α cells (Kwon-Chung 1976; Nielsen *et al.* 2003), while *C. deneoformans* also undergoes robust unisexual reproduction in the absence of an opposite mating type partner (Lin *et al.* 2005). During *Cryptococcus* sexual reproduction, yeast cells undergo a morphological transition to a hyphal state, and this process is regulated by the pheromone response pathway and the transcription factors Mat2 and Znf2 (Wang *et al.* 2012; Feretzaki and Heitman 2013). In the absence of *CNA1* or *CNB1*, hyphal production is blocked during both unisexual and bisexual reproduction (Cruz *et al.* 2001), and this calcineurin-dependent filamentation is independent of the pheromone response pathway under some conditions (Gyawali *et al.* 2017). A multicopy suppressor screen identified a calcineurin temperature suppressor Cts1, which is also required for filamentation during *Crytopcoccus* sexual reproduction (Fox *et al.* 2003), and a yeast two-hybrid screen identified a calcineurin-binding protein Cbp1/Calcipressin, which is only required for filamentation during bisexual but not unisexual reproduction (Görlach *et al.* 2000; Fox and Heitman 2005). In a phosphoproteomic study, Park *et al.* (2016) identified 44 putative calcineurin targets and demonstrated that calcineurin regulates bisexual reproduction in *C. neoformans* through three Cna1 downstream targets: the poly(A)-binding protein-binding protein Pbp1 and the translation initiation factor 4B Tif3, which are both required for hyphal production, and the pumillio-family RNA-binding protein Puf4, which negatively regulates hyphal production (Park *et al.* 2016).

Growth at host temperature is a prerequisite for fungal virulence (Fox and Heitman 2002). In *Cryptococcus*, calcineurin is essential for growth at 37° and virulence in mice (Odom *et al.* 1997; Fox *et al.* 2001). The temperature-sensitive phenotype exhibited by the *cna1*Δ mutant is suppressed by a high copy number of the *CTS1* gene (Fox *et al.* 2003). Cts1 is dephosphorylated by calcineurin at 37°, suggesting that Cts1 functions as an effector for the calcineurin pathway during high-temperature stress (Aboobakar *et al.* 2011). Cts1 is also required for high-temperature tolerance and virulence in *Cryptococcus* (Fox *et al.* 2003). The calcineurin-binding protein Cbp1 is not required for calcineurin-dependent high-temperature growth, and deletion of *CBP1* only attenuates but does not abolish virulence (Görlach *et al.* 2000). Based on the phenotypes of *cna1*Δ, *cts1*Δ, and *cbp1*Δ mutants, virulence is correlated with the ability to grow at 37°. On the contrary, the calcineurin downstream targets Puf4 and Pbp1 regulate thermosensitivity and virulence in an opposite fashion in *C. neoformans* (Glazier *et al.* 2015; Park *et al.* 2016). Puf4 is required for high-temperature stress tolerance but not required for virulence (Glazier *et al.* 2015). Whereas deletion of *PBP1* confers high-temperature resistance, the virulence of *pbp1*Δ mutants is, however, attenuated (Park *et al.* 2016). This opposite correlation between virulence and thermosensitivity in *puf4* and *pbp1* mutants suggests that virulence is a complex trait, impacted by many factors in addition to thermotolerance to host temperature.

In *Saccharomyces cerevisiae* and *Ca. albicans*, calcineurin orchestrates stress responses via the calcineurin-responsive zinc finger transcription factor 1 (Crz1) (Stathopoulos and Cyert 1997; Yoshimoto *et al.* 2002; Onyewu *et al.* 2004; Santos and de Larrinoa 2005; Karababa *et al.* 2006). A Crz1 ortholog identified in *C. neoformans* regulates cell wall integrity (Lev *et al.* 2012; Moranova *et al.* 2014), and transcriptional profiling suggests the calcineurin-Crz1 regulatory network has been extensively rewired in *C. neoformans* compared to *S. cerevisiae* (Chow *et al.* 2017). Deletion of *CRZ1* does not impact bisexual reproduction in *C. neoformans*, suggesting that calcineurin regulates mating through a Crz1-independent pathway (Park *et al.* 2016; Chow *et al.* 2017). *crz1*Δ mutants display similar but less-severe stress response phenotypes compared to *cna1*Δ mutants, and the virulence of *crz1*Δ mutants is only attenuated rather than abolished in contrast to *cna1*Δ mutants, indicating that Crz1 functions in a branched pathway downstream of calcineurin (Chow *et al.* 2017). Similarly, in *Ca. albicans*, Crz1 is involved in azole tolerance but not required for virulence (Onyewu *et al.* 2004), and in *A. fumigatus*, the Crz1 ortholog CrzA functions downstream of calcineurin and plays contributory but not essential roles in regulating hyphal growth, conidial germination, and virulence compared to calcineurin (Cramer *et al.* 2008). Thus, it is a common theme in fungal pathogens that calcineurin regulates morphogenesis, stress tolerance, and virulence through branched downstream pathways.

To better understand how the calcineurin pathway regulates sexual reproduction, stress responses, and virulence, we conducted genetic analyses in the self-filamentous *C. deneoformans* strain XL280α. We found that the yeast–hyphal morphological transition during unisexual reproduction is independent of

Crz1, but that the sporulation process is dependent upon Crz1. We demonstrated that the calcineurin downstream targets synergistically interact with each other in regulating stress responses, and functional redundancy among these genes enables plasticity in the calcineurin pathway and allows rapid signaling network rewiring during evolution. Finally, we showed that high-temperature-tolerant *cna1*Δ *pbp1*Δ and *cna1*Δ *tif3*Δ mutants are avirulent, revealing that conferring thermotolerance to host temperature is not the only way in which the calcineurin pathway controls virulence.

Materials and Methods

Strains, media, and growth conditions

Strains used in this study are listed in Supplemental Material, Table S1. All gene deletion mutants were generated in the congenic strain pair *MAT*α XL280 and *MAT***a** XL280 backgrounds (Lin *et al.* 2005; Zhai *et al.* 2013). The congenic strain pair *MAT*α JEC21 and *MAT***a** JEC20 was used for wild-type bisexual mating (Kwon-Chung *et al.* 1992). Yeast cells were grown at 30° on Yeast extract Peptone Dextrose (YPD) medium. Strains harboring dominant selectable markers were grown on YPD medium supplemented with nourseothricin (NAT) or G418 (NEO) for selection. Mating assays were performed on either 5% V8 juice agar medium (pH 7.0) or Murashige and Skoog (MS) medium without sucrose (Sigma [Sigma Chemical], St. Louis, MO) in the dark at room temperature for the designated time period.

Bioinformatics

BLASTP searches using *C. neoformans* Cna1, Cnb1, Cbp1, Cts1, Crz1, Pbp1, Tif3, and Puf4 protein sequences against the *C. deneoformans* JEC21 genome on FungiDB (www.fungidb.org) identified the calcineurin pathway orthologous genes CNJ02230 (*CNA1*), CND00260 (*CNB1*), CNA07790 (*CBP1*), CNG01630 (*CTS1*), CNA01450 (*CRZ1*), CNE04890 (*PBP1*), CNK00210 (*TIF3*), and CNC04280 (*PUF4*) (Stajich *et al.* 2012; Park *et al.* 2016). To test whether the putative Cna1-targeted dephosphorylation sites in Pbp1, Tif3, and Puf4 are conserved between *C. neoformans* and *C. deneoformans*, protein sequences between these two species were aligned using Clustal ω (Sievers *et al.* 2011).

Gene disruption and generation of double mutants

Table S2 lists the primers used in this study. To generate deletion mutants for the genes of interest, deletion constructs consisting of the 5′ and 3′ regions of the targeted genes flanking an appropriate selection marker (*NAT* or *NEO* cassette) were generated by overlap PCR as previously described (Davidson *et al.* 2002). The deletion constructs were introduced into the congenic strain pair XL280α and XL280**a** via biolistic transformation, as previously described (Toffaletti *et al.* 1993). Transformants were selected on YPD medium supplemented with NAT (100 mg/liter) or NEO (200 mg/liter). Gene replacements by homologous recombination were confirmed by PCR. Two independent deletion mutants were obtained for *CNA1* (CF637 and CF645), *CNB1* (CF1095 and CF1099), *CBP1* (CF799 and CF802), *CTS1* (CF787 and CF794), and *CRZ1* (CF684 and CF688). Only one gene deletion mutant was obtained for *PBP1* (CF1100), *TIF3* (CF1113), and *PUF4* (CF1112). Additional deletion mutants were obtained for *PBP1* (CF1123 and CF1126), *TIF3* (CF1219 and CF1220), and *PUF4* (CF1194 and CF1195) by microscopically dissecting meiotic spores from bisexual crosses between, respectively, CF1100, CF1113, or CF1112 and the XL280 strain of the opposite mating type. A *MAT***a** *crz1*Δ mutant (CF863) was obtained from mating cross between strains CF688 and XL280**a**.

