The Vacuolar Ca\textsuperscript{2+} ATPase Pump Pmc1p Is Required for Candida albicans Pathogenesis

Arturo Luna-Tapia,\textsuperscript{a} Christian DeJarnette,\textsuperscript{b} Emily Sansevere,\textsuperscript{a} Parker Reitler,\textsuperscript{b} Arielle Butts,\textsuperscript{a} Kirk E. Hevener,\textsuperscript{c} Glen E. Palmer\textsuperscript{a}

\textsuperscript{a}Department of Clinical Pharmacy and Translational Science, College of Pharmacy, University of Tennessee Health Sciences Center, Memphis, Tennessee, USA
\textsuperscript{b}Department of Molecular Immunology and Biochemistry, College of Graduate Health Sciences, University of Tennessee Health Sciences Center, Memphis, Tennessee, USA
\textsuperscript{c}Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Sciences Center, Memphis, Tennessee, USA

ABSTRACT  Calcium is a critically important secondary messenger of intracellular signal transduction in eukaryotes but must be maintained at low levels in the cytoplasm of resting cells to avoid toxicity. This is achieved by several pumps that actively transport excess cytoplasmic Ca\textsuperscript{2+} out of the cell across the plasma membrane and into other intracellular compartments. In fungi, the vacuole serves as the major storage site for excess Ca\textsuperscript{2+}, with two systems actively transporting cytoplasmic calcium ions into the vacuole. The H\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, Vcx1p, harnesses the proton-motive force across the vacuolar membrane (generated by the V-ATPase) to drive Ca\textsuperscript{2+} transport, while the P-type ATPase Pmc1p uses ATP hydrolysis to translocate Ca\textsuperscript{2+} into the vacuole. Ca\textsuperscript{2+}-dependent signaling is required for the prevalent human fungal pathogen Candida albicans to endure exposure to the azole antifungals and to cause disease within the mammalian host. The purpose of this study was to determine if the Pmc1p or Vcx1p Ca\textsuperscript{2+} pumps are required for C. albicans pathogenicity and if these pumps impact antifungal resistance. Our results indicate that Pmc1p is required by C. albicans to transition from yeast to hyphal growth, to form biofilms in vitro, and to cause disease in a mouse model of disseminated infection. Moreover, loss of Pmc1p function appears to enhance C. albicans azole tolerance in a temperature-dependent manner.

IMPORTANCE  Maintenance of Ca\textsuperscript{2+} homeostasis is important for fungal cells to respond to a multitude of stresses, as well as antifungal treatment, and for virulence in animal models. Here, we demonstrate that a P-type ATPase, Pmc1p, is required for Candida albicans to respond to a variety of stresses, affects azole susceptibility, and is required to sustain tissue invasive hyphal growth and to cause disease in a mouse model of disseminated infection. Defining the mechanisms responsible for maintaining proper Ca\textsuperscript{2+} homeostasis in this important human pathogen can ultimately provide opportunities to devise new chemotherapeutic interventions that dysregulate intracellular signaling and induce Ca\textsuperscript{2+} toxicity.

KEYWORDS  Candida albicans, Pmc1p, Vcx1p, calcium, pathogenesis, vacuole

The fungal vacuole is an acidic intracellular compartment that plays a central role in the maintenance of cellular homeostasis. This includes facilitating the degradation of macromolecules, the storage of crucial metabolites, and the sequestration of potentially toxic substances (1, 2). The vacuole also serves as the major storage site for metal ions, including calcium, a critically important secondary messenger of intracellular signal transduction in eukaryotes. To fulfill its role as a second messenger, Ca\textsuperscript{2+} must be maintained at low levels in the cytoplasm of resting cells (3). This is achieved by several pumps that actively transport excess cytoplasmic Ca\textsuperscript{2+} out of the cell across the plasma membrane or into intracellular compartments, including the endoplasmic...
reticulum and Golgi apparatus (4). In yeast, more than 90% of the intracellular Ca2+ is sequestered in the vacuole (4). Upon appropriate stimulation, Ca2+ ions are rapidly released into the cytoplasm from extracellular sources as well as intracellular stores to activate Ca2+-dependent proteins such as calmodulin (5, 6). Resting state equilibrium is subsequently restored through calcium efflux, resulting in transient cytoplasmic Ca2+ fluxes that relay the extracellular signal. Failure to remove excess intracellular Ca2+ from the cytoplasm or to restore presignal equilibrium dysregulates intracellular signaling and can lead to cell death (7, 8). As such, the mechanisms responsible for Ca2+ detoxification are of critical importance to eukaryotic cell viability.