Double mutants were obtained from mating crosses between two single mutants of opposite mating types. Spore progeny of each mating cross were isolated by microscopic manipulation using a fiber optic needle spore dissection system, as previously described (Idnurm 2010). Gene deletions of the double mutants were verified by PCR. The *crz1*Δ *cbp1*Δ (CF1025 and CF1026), *crz1*Δ *cts1*Δ (CF998 and CF1001), and *crz1*Δ *puf4*Δ (CF1200 and CF1203) double mutants were generated from mating crosses of CF863 with CF802, CF787, or CF1112, respectively. The *crz1*Δ *pbp1*Δ (CF1143 and CF1145) and *crz1*Δ *tif3*Δ (CF1231 and CF1239) double mutants were generated from mating crosses of CF688 with CF1100 or CF1113, respectively. The *cna1*Δ *pbp1*Δ (CF1288 and CF1289) and *cna1*Δ *tif3*Δ (CF1252 and CF1256) double mutants were generated from mating crosses of CF637 with CF1100 or CF1113, respectively. The *cbp1*Δ *pbp1*Δ (CF1260 and CF1268) double mutants were generated from mating crosses of CF802 with CF1100.

Phenotypic assays

To test whether the constructed deletion mutants undergo unisexual reproduction, strains were grown overnight in liquid YPD medium at 30°, cells were washed twice with ddH_2O, and then diluted to a final density of OD_{600} = 2. For each strain, 10 μl of the diluted culture was inoculated onto MS and V8 medium, and incubated at room temperature in the dark for 5 and 14 days. To test whether the deletion mutants undergo pheromone-independent unisexual reproduction, cells were inoculated onto V8 medium supplemented with 200 μM $CuSO_4$. Hyphal growth on the edge of the mating patches, basidia, and spore chains were captured by using a Nikon (Garden City, NY) Eclipse E400 microscope equipped with a Nikon DXM1200F camera.

For spotting assays, strains were grown overnight in liquid YPD medium at 30°, and cells were washed twice with ddH_2O and diluted to a final density of OD_{600} = 0.8. Five 10-fold serial dilutions for each strain were made and spot inoculated onto solid media. To test cell viability of the deletion mutants at high temperature, 5 μl of 10-fold serial dilutions were spotted on YPD medium and incubated at different temperatures (30, 37, or 39°) for 2–3 days. To test stress-related phenotypes, 3–5 μl of 10-fold serial dilutions were spotted on YPD medium supplemented with 20 mM DTT (Sigma), 0.35 M $CaCl_2$, 0.5% calcofluor white (CFW) (Sigma), or 1% Congo red (CR) (Sigma), and incubated at 30° for 2–3 days.

*crz1*Δ *cbp1*Δ, *crz1*Δ *pbp1*Δ, and *cbp1*Δ *pbp1*Δ double mutants were also tested on YPD medium supplemented with 0.75 and 1% CFW, and 1.5 and 2% CR. To test cell susceptibility to the calcineurin inhibitor FK506 at different temperatures, 5 μl of 10-fold serial dilutions were spotted on YPD medium supplemented with 5 ng/ml FK506 (Astella Pharma), and incubated at a range of temperatures (17, 25, 30, 35, 36, or 37°) for 2–3 days.

RNA extraction and real-time PCR

For unisexual reproduction, wild-type XL280α and the deletion mutants (*cna1*Δ, *cnb1*Δ, *cbp1*Δ, *cts1*Δ, and *crz1*Δ) were grown overnight in liquid YPD medium. Cells were washed twice with ddH_2O and diluted to $OD_{600} = 2$, and 250 μl of the diluted cells were spotted on V8 or YPD agar medium. For bisexual reproduction, wild-type JEC20**a** and JEC21α were prepared the same way as described above, and then 250 μl of an equal-volume-mixture of cells were spotted on V8 or YPD agar medium. Mating patches were harvested after incubation at room temperature in the dark for 36 hr, and flash frozen in liquid nitrogen. RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's instructions. RNA was treated with Turbo DNAse (Ambion), and single-stranded cDNA was synthesized by AffinityScript Reverse Transcriptase (RT)-RNAse (Stratagene, La Jolla, CA). For each sample, cDNA synthesized without the RT-RNAse block enzyme mixture was used as a "no RT control" to exclude genomic DNA contamination. The relative expression level of target genes was measured by quantitative real-time PCR using Brilliant III ultrafast SYBR green QPCR mix (Stratagene) in an Applied Biosystems 7500 real-time PCR System. For each target, a no template control was performed to analyze melting curves to exclude primer artifacts. Technical triplicates and biological triplicates were performed for each sample. Gene expression levels were normalized using the endogenous reference gene *GPD1* and determined by using the comparative ΔΔCt method. The primers used for real-time PCR are listed in Table S2. The Student's *t*-test was used to determine if the relative gene expression levels between different strains exhibited statistically significant differences ($P < 0.05$).

Virulence studies

An intranasal inhalation mouse model of systemic cryptococcosis was used, as described previously, to study the virulence of *pbp1*Δ and *tif3*Δ deletion mutants (Ni *et al.* 2013; Zhai *et al.* 2013). For each gene of interest, a wild-type XL280α, a *cna1*Δ mutant (CF637), *tif3* or *pbp1* single mutants (CF1219 and CF1220 for *tif3*Δ, and CF1123 for *pbp1*Δ), and two double mutants (CF1252 and CF1256 for *cna1*Δ *tif3*Δ, and CF1288 and CF1289 for *cna1*Δ *pbp1*Δ) were tested as a group. For the *tif3*Δ group, mice were sedated with Nembutal (sodium pentobarbital) (37.5 mg/kg). For the *pbp1*Δ group, mice were sedated with isoflurane, a less invasive inhalation anesthetic, in compliance with animal welfare protocols to limit the use of a controlled substance. For each strain, 9 or 10 8- to 10-week-old female A/J mice (Jackson Laboratory) were inoculated with 5×10^6 cells in 50 μl PBS. After infection, mice were weighed daily and monitored twice a day. Moribund mice were killed. On day 20, four or five mice per strain were killed and dissected to study the fungal burden in the lung and the brain, and the remaining five mice for each strain were monitored for up to 60 days for survival studies. For the *PBP1* group, mice that had survived cryptococcosis when inoculated with the *cna1*Δ mutant (CF637), the *pbp1*Δ mutant (CF1123), and the two *cna1*Δ *pbp1*Δ mutants (CF1288 and CF1289) were dissected at the end of the experiment for fungal burden studies. The dissected organ tissues were homogenized in 2 ml PBS, serially diluted, and plated on YPD agar medium supplemented with 50 μg/ml ampicillin and 30 μg/ml chloramphenicol. After incubation at 30° for 2–3 days, colonies were counted to calculate CFUs for each organ.

Statistical analysis

Survival curves were plotted according to the Kaplan–Meier method, and statistical significance between two survival curves was assessed with the log-rank test, with a *P*-value < 0.05 considered statistically significant. Welch's *t*-test was used to determine if the fungal burden between different strains exhibited statistically significant differences ($P < 0.05$). All statistical analyses were performed using the Graphpad Prism 7 program.

Ethics statement

All experiments and animal care were conducted in accordance with the ethical guidelines of the Institutional Animal Care and Use Committee (IACUC) of Duke University Medical Center (DUMC). The DUMC IACUC approved all of the vertebrate studies under protocol number A245-13-09. Mice studies were conducted in the Division of Laboratory Animal Resources facilities, which are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Data availability

Strains are available upon request. Table S1 lists all of the strains and their genotypes, and Table S2 lists all of the primer sequences used in this study.

Results

Calcineurin regulates unisexual reproduction independent of the transcription factor Crz1

To test how the calcineurin pathway governs unisexual reproduction, we generated deletion mutants in the self-filamentous strain XL280α for eight genes (*CNA1*, *CNB1*, *CBP1*, *CTS1*, *CRZ1*, *PBP1*, *TIF3*, and *PUF4*) in the calcineurin pathway. These genes have been previously shown to regulate bisexual reproduction in *C. neoformans* and *C. deneoformans* (Cruz *et al.* 2001; Fox *et al.* 2003; Fox and Heitman 2005; Park *et al.* 2016; Chow *et al.* 2017). The calcineurin-dependent dephosphorylation sites in the three calcineurin targets Pbp1, Tif3, and Puf4 are conserved between *C. neoformans*

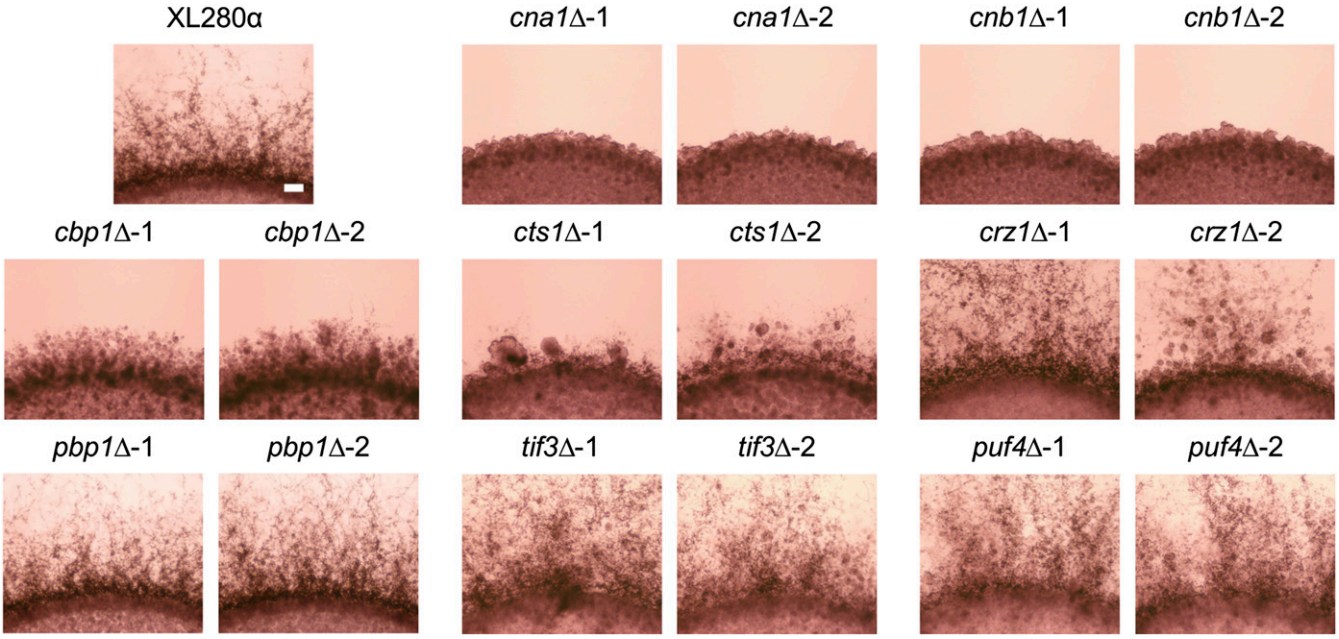


Figure 1 Unisexual mating phenotypes of calcineurin pathway deletion mutants. A wild-type (XL280α) strain and two independent *cna1*Δ (CF637 and CF645), *cnb1*Δ (CF1095 and CF1099), *cbp1*Δ (CF799 and CF802), *cts1*Δ (CF787 and CF794), *crz1*Δ (CF684 and CF688), *pbp1*Δ (CF1123 and CF1126), *tif3*Δ (CF1219 and CF1220), and *puf4*Δ (CF1194 and CF1195) deletion mutants were inoculated onto Murashige and Skoog medium, and incubated in the dark at room temperature for 14 days. Bar represents 200 μm.

and *C. deneoformans* (Figure S1). Deletion of the *CNA1* or *CNB1* gene completely abolished filamentation during early mating, and deletion of *CBP1* or *CTS1* delayed filamentation, while deletion of *CRZ1*, *PBP1*, *TIF3*, or *PUF4* did not impact filamentation (Figure 1 and Figure S2). The unisexual mating phenotypes were consistent for the deletion mutants on both MS and V8 medium, with the exception of *puf4*Δ mutants, which exhibited a wild-type filamentation phenotype on MS medium but reduced filamentation on V8 medium (Figure 1 and Figure S3A). Under pheromone-independent unisexual mating-inducing conditions (Gyawali *et al.* 2017), *cna1*Δ, *cnb1*Δ, *cbp1*Δ, *cts1*Δ, and *puf4*Δ mutants displayed similar filamentation defects on V8 medium in the presence of 200 μM Cu^{2+} (Figure S3B). Although *cna1*Δ and *cnb1*Δ mutants eventually produced rare, short, blunted hyphae along the edges of the mating patch, these hyphae failed to form long mature hyphae and basidia (Figure S4). *pbp1*Δ and *tif3*Δ mutants produced basidia with four chains of spores similar to wild-type, while all other single mutants produced basidia lacking the characteristic four spore-chain structures (Figure S4).

To test whether the transcription factor Crz1 is functionally redundant with other calcineurin target genes in governing unisexual reproduction, we generated double mutants of *crz1* with each other calcineurin downstream gene by crossing the *crz1*Δ mutant with *cbp1*Δ, *cts1*Δ, *pbp1*Δ, *tif3*Δ, and *puf4*Δ mutants, individually. The double mutants exhibited the same mating phenotype during unisexual reproduction as the non-*crz1* parental single mutants (Figure 2). All double mutants, including *crz1*Δ *pbp1*Δ and *crz1*Δ *tif3*Δ double mutants, produced bald basidia lacking spores, similar to the parental *crz1*Δ mutants (Figure S4). These results suggest that Crz1 is dispensable for filamentation but required for sporulation during unisexual reproduction.

Calcineurin regulates unisexual reproduction independent of the pheromone response pathway

To further investigate the involvement of the calcineurin pathway in sexual reproduction, we performed real-time PCR to measure the expression levels of the genes encoding calcineurin and the calcineurin downstream components. During bisexual reproduction between JEC20**a** and JEC21α, expression levels of *CNA1*, *CNB1*, *CBP1*, *CTS1*, and *CRZ1* increased significantly to approximately twice the expression level on nonmating-inducing YPD medium (2.1-fold for *CNA1*, $P < 0.005$; 3.1-fold for *CNB1*, $P < 0.005$; 1.9-fold for *CBP1*, $P < 0.005$; 1.7-fold for *CTS1*, $P < 0.05$; and 2.3-fold for *CRZ1*, $P < 0.005$) (Figure 3). *PBP1*, *TIF3*, and *PUF4* were not upregulated during bisexual reproduction (Figure 3). During unisexual reproduction in XL280α, expression levels of *CNA1*, *CNB1*, *CBP1*, *CTS1*, and *CRZ1* were also significantly increased, but at a range of 1.4–4.3-fold increase compared to the expression levels under nonmating-inducing conditions (1.7-fold for *CNA1*, $P < 0.005$; 4.3-fold change for *CNB1*, $P < 0.005$; 1.4-fold for *CBP1*, $P < 0.005$; 2.2-fold change for *CTS1*, $P < 0.005$; and 2.4-fold change for *CRZ1*, $P < 0.005$) (Figure 3). Interestingly, *PBP1* and *PUF4* showed no significant expression changes during bisexual reproduction, but were modestly downregulated during unisexual reproduction (1.9-fold decrease for *PBP1* during unisex, $P < 0.005$, and 2.2-fold decrease for *PUF4* during unisex, $P < 0.05$) (Figure 3). *TIF3* was expressed at the same level

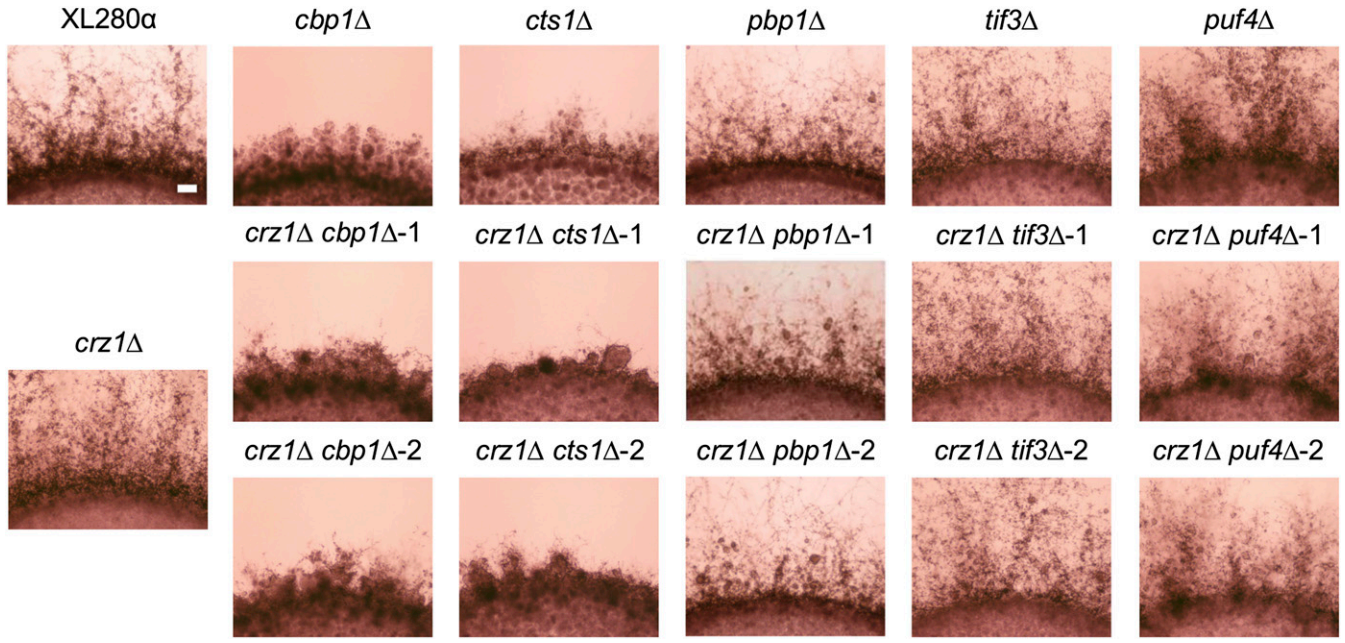


Figure 2 Unisexual mating phenotypes of double mutants of *crz1* with calcineurin downstream target genes. A wild-type (XL280α) strain; *crz1*Δ (CF688), *cbp1*Δ (CF799), *cts1*Δ (CF787), *pbp1*Δ (CF1123), *tif3*Δ (CF1219), and *puf4*Δ (CF1194) single mutants; and *crz1*Δ *cbp1*Δ (CF1025 and CF1026), *crz1*Δ *cts1*Δ (CF998 and CF1001), *crz1*Δ *pbp1*Δ (CF1143 and CF1145), *crz1*Δ *tif3*Δ (CF1231 and CF1239), and *crz1*Δ *puf4*Δ (CF1200 and CF1203) double mutants were inoculated onto Murashige and Skoog medium, and incubated in the dark at room temperature for 14 days. Bar represents 200 μm.

during bisexual and unisexual reproduction compared to non-mating-inducing conditions (Figure 3).