In fungi, two systems are responsible for sequestering cytoplasmic Ca2+ ions into the vacuole, namely, the H+/Ca2+ exchanger Vcx1p (9–11), which uses the proton gradient across the vacuolar membrane (generated by the V-ATPase) to drive Ca2+ transport, and the P-type ATPase Pmc1p (11, 12). Both Pmc1p and Vcx1p are required for Cryptococcus neoformans to colonize lung or brain tissue in a mouse model of infection (13–15). While Aspergillus fumigatus, a major cause of pulmonary as well as disseminated infections of humans, has three Pmc1p homologues (PMC1, PMCA, and PMCC), with PMCC seemingly essential for viability and PMCA required for virulence in a neutropenic mouse model of invasive pulmonary aspergillosis (16). Ca2+-dependent signaling, particularly through the calcineurin signaling pathway, is also required for Candida albicans to tolerate the azole antifungals (17, 18). However, deletion of the PMC1 gene has been reported to result in fluconazole resistance in C. albicans (18). The purpose of this study was to determine if Pmc1p or Vcx1p is required for C. albicans pathogenicity and how these pumps impact antifungal resistance.

RESULTS

Pmc1p is required for Candida albicans stress tolerance. To determine if Pmc1p or Vcx1p is required for C. albicans pathogenesis, we constructed pmc1Δ/Δ and vcx1Δ/Δ mutants using a PCR-based approach (19). Complemented strains were made by reintroducing a wild-type PMC1 or VCX1 allele into the pmc1Δ/Δ or vcx1Δ/Δ mutant, respectively, using an integrating vector that fully restores the IRO1-URA3 locus. We initially examined phenotypes that have been associated with loss of Pmc1p or Vcx1p function in fungi (10–12, 15, 16, 18). Each of the described phenotypes was verified using at least two independently derived clones for each genotype. While the pmc1Δ/Δ and vcx1Δ/Δ mutants grew to a similar extent as the wild-type control strain on yeast extract-peptone-dextrose (YPD) agar plates, the pmc1Δ/Δ mutant was severely impaired by high concentrations of CaCl2 (Fig. 1A; see also Fig. S1 in the supplemental material). The pmc1Δ/Δ mutant was more resistant than the wild type to LiCl and CdSO4 but hypersensitive to the membrane stressor SDS (Fig. 1A). Reintroduction of PMC1 into the pmc1Δ/Δ mutant only partially restored wild-type phenotypes with respect to LiCl, CaCl2, and CdSO4 sensitivity, suggesting that PMC1 may be haploinsufficient. The PMC1 reconstituted strain also remained completely sensitive to SDS at the concentration tested (0.05%), suggesting that the capacity of C. albicans to survive plasma membrane stress is especially sensitive to Pmc1p deficiencies. The growth of the vcx1Δ/Δ mutant, on the other hand, was unaffected by CaCl2, LiCl, or CdSO4 supplements (Fig. 1A). The vcx1Δ/Δ mutant was, however, sensitive to SDS, albeit to a lesser extent than the pmc1Δ/Δ mutant. These results confirm the importance of C. albicans Pmc1p for Ca2+ homeostasis and resistance to ionic as well as membrane stress.

Pmc1p influences Candida albicans antifungal susceptibility. A previous study reported that a C. albicans pmc1Δ/Δ mutant was resistant to the antifungal drug fluconazole (18). Similarly, we found our pmc1Δ/Δ mutant to be more resistant than the wild type to fluconazole when grown at 30°C on YPD agar supplemented with the drug (Fig. 1A) or using Etest strips (see Fig. S2). However, when tested in liquid RPMI medium at 35°C according to the standards of the CLSI protocol (20), or on RPMI agar with Etest strips (Fig. S2), the pmc1Δ/Δ mutant was as susceptible as the wild type. This indicated that the fluconazole resistance phenotype of the pmc1Δ/Δ mutant was dependent on the medium or some other growth condition. To determine how temperature influ-
ences the pmc1Δ/Δ mutants’ susceptibility to fluconazole, we compared the sensitivity of our C. albicans strains on YPD agar using spot dilution assays, following incubation at either 30, 35, 37, or 42°C. This revealed that while the pmc1Δ/Δ mutant is more resistant to fluconazole than the wild type at 30°C, it was not significantly different at 35 or 37°C and, paradoxically, was more sensitive at 42°C (see Fig. S3). Thus, the effect of Pmc1p on C. albicans sensitivity to fluconazole was temperature dependent. Notably, the vcx1Δ/Δ mutants’ sensitivity to fluconazole was indistinguishable from that of the wild type under all conditions tested.