Because the pheromone response pathway regulates sexual reproduction in *Cryptococcus*, we examined whether the genes encoding pheromone (*MF*α), the pheromone receptor (*STE3*α), MAP kinase (*CPK1*), transcription factors (*MAT2* and *ZNF2*), and a homeodomain protein (*SXI1*α) were misregulated in calcineurin pathway mutants. Under mating-inducing conditions, these genes were expressed at similar levels between wild-type and calcineurin pathway single mutants (*cna1*Δ, *cnb1*Δ, *cbp1*Δ, and *crz1*Δ), except in the *cts1*Δ mutants (Figure S5). *MF*α, *STE3*α, *CPK1*, and *MAT2* were significantly upregulated in *cts1*Δ mutants compared to wild-type (2.5-fold for *MF*α, $P < 0.05$; twofold for *STE3*α, $P < 0.05$; twofold for *CPK1*, $P < 0.05$; and 3.6-fold for *MAT2*, $P < 0.005$) (Figure S5). *ZNF2* and *SXI1*α were upregulated in *cts1*Δ compared to wild-type, although these were not statistically significantly different (4.3-fold for *ZNF2*, $P = 0.0542$, and 1.4-fold for *SXI1*α, $P = 0.1229$) (Figure S5). These expression patterns support the hypothesis that calcineurin does not regulate the pheromone response pathway during unisexual reproduction, with the exception of Cts1, which suppresses expression of the pheromone pathway genes.

Calcineurin downstream targets function synergistically in response to different environmental stresses

The calcineurin pathway governs *Cryptococcus* tolerance to various environmental stresses (Park *et al.* 2016; Chow *et al.* 2017). We tested calcineurin pathway mutants in the *C. deneoformans* XL280α strain background in the presence of high temperature (37 and 39°), a calcineurin inhibitor (5 ng/ml FK506 at 30 and 37°), an ER stress reagent (20 mM DTT), high calcium (0.35 M $CaCl_2$), or cell wall stress reagents (0.5% CFW and 1% CR) (Figure 4).

The calcineurin phosphatase catalytic subunit *cna1*Δ mutants and the regulatory subunit *cnb1*Δ mutants were sensitive to high-temperature (37 and 39°), ER, and high-calcium stress, and exhibited intermediate sensitivity to cell wall stress (Figure 4A and Table 1). Other calcineurin pathway mutants were less sensitive to one or more stress conditions compared to the *cna1*Δ and *cnb1*Δ mutants. The *cbp1*Δ mutants were only mildly sensitive to high temperature at 39° (Figure 4A). The *cts1*Δ mutants were less sensitive to all stress conditions except the calcineurin inhibitor FK506 compared to the *cna1*Δ and *cnb1*Δ mutants, and the *cts1*Δ mutants exhibited hypersensitivity to FK506 at 30° (Figure 4A). The *crz1*Δ mutants were only mildly sensitive to high temperature at 39° and high calcium (Figure 4A). The *pbp1*Δ mutants were not sensitive to any stress tested except FK506 (Figure 4B). The *tif3*Δ mutants were only mildly sensitive to high temperature at 39° and cell wall stresses. Both the *pbp1*Δ and the *tif3*Δ mutants exhibited an enhanced high-temperature tolerance phenotype in the presence of FK506 compared to wild-type (Figure 4B). The *puf4*Δ mutants were sensitive to high temperature at both 37 and 39° at similar levels, and less sensitive to ER stress and high calcium compared to the *cna1*Δ and *cnb1*Δ mutants (Figure 4B).

Due to the nearly wild-type stress tolerance phenotypes of the *cbp1*Δ and *pbp1*Δ mutants under most stress conditions tested, we sought to determine whether or not *CBP1* and

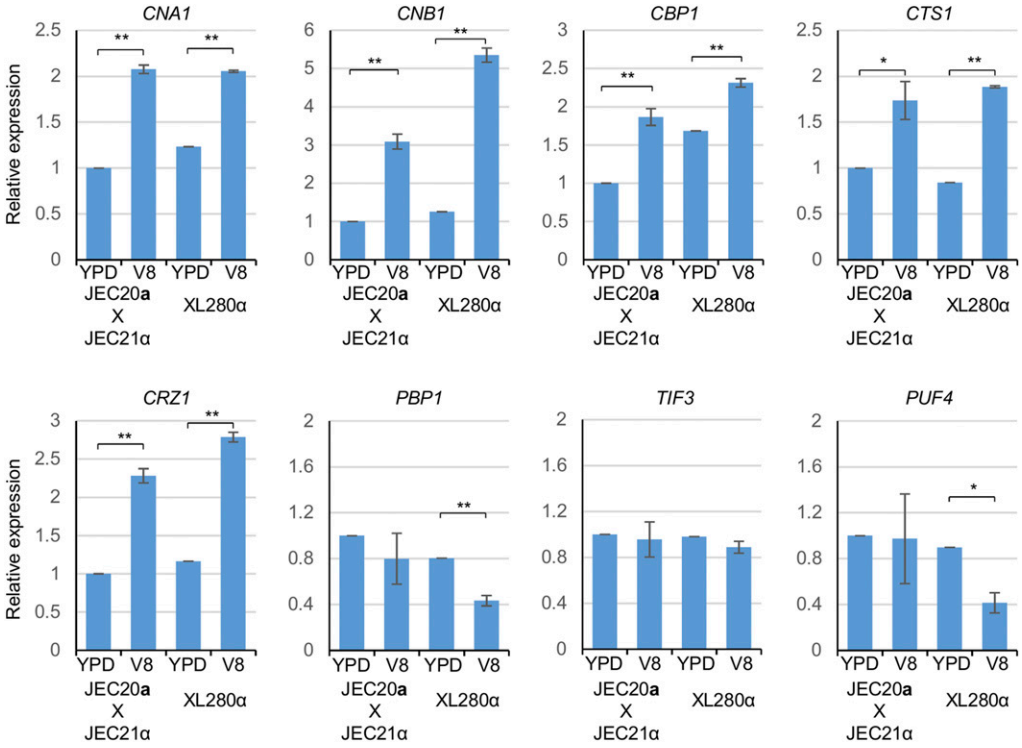


Figure 3 The calcineurin pathway is upregulated during sexual reproduction. Gene expression patterns for *CNA1*, *CNB1*, *CBP1*, *CTS1*, *CRZ1*, *PBP1*, *TIF3*, and *PUF4* were examined by real-time PCR (* indicates $P < 0.05$ and ** indicates $P < 0.005$ for each pairwise comparison). A wild-type cross between JEC20a and JEC21α for bisexual reproduction and the wild-type strain XL280α for unisexual reproduction was grown on YPD and V8 agar medium for 36 hr. The expression levels of the cross between strains JEC20a and JEC21α on YPD medium were set to 1, and the remaining values were normalized to this. The error bars represent the SD of the mean for three biological replicates.

PBP1 are functionally redundant. Interestingly, the *cbp1*Δ *pbp1*Δ double mutants displayed reduced filamentation during unisexual reproduction compared to the *cbp1*Δ and *pbp1*Δ single mutants (Figure 5A). The *cbp1*Δ *pbp1*Δ double mutants were also more sensitive than the single mutants to high-temperature (37 and 39°), ER, and high-calcium stress, but were not sensitive to cell wall stress (CR and CFW) (Figure 5B and Figure S6A). The *cbp1*Δ single and *cbp1*Δ *pbp1*Δ double mutants exhibited mild sensitivity to 1% CFW compared to the wild type and the *pbp1*Δ single mutant, confirming that *PBP1* does not synergistically interact with *CBP1* in response to cell wall stresses (Figure S6A). These phenotypic analyses suggest that *CBP1* and *PBP1* function redundantly downstream of calcineurin in regulating high-temperature and ER stress tolerance, and filamentation, during unisexual reproduction.

To test whether the transcription factor Crz1 synergistically interacts with other calcineurin downstream targets in regulating stress tolerance, we examined stress response phenotypes for *crz1* double mutants. The *crz1*Δ *cbp1*Δ double mutants were more sensitive to ER stress and high calcium compared to *crz1*Δ and *cbp1*Δ single mutants (Figure 4C). At lower concentrations of CFW and CR, the double mutants and the single mutants exhibited a wild-type phenotype. However, at higher concentrations of cell wall stressors, the *crz1*Δ *cbp1*Δ double mutants displayed sensitive phenotypes similar to that of the *crz1*Δ single mutant (Figure 4C and Figure S6A). The *crz1*Δ *cts1*Δ double mutants were more sensitive to all stresses tested (except FK506 at 30°) compared to the *crz1*Δ and *cts1*Δ single mutants (Figure 4D). At lower temperatures of 17 and 25°, the *crz1*Δ *cts1*Δ double mutants were more sensitive to FK506 compared to the single mutants (Figure S6B). The *crz1*Δ *puf4*Δ double mutants were more sensitive to ER, high-calcium, and cell wall stresses compared to *crz1*Δ and *puf4*Δ single mutants (Figure 4E). The *crz1*Δ *pbp1*Δ and *crz1*Δ *tif3*Δ double mutants were more sensitive to ER, high-calcium, and cell wall stresses compared to the single mutants, but less sensitive to high temperature at 39° than *crz1*Δ mutants (Figure 4, F and G). The *crz1*Δ *tif3*Δ and *crz1*Δ *pbp1*Δ double mutants displayed a more resistant phenotype at high temperature in the presence of FK506 (Figure 4, F and G and Figure S6C). Taken together, these results support a model in which Crz1 functions together with Cbp1, Cts1, Pbp1, Tif3, and Puf4 in branched pathways downstream of calcineurin to control stress responses.

PBP1 and TIF3 are required for virulence in C. deneoformans

Growth at the host temperature of 37° is a key virulence factor for *Cryptococcus* infection (Odom *et al.* 1997). Inhibition of the calcineurin pathway by FK506, or deletion of *CNA1* or *CNB1*, prevents *C. deneoformans* growth at 37°; however, *cna1*Δ *pbp1*Δ and *cna1*Δ *tif3*Δ double mutants were partially suppressed for the high-temperature-sensitive phenotype compared to the *cna1*Δ mutants (Figure 4, F and G). To analyze the thermotolerant phenotype in further detail, *pbp1*Δ and *tif3*Δ single mutants, *cna1*Δ *pbp1*Δ and *cna1*Δ *tif3*Δ double mutants, and *crz1*Δ *pbp1*Δ and *crz1*Δ *tif3*Δ double mutants were tested at high temperatures ranging from 35 to 37° in the presence of the calcineurin inhibitor FK506. Growth of the *cna1*Δ and *crz1*Δ mutants was completely

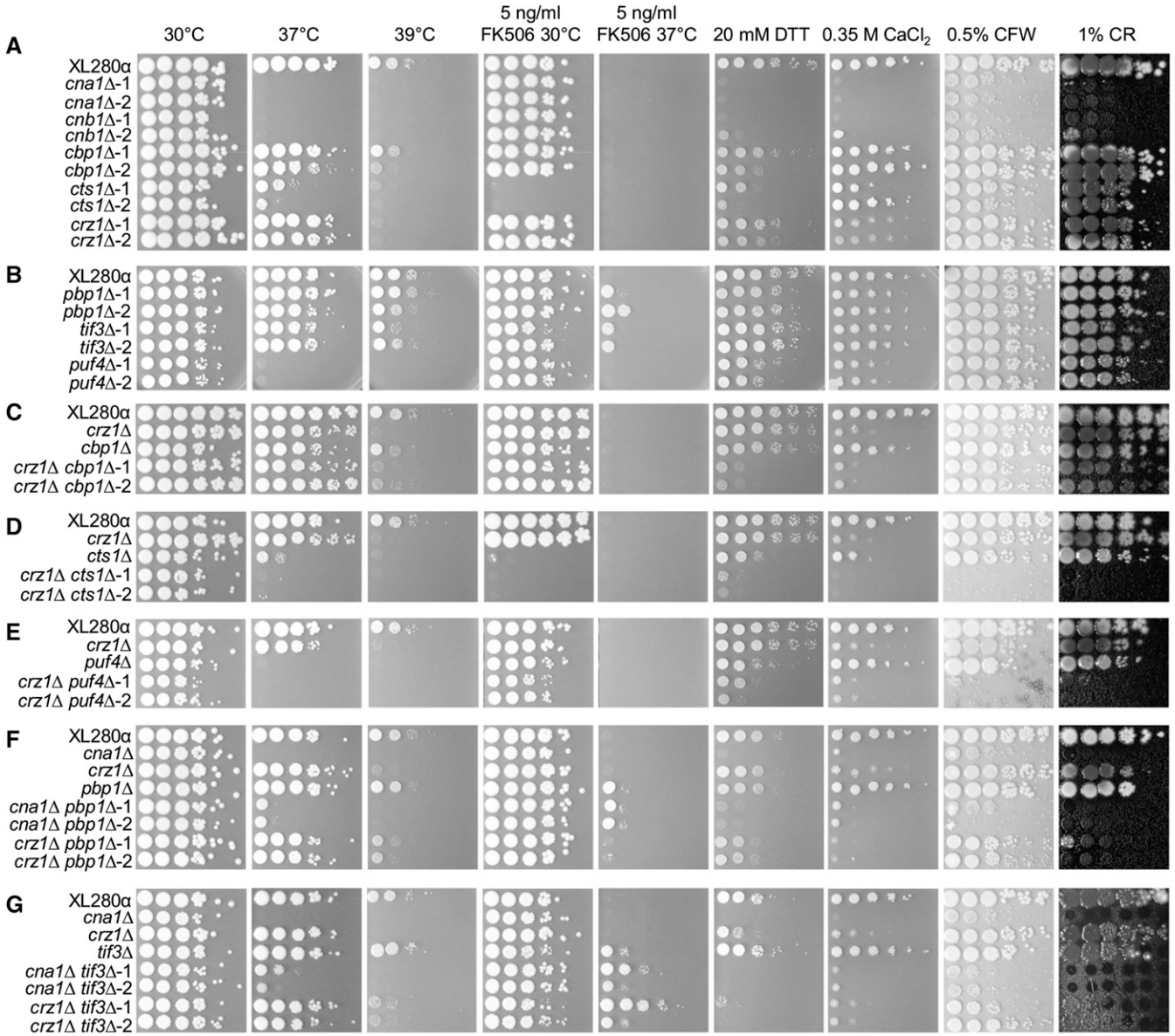


Figure 4 Stress response phenotypes of the calcineurin pathway single and double mutants. Stress response phenotypes were tested for: (A) XL280α and *cna1*Δ (CF637 and CF645), *cnb1*Δ (CF1095 and CF1099), *cbp1*Δ (CF799 and CF802), *cts1*Δ (CF787 and CF794), and *crz1*Δ (CF684 and CF688) mutants; (B) XL280α and *pbp1*Δ (CF1123 and CF1126), *tif3*Δ (CF1219 and CF1220), and *puf4*Δ (CF1194 and CF1195) mutants; (C) XL280α, *crz1*Δ (CF688) and *cbp1*Δ (CF799) single mutants, and *crz1*Δ *cbp1*Δ double mutants (CF1025 and CF1026); (D) XL280α, *crz1*Δ (CF688) and *cts1*Δ (CF787) single mutants, and *crz1*Δ *cts1*Δ double mutants (CF998 and CF1001); (E) XL280α, *crz1*Δ (CF688) and *puf4*Δ (CF1194) single mutants, and *crz1*Δ *puf4*Δ double mutants (CF1200 and CF1203); (F) XL280α, *cna1*Δ (CF637), *crz1*Δ (CF688) and *pbp1*Δ (CF1123) single mutants, and *cna1*Δ *pbp1*Δ (CF1288 and CF1289) and *crz1*Δ *pbp1*Δ (CF1143 and CF1145) double mutants; and (G) XL280α, *cna1*Δ (CF637), *crz1*Δ (CF688) and *tif3*Δ (CF1219) single mutants, and *cna1*Δ *tif3*Δ (CF1252 and CF1256) and *crz1*Δ *tif3*Δ (CF1231 and CF1239) double mutants. Strains were grown overnight at 30°, serially diluted 10-fold, and spot inoculated onto YPD and YPD agar medium supplemented with 5 ng/ml FK506, 20 mM DTT, 0.35 M $CaCl_2$, 0.5% calcofluor white (CFW), and 1% Congo red (CR). Thermosensitivity of these strains was tested by comparing growth at 30, 37, and 39°.

inhibited at 37°, and the mutants were sensitive to high temperature at 35 and 36° Compared to wild-type (Figure 4 and Figure S6C). In comparison, the *pbp1*Δ and *tif3*Δ mutants, the *crz1*Δ *pbp1*Δ and *crz1*Δ *tif3*Δ double mutants, and the *cna1*Δ *pbp1*Δ and *cna1*Δ *tif3*Δ double mutants were resistant to high temperature at 35, 36, and 37° in the presence of FK506 compared to the wild type or the *cna1*Δ and *crz1*Δ single mutants (Figure 4 and Figure S6C).