Interestingly, the pmc1Δ/Δ mutant was slightly more sensitive than the wild type to the morpholine antifungal amorolfine (see Fig. S4). Amorolfine inhibits both C-8 sterol isomerase (Erg2p) and C-14 sterol reductase (Erg24p), both of which act downstream of Erg11p in the ergosterol biosynthetic pathway (21). Again, the susceptibility of the vcx1Δ/Δ mutant to amorolfine was indistinguishable from that of the wild-type control (data not shown).

**Loss of Pmc1p impairs Candida albicans hyphal formation.** The ability to form hyphae is important for C. albicans pathogenicity (22, 23). We therefore examined the pmc1Δ/Δ and vcx1Δ/Δ mutants’ capacity to form hyphae. The pmc1Δ/Δ mutant’s ability to form hyphae was severely impaired on M199 or 10% fetal bovine serum (FBS) agar (Fig. 2A), and it remained as yeast cells in liquid FBS (Fig. 3). While a significant fraction of pmc1Δ/Δ cells produced short filaments in liquid M199 (Fig. 3), these were substan-
tially shorter than for the wild type, and many cells remained in the yeast form under these conditions. In contrast, the \textit{vcx1}Δ/Δ mutant exhibited no detectable defects in hyphal growth under any of these conditions, indicating that Vcx1p is not required for \textit{C. albicans}.

Since the \textit{pmc1}Δ/Δ mutant was hypersensitive to high levels of Ca\textsuperscript{2+} and unable to form normal hyphae in FBS, we next evaluated if the defects in hyphal formation were due to the high levels of Ca\textsuperscript{2+} found in serum (3.5 to 4 mM) (24). This was tested using the Ca\textsuperscript{2+} chelator EGTA to sequester free Ca\textsuperscript{2+} in the FBS. The addition of 5 mM EGTA to the FBS largely restored the ability of the \textit{pmc1}Δ/Δ mutant to form filaments (Fig. 4). Interestingly, the ability of the wild type and the complemented control strain to form hyphae was reduced by the addition of EGTA to the FBS. These results establish that the calcium concentrations within host tissues and fluids, as well as the calcium detoxification functions performed by Pmc1p, are an essential determinant of fungal morphogenesis and therefore \textit{C. albicans} pathogenicity.

Finally, since hyphal growth is intimately linked to the capacity of \textit{C. albicans} to form biofilms (25), we compared the \textit{pmc1}Δ/Δ, \textit{vcx1}Δ/Δ, and wild-type control strains abilities to form biofilms using a simple \textit{in vitro} assay. Again, the \textit{pmc1}Δ/Δ mutant was significantly impaired in its capacity to form biofilms, while the \textit{vcx1}Δ/Δ mutant was able to form biofilms to the same extent as the wild-type control and complemented strains (Fig. 2B).
Pmc1p is required for *Candida albicans* virulence in a mouse model of disseminated infection. To determine if either Pmc1p or Vcx1p is required for *C. albicans* to cause disease within its mammalian host, we compared the virulence of the *pmc1*Δ/Δ and *vcx1*Δ/Δ mutants with that of the wild type in a mouse model of disseminated infection (Fig. 5). All mice infected with wild-type *C. albicans* succumbed within 7 days of infection. However, all 7 of the mice infected with the *pmc1*Δ/Δ mutant survived the duration of the experiment (14 days), with 3 having undetectable levels of fungal colonization within their kidneys and the remainder having relatively low levels (ranging from $3.32 \times 10^3$ to $2.34 \times 10^4$ CFU/g of kidney). In contrast, the *vcx1*Δ/Δ mutant was as virulent as the wild-type and revertant control strains, as determined by the comparable survival times of mice infected with each strain. These data indicate that Pmc1p, but not Vcx1p, is required for *C. albicans* pathogenicity following dissemination through the bloodstream (Fig. 5).

**DISCUSSION**

In this study, we explored the contributions of the Pmc1p Ca$^{2+}$ pump and the Vcx1p Ca$^{2+}$ exchanger to stress response and pathogenesis of *C. albicans*. It is evident from these results that the inability of the *pmc1*Δ/Δ mutant to properly remove Ca$^{2+}$ from the cytoplasm into the vacuole renders the excess Ca$^{2+}$ toxic to the mutant in a Ca$^{2+}$-rich medium.