To determine whether high-temperature tolerance impacts *Cryptococcus* virulence, *pbp1*Δ and *tif3*Δ single mutants, and *cna1*Δ *pbp1*Δ and *cna1*Δ *tif3*Δ double mutants, were tested in the murine inhalation infection model. The *pbp1*Δ and *tif3*Δ virulence experiments were performed with different anesthetic methods (isoflurane for the *pbp1*Δ mutant group and pentobarbital for the *tif3*Δ group). The wild-type strain, XL280α, produced a 60% mortality rate using the inhalation

Table 1 Summary of stress phenotypes for calcineurin pathway mutants

	37°	39°	5 ng/ml FK506 30°	5 ng/ml FK506 37°	20 mM DTT	0.35 M $CaCl_2$	5 mg/ml CFW	1% Congo red
Wild type	NS	S	NS	SSS	NS	NS	NS	NS
*cna1*Δ	SSS	SSS	WT	WT	SSS	SSS	SS	SS
*cnb1*Δ	SSS	SSS	WT	WT	SSS	SSS	SS	SS
*cbp1*Δ	WT	S	WT	WT	WT	WT	WT	WT
*cts1*Δ	SS	SS	SSS	WT	S	S	S	S
*crz1*Δ	WT	SS	WT	WT	WT	S	WT	WT
*pbp1*Δ	WT	WT	WT	R	WT	WT	WT	WT
*tif3*Δ	WT	S	WT	R	WT	WT	S	S
*puf4*Δ	SSS	SSS	WT	WT	S	S	WT	WT
*crz1*Δ *cbp1*Δ	WT	S	WT	WT	SS	SS	WT	WT
*crz1*Δ *cts1*Δ	SSS	SSS	SSS	WT	SS	SSS	SSS	SSS
*crz1*Δ *puf4*Δ	SSS	SSS	WT	WT	SS	SS	SSS	SSS
*cna1*Δ *pbp1*Δ	SS	SSS	WT	R	SSS	SSS	SS	SS
*crz1*Δ *pbp1*Δ	WT	S	WT	WT	S	SSS	S	S
*cna1*Δ *tif3*Δ	SS	SSS	WT	R	SSS	SSS	SS	SS
*crz1*Δ *tif3*Δ	WT	S	WT	R	SS	SS	S	S
*cbp1*Δ *pbp1*Δ	SS	SS	WT	WT	S	S	WT	WT

NS, the strain exhibited no sensitive phenotype under the stress; S, the strain exhibited a mild sensitivity phenotype under the stress; SS, the strain exhibited an intermediate sensitivity phenotype under the stress; SSS, the strain failed to grow under the stress; WT, deletion mutant exhibited a similar stress tolerance phenotype as the wild type; R, deletion mutant exhibited an enhanced stress tolerance phenotype compared to the wild type.

anesthetic isoflurane, and a 100% mortality rate was observed using subcutaneous injection of the anesthetic sodium pentobarbital (Figure 6, A and B). The different anesthetic methods may vary in the depths of sedation in mice, which may influence lodging of fungal cells in the host lungs and ultimately impact fungal virulence outcome. Infection with the *pbp1*Δ mutant gave a similar survival curve to infection with the wild type (Figure 6A). The *cna1*Δ *pbp1*Δ double mutants were avirulent, similar to a *cna1*Δ mutant (Figure 6A). Fungal burden in the lung on day 20 postinfection was significantly lower in the *pbp1*Δ mutant compared to wild-type, suggesting that the virulence of the *pbp1*Δ mutant is attenuated in mice; however, fungal burden in the brain was only detected in fewer animals (three for wild-type, none for *cna1*Δ, two for *pbp1*Δ, one for *cna1*Δ *pbp1*Δ-1, and one for *cna1*Δ *pbp1*Δ-2, out of five tested for each group), therefore the data were not statistically significant between different groups due to the sample size (Figure S7A). At the end of the virulence experiment, the fungal burden in both the lungs and brain were detected in all mice that survived cryptococcal infection with the *pbp1*Δ mutant (two out of two), but not the *cna1*Δ mutant or the *cna1*Δ *pbp1*Δ-2 double mutant (Figure S8). Only one out of five mice infected with the *cna1*Δ *pbp1*Δ-1 double mutant was detected with fungal burden in the lung. The virulence of the *tif3*Δ mutants was attenuated compared to wild-type, and the *cna1*Δ *tif3*Δ double mutants were as avirulent as the *cna1*Δ mutant (Figure 6B). The fungal burden in the lung on day 20 postinfection was significantly lower in the *cna1*Δ and *tif3*Δ mutants compared to wild-type, and the fungal burden in the *cna1*Δ *tif3*Δ double mutants was higher than the *cna1*Δ mutant, but only statistically significant for one double mutant (Figure S7B). The fungal burden in the brain was detectable in mice infected with wild-type (five out of five mice), *tif3*Δ mutants (four out of four mice for one mutant and three out five for the other), and one *cna1*Δ *tif3*Δ double mutant (two out of five mice), but not detectable in mice infected with the *cna1*Δ mutant and the other *cna1*Δ *tif3*Δ double mutant. The fungal burden for the *tif3*Δ mutants and the *cna1*Δ *tif3*Δ double mutant was lower than wild-type, but not statistically significant due to the small sample size (Figure 7B). Despite the fact that this finding did not meet statistical significance ($P > 0.05$), the fungal burdens in mice brains infected with the *pbp1*Δ and *tif3*Δ single mutants did exhibit a trend and were consistently lower than with the wild-type strains. This may indicate a potential dissemination defect of the *pbp1*Δ and *tif3*Δ mutants into the central nervous system during cryptococcal infection (Figure S7). The virulence experiments showed that the virulence of the thermotolerant *pbp1*Δ and *tif3*Δ mutants was attenuated, while the *cna1*Δ *pbp1*Δ and *cna1*Δ *tif3*Δ double mutants were avirulent in mice. In summary, these results revealed that both Pbp1 and Tif3 play significant roles in *C. deneoformans* pathogenesis.

Discussion

Calcineurin orchestrates mating, stress responses, and virulence in *Cryptococcus* via distinct pathways (Cruz *et al.* 2001; Park *et al.* 2016; Chow *et al.* 2017). To elucidate the mechanisms via which calcineurin elicits unisexual reproduction, we deleted individual genes involved in the calcineurin pathway in the self-filamentous strain XL280α (Lin *et al.* 2005, 2006) and analyzed the mating phenotypes during unisexual reproduction. The calcineurin phosphatase catalytic subunit Cna1, regulatory subunit Cnb1, calcineurin-binding protein Cbp1, and calcineurin temperature suppressor Cts1 are each required for filamentation during both bisexual and unisexual reproduction (Cruz *et al.* 2001; Fox *et al.* 2003; Fox and Heitman 2005). In the wild-type strains, the expression levels for the *CNA1*, *CNB1*, *CBP1*, and *CTS1* genes were upregulated

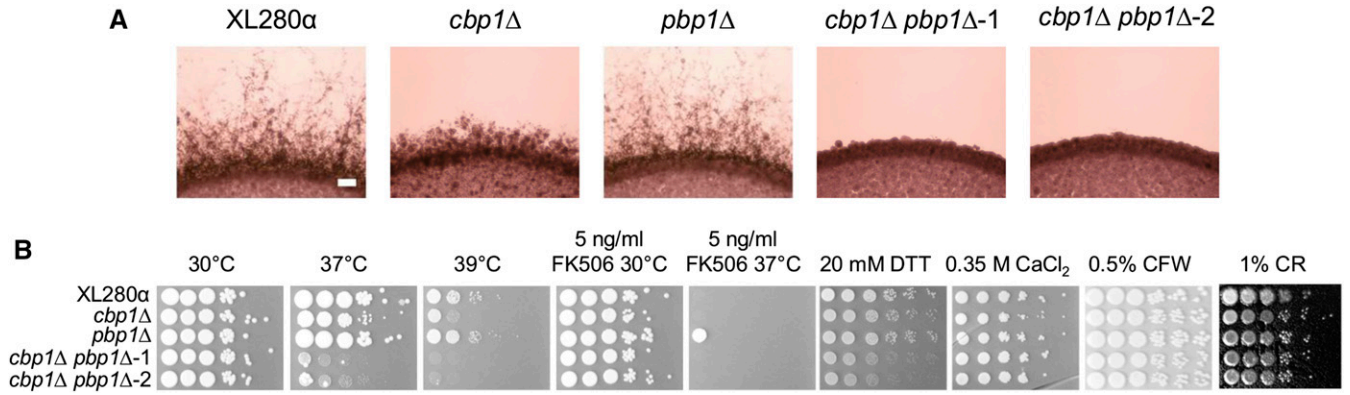


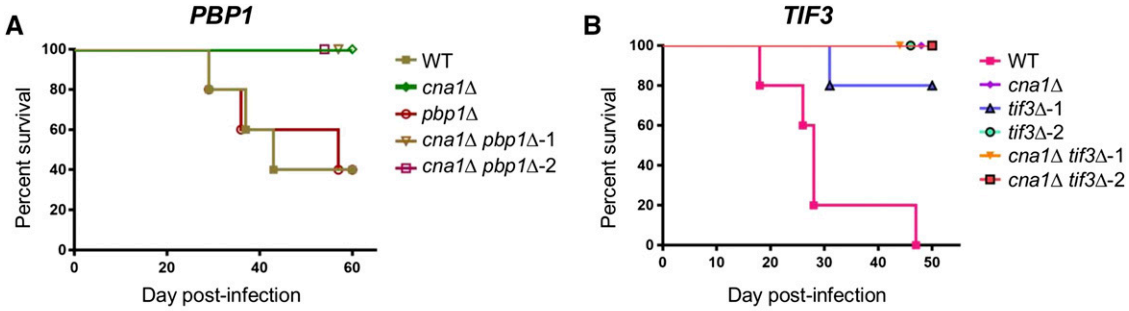
Figure 5 *CBP1* and *PBP1* synergistically regulate unisexual reproduction and stress responses in *C. deneoformans*. (A) Wild-type XL280α, *cbp1*Δ (CF799) and *pbp1*Δ (CF1123) single mutants, and two *cbp1*Δ *pbp1*Δ double mutants (CF1260 and CF1268) were grown on Murashige and Skoog agar medium for 14 days. Bar represents 200 μm. (B) Stress response phenotypes were tested for wild-type XL280α, *cbp1*Δ (CF799), *pbp1*Δ (CF1123), and two *cbp1*Δ *pbp1*Δ double mutants (CF1260 and CF1268). Strains were grown overnight at 30°, serially diluted 10-fold, and spot inoculated onto YPD and YPD agar medium supplemented with 5 ng/ml FK506, 20 mM DTT, 0.35 M $CaCl_2$, 0.5% calcofluor white (CFW), and 1% Congo red (CR). Thermosensitivity of these strains was tested at 30, 37, and 39°.

during both unisexual and bisexual reproduction, confirming that the calcineurin pathway is involved in mating of *C. deneoformans*. Deletion of these genes caused defects in hyphal production to varying degrees during unisexual reproduction. Compared to other calcineurin pathway mutants, the *cna1*Δ and *cnb1*Δ mutants produced few blunted hyphae after a prolonged incubation period, suggesting that Cna1 and Cnb1 play central roles in the calcineurin pathway promoting unisexual reproduction.

The three calcineurin downstream targets Pbp1, Tif3, and Puf4, identified by a phospho-proteomic study, are involved in *C. neoformans* bisexual reproduction (Park *et al.* 2016). In *S. cerevisiae*, Pbp1, Tif3, and Puf4 are involved in controlling the extent of mRNA polyadenylation, translation, and stability, respectively (Altmann *et al.* 1995; Gerber *et al.* 2004; Mangus *et al.* 2004). The poly(A)-binding protein-binding protein Pbp1 has also been shown to be required for mating type switch in *S. cerevisiae* and for normal sexual development in *A. nidulans* (Tadauchi *et al.* 2004; Soukup *et al.* 2017). In *C. neoformans*, deletion of *PBP1* or *TIF3* leads to a reduction in dikaryotic hyphal production, and deletion of *PUF4* leads to a hyperfilamentous phenotype (Park *et al.* 2016). In contrast to bisexual reproduction in *C. neoformans*, deletion of *PBP1*, *TIF3*, and *PUF4* did not impact unisexual reproduction in *C. deneoformans*, and these genes were not upregulated during unisexual reproduction, indicating that the calcineurin pathway may govern unisexual and bisexual reproduction in these related *Cryptococcus* species via different downstream targets. Interestingly, *C. deneoformans puf4*Δ mutants exhibited different filamentation phenotypes on MS and V8 medium, suggesting that Puf4 plays a role in driving unisexual reproduction on V8 medium. *pbp1*Δ mutants did not display any defect in filamentation during unisexual reproduction, and the *cbp1*Δ *pbp1*Δ double mutants exhibited a severe filamentation defect phenotype compared to the *cbp1*Δ and *pbp1*Δ single mutants, implying that Pbp1 plays a role in promoting unisexual reproduction that is functionally redundant with Cbp1. Similarly, Tif3 may also play a role in unisexual reproduction, but have redundant functions with other calcineurin downstream factors, as both proteins have been shown to regulate *C. neoformans* bisexual reproduction (Park *et al.* 2016).

The calcineurin-responsive zinc finger transcription factor Crz1 is not required for bisexual reproduction in *C. neoformans* (Park *et al.* 2016; Chow *et al.* 2017). Similarly, deletion of *CRZ1* did not cause any filamentation defect during unisexual reproduction in XL280α, and all double mutants of *crz1* with other calcineurin downstream target genes exhibited the non-*crz1* parental deletion mutant unisexual mating phenotype, suggesting that hyphal production during unisexual reproduction is independent of the transcription factor Crz1. During pheromone-independent unisexual reproduction in the presence of high copper, Cna1 and Cnb1 are required for filamentation, but the transcription factor Crz1 is not (Gyawali *et al.* 2017), further demonstrating that Crz1 is dispensable for hyphal production during this process. In our study, the expression of the pheromone response pathway genes, including *MF*α (pheromone), *STE3* (pheromone receptor), *CPK1* (MAP kinase), and *MAT2* and *ZNF2* (transcription factors downstream of the pheromone pathway) (Feretzaki and Heitman 2013), was not affected by the deletion of *CNA1*, *CNB1*, *CBP1*, or *CRZ1* during unisexual reproduction, supporting the hypothesis that the calcineurin pathway regulates filamentation independently of the pheromone response pathway. Interestingly, deletion of *CTS1* upregulated the pheromone response pathway; however, the elevated pheromone signaling failed to rescue the filamentation defect of the *cts1*Δ mutants. This indicates that Cts1 suppresses the pheromone response pathway and that Cts1 may serve as a conduit between these two pathways during unisexual reproduction.

Although the calcineurin pathway mutants produced different amounts of hyphae, the *cna1*Δ, *cnb1*Δ, *cts1*Δ, and



	p-Value
WT vs *cna1*Δ	0.0494*
WT vs *pbp1*Δ	0.9809
WT vs *cna1*Δ *pbp1*Δ-1	0.0494*
WT vs *cna1*Δ *pbp1*Δ-2	0.0494*
*cna1*Δ vs *cna1*Δ *pbp1*Δ-1	>0.9999
*cna1*Δ vs *cna1*Δ *pbp1*Δ-2	>0.9999

	p-Value
WT vs *cna1*Δ	0.0021**
WT vs *tif3*Δ-1	0.0072*
WT vs *tif3*Δ-2	0.0021**
WT vs *cna1*Δ *tif3*Δ-1	0.0021**
WT vs *cna1*Δ *tif3*Δ-2	0.0021**
*cna1*Δ vs *cna1*Δ *tif3*Δ-1	>0.9999
*cna1*Δ vs *cna1*Δ *tif3*Δ-2	>0.9999

Figure 6 Deletion of *PBP1* and *TIF3* attenuates *C. deneoformans* virulence in a murine infection model. (A) Virulence was tested for the wild-type (WT) XL280α, *cna1*Δ (CF637) and *pbp1*Δ (CF1123) single mutants, and *cna1*Δ *pbp1*Δ double mutants (CF1288 and CF1289). (B) Virulence was tested for the WT XL280α, *cna1*Δ (CF637) and *tif3*Δ (CF1219 and CF1220) single mutants, and *cna1*Δ *tif3*Δ double mutants (CF1252 and CF1256). Strains were grown overnight in YPD liquid medium at 30° and washed with PBS. For each strain, five female A/J mice were inoculated with 5×10^6 cells via intranasal infection, and animal survival was monitored up to 60 days postinfection. *P*-values for the virulence experiments are provided in the bottom panels.

*cbp1*Δ mutants were defective in sporulation. Additionally, *crz1*Δ and *puf4*Δ mutants, which produced wild-type levels of hyphae, formed basidia without spore chains, suggesting that the calcineurin pathway may play a role during the meiotic process or in spore chain formation, and that this function is dependent on Crz1 and Puf4. Taken together, calcineurin regulates unisexual reproduction at different stages: the Crz1-independent stage during initial filamentation and hyphal production, and the Crz1- and Puf4-dependent sporulation stage (Figure 7).

Besides the regulation of mating, calcineurin functions as a hub for the calcineurin signaling network in regulating various stress responses, and Crz1 and other calcineurin downstream targets function in branched pathways (Park *et al.* 2016; Chow *et al.* 2017). Supporting this model, the *cna1*Δ and *cnb1*Δ mutants in the XL280α background exhibited the most sensitive phenotypes against ER, high-temperature, high-calcium, and cell wall stresses, while mutants of calcineurin downstream targets, including Cbp1, Cts1, Crz1, Pbp1, Tif3, and Puf4, displayed intermediate to nonsensitive phenotypes toward these stresses. These results suggest that Cna1 and Cnb1 function early on in the calcineurin pathway to control stress responses, and that the downstream targets share redundant functions in stress tolerance (Figure 7).

In *S. cerevisiae*, inactivation of calcineurin promotes the activity of the vacuolar H^+/Ca^{2+} exchanger (Vcx1), which in turn enables yeast cells to tolerate high concentrations of calcium (Cunningham and Fink 1996). Moreover, calcineurin regulates calcium homeostasis through the transcription factor Crz1 in yeast (Matheos *et al.* 1997; Stathopoulos and Cyert 1997). In *C. neoformans*, calcineurin and Crz1 positively regulate expression of the *VCX1* gene and the *PMC1* gene encoding the vacuolar calcium transporter Pmc1 to confer higher tolerance to calcium (Kmetzsch *et al.* 2010, 2013; Chow *et al.* 2017; Squizani *et al.* 2017). Several studies have shown that the calcineurin/Crz1 regulatory network has been extensively rewired in different fungal species (Yoshimoto *et al.* 2002; Kim *et al.* 2010; Soriani *et al.* 2010; Chatfield-Reed *et al.* 2016; Chow *et al.* 2017), which may account for the high calcium sensitivity observed in *cna1*Δ and *cnb1*Δ mutants in *Cryptococcus*.

Based on the ER and high-calcium stress tolerance phenotypes, Crz1 synergistically interacts with all of the other calcineurin downstream targets. However, all of the *crz1* double mutants were less sensitive to these stresses than the *cna1*Δ and *cnb1*Δ mutants, suggesting that Crz1 and these downstream targets function in multiple parallel pathways for ER stress and high-calcium tolerance. In addition, *CBP1* and *PBP1* synergistically regulate ER and high-calcium stress responses, but the double mutants were only mildly sensitive to these stresses, supporting the hypothesis that complex multiple branched pathways function downstream of calcineurin to regulate responses to ER and high-calcium stresses (Figure 7).

In response to the cell wall stressors CR, known to interact with β-glucans (Teather and Wood 1982), and CFW, which binds to chitin (Elorza *et al.* 1983), Crz1 synergistically

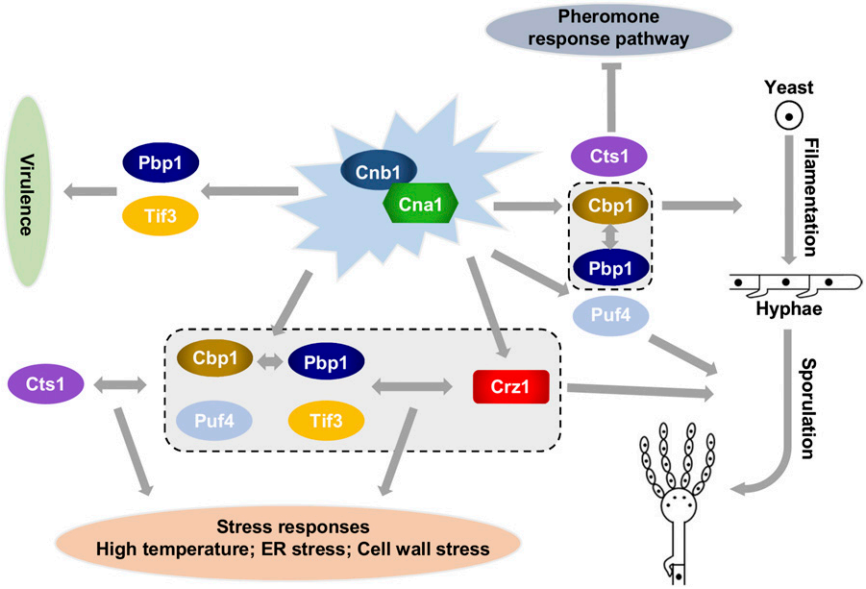


Figure 7 The calcineurin pathway controls unisexual reproduction, stress responses, and virulence in *C. deneoformans*. Calcineurin Cna1/Cnb1 functions as the central hub in the calcineurin pathway. During unisexual reproduction, calcineurin regulates the yeast–hyphal morphological transition through Cbp1 and Cts1, and via a pathway in which Cbp1 synergistically interacts with Pbp1, whereas Cts1 inhibits the pheromone response pathway. Crz1 and Puf4 are required for sporulation. In response to different environmental stresses, Crz1 synergistically interacts with other calcineurin downstream targets via complex branched pathways. Finally, Pbp1 and Tif3 are key virulence factors downstream of calcineurin in *C. neoformans*.

interacts with Cts1, Puf4, Tif3, and Pbp1, but not Cbp1. Interestingly, *cbp1*Δ single mutants and *cbp1*Δ *pbp1*Δ double mutants exhibited largely wild-type tolerance to CR and CFW, except for a mild sensitivity to 2% CFW, indicating that Cbp1 does not play a significant role in response to these cell wall stresses. Double mutants of *crz1* with *tif3* or *pbp1* were less sensitive to cell wall stresses than the *cna1*Δ and *cnb1*Δ mutants, indicating that these three genes are functionally redundant and that additional factors may contribute to cell wall stress tolerance. Double mutants of *crz1* with *cts1* or *puf4* were more sensitive to cell wall stresses compared to the *cna1*Δ and *cnb1*Δ mutants, suggesting that Cts1 and Puf4 may function independently of the calcineurin pathway in regulating cell wall stress tolerance.

Calcineurin is essential for survival at high temperature at 37°. Deletion of *CNA1* or *CNB1*, or inhibition of the calcineurin pathway by the inhibitor FK506, leads to high-temperature sensitivity to 37° (Odom *et al.* 1997). Among the calcineurin downstream targets analyzed in this study, only the *puf4* mutants exhibited the same high-temperature-sensitive phenotype as the *cna1*Δ and *cnb1*Δ mutants, and the *crz1*Δ *puf4*Δ double mutants had similar thermosensitivity to the *puf4*Δ single mutants, indicating that Puf4 plays a key role downstream of calcineurin in governing high-temperature stress tolerance in *C. deneoformans*. Interestingly, Puf4 was previously shown to synergistically interact with Crz1 in *C. neoformans* in orchestrating high-temperature stress responses (Park *et al.* 2016), suggesting that the calcineurin pathway has been rewired in the *Cryptococcus* sister species. Furthermore, deletion of *PUF4* caused a defect in hyphal production in *C. neoformans* during bisexual reproduction (Park *et al.* 2016), but not in *C. deneoformans* during unisexual reproduction. Puf4 belongs to the pumilio-FBF (fem-3 mRNA binding factor) family of mRNA-binding proteins and it controls the turnover of the unfolded protein response pathway transcription factor Hxl1 in *C. neoformans* (Glazier *et al.* 2015). Puf4 may impact thermosensitivity in *C. deneoformans* via similar mechanisms by promoting the stability of calcineurin pathway mRNAs.

Overexpression of *CTS1* was previously shown to suppress the thermosensitive phenotype of *cna1*Δ mutants (Fox *et al.* 2003). Cts1 colocalizes with Cna1 in mRNA-processing P-body sites, and dephosphorylation of Cts1 is dependent on calcineurin, suggesting that Cts1 functions as an effector downstream of Cna1 in the calcineurin pathway (Aboobakar *et al.* 2011). However, in this study, deletion of *CTS1* caused hypersensitivity to 30° in the presence of FK506, and *crz1*Δ *cts1*Δ double mutants were more sensitive to cell wall stresses compared to the *cna1*Δ, *cnb1*Δ, and *crz1*Δ single mutants, suggesting that Cts1 may function in a pathway parallel, with calcineurin enabling responses to various stress conditions.

In contrast to the *cna1*Δ and *cnb1*Δ mutants, the *pbp1*Δ and *tif3*Δ single mutants, and the *cna1*Δ *pbp1*Δ and *cna1*Δ *tif3*Δ double mutants, were resistant to 37°, both in the absence and in the presence of the calcineurin inhibitor FK506, suggesting that Pbp1 and Tif3 function as suppressors in the calcineurin pathway regulating the stress responses to high temperature. Thermosensitivity at 37° is considered a key virulence factor that contributes to the avirulent phenotype in mice for the *cna1*Δ mutants (Odom *et al.* 1997; Cruz *et al.* 2000). However, the thermosensitive *puf4*Δ mutants were reported to be as virulent as the wild type in mice (Glazier *et al.* 2015), and the virulence for the high-temperature-tolerant *pbp1*Δ mutants in *C. neoformans* was attenuated (Park *et al.* 2016), indicating that thermosensitivity may not be entirely predictive of virulence potential. Supporting this hypothesis, the high-temperature-resistant phenotype for *pbp1*Δ and *tif3*Δ mutants in *C. deneoformans* did not confer hypervirulence in mice, as both the survival curve and fungal burden in the lung and brain for the single mutants were attenuated compared to wild-type. The modestly thermotolerant *cna1*Δ *pbp1*Δ and *cna1*Δ *tif3*Δ double mutants were

avirulent like the *cna1*Δ mutants, and fungal burdens were comparable between the double mutants and the *cna1*Δ single mutants, further suggesting that host temperature sensitivity at 37° alone does not explain the avirulent phenotype observed for the *cna1*Δ mutants. Interestingly, the thermosensitive *C. neoformans puf4*Δ mutants exhibited lower fungal burden than the wild-type, suggesting that growth at host temperature contributes to fungal survival in the host, but may not predict the overall virulence outcome (Glazier *et al.* 2015). *C. deneoformans puf4*Δ mutants are more sensitive to 37° than *C. neoformans puf4*Δ mutants, which is likely due to species-specific responses, in that the *C. deneoformans* calcineurin mutant is more sensitive to higher temperature than the *C. neoformans cna1*Δ mutant (Cruz *et al.* 2000; Glazier *et al.* 2015; Park *et al.* 2016). The severe thermosensitive phenotype may further influence growth of the *C. deneoformans puf4*Δ mutant in the host. Our studies indicate that Pbp1 and Tif3 are key virulence factors, which regulate virulence downstream of calcineurin in a temperature-independent manner in *C. deneoformans*. Pbp1 and Tif3 synergistically interact with Crz1 in responses to ER, high-calcium, and cell wall stresses, indicating that Pbp1 and Tif3 play additional roles in regulating multiple stress responses downstream of calcineurin, thereby contributing to virulence in mice.

In summary, by utilizing the self-filamentous *C. deneoformans* strain XL280α, we elucidated the functions of the calcineurin pathway in the control of unisexual reproduction, stress responses, and virulence (Figure 7). The calcineurin-responsive transcription factor Crz1 is not required for the yeast–hyphal morphological transition, but it is important for spore chain formation. Crz1 synergistically interacts with other calcineurin downstream targets in coordinating responses to high-temperature, ER, high-calcium, and cell wall stresses. The widespread synergy among calcineurin downstream targets suggests that the calcineurin pathway is highly plastic, which allows for rapid signaling network rewiring in adaptation to changing environments during evolution.

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