Another interesting finding was the impairment of hyphal formation in the *pmc1*Δ/Δ mutant in M199 medium and FBS. This suggests that the regulation of Ca$^{2+}$ fluxes by Pmc1p into the vacuole is important for the yeast-to-hyphae transition and follows the findings of previous work indicating that Ca$^{2+}$ release from the fungal vacuole into the cytoplasm via the Ca$^{2+}$ channel Yvc1p is important for hyphal formation (26).
highlights the importance of Ca\(^{2+}\) uptake into and release from the fungal vacuole in morphogenesis. Interestingly, removal of free Ca\(^{2+}\) from the FBS allowed the \(\text{pmc1}\Delta/\Delta\) mutant to form filaments. Thus, while the concentration of calcium in FBS (3.5 to 4 mM) (24) was substantially lower than that used to test the calcium toxicity herein, it was nonetheless sufficient to impair the mutant’s capacity to form hyphae, a characteristic that is intimately associated with the ability of \(C.\ albicans\) to cause disease (22, 23). Our results further suggest that the \(\text{pmc1}\Delta/\Delta\) mutant does not have a mechanical defect in its ability to form hyphae, but rather its inability to remove excess Ca\(^{2+}\) from the cytoplasm interferes with the requisite signaling events. It is likely that the dysregulation of Ca\(^{2+}\)-based signaling also underlies the wide range of physiological and stress-related phenotypes of the \(\text{pmc1}\Delta/\Delta\) mutant.

\(C.\ albicans\) mutants deficient in calcineurin signaling were previously reported as exquisitely sensitive to Ca\(^{2+}\) levels in serum (18, 24) and had severe defects in the colonization of kidney tissue in a mouse model of disseminated infection (18, 27). This makes sense, since Pmc1p is a downstream effector of calcineurin signaling (18, 28); therefore, a loss of Pmc1p would be expected to produce a similar phenotypic profile.

**FIG 4** The \(C.\ albicans\) \(\text{pmc1}\Delta/\Delta\) mutant’s hyphal growth defect is calcium dependent. Wild-type (GP1), \(\text{pmc1}\Delta/\Delta\), and revertant strains of \(C.\ albicans\) were subcultured in FBS or FBS plus 10 mM EGTA at \(\sim 1 \times 10^{6}\) cells/ml and incubated at 37°C with shaking. Samples were taken after 6 h of incubation, and cells were fixed with formalin. Cell morphologies were subsequently observed by light microscopy using a 40× objective. Bar, 50 μm.

**FIG 5** \(C.\ albicans\) \(\text{pmc1}\Delta/\Delta\) mutant is avirulent in a mouse model of disseminated candidiasis. Groups of BALB/c mice (N = 7) were inoculated with \(\sim 5 \times 10^{6}\) CFU of either wild-type (GP1), \(\text{pmc1}\Delta/\Delta\), \(\text{vcx1}\Delta/\Delta\), or revertant strains via lateral tail vein injections. The mice were then monitored 3 times daily for 14 days, and those showing signs of distress were humanely euthanized. The survival of each group was compared using the log rank test (\(P < 0.0001\)).
to that resulting from calcineurin dysfunction. Collectively, the phenotypic deficiencies of the \textit{pmc1\textDelta/\textDelta} mutant resulting from its reduced capacity to sequester excess Ca\textsuperscript{2+} into the vacuole likely underlie its inability to colonize or invade mammalian tissue in the mouse model of disseminated infection. Interestingly, loss of Vcx1p function had little consequence on \textit{C. albicans} physiology \textit{in vitro} or pathogenicity \textit{in vivo}. Given that Pmc1p is a low-capacity high-affinity Ca\textsuperscript{2+} transporter, while Vcx1p is a high-capacity low-affinity transporter, our results suggest that restoring a low cytoplasmic concentration of Ca\textsuperscript{2+} following a signaling event may be more important with respect to avoiding toxicity than the rate at which Ca\textsuperscript{2+} is initially removed.

While most Pmc1p homologs are not essential for fungal viability \textit{in vitro}, their function appears to be essential for the survival of \textit{C. albicans}, \textit{C. neoformans} and \textit{A. fumigatus} \textit{in vitro}, i.e., within mammalian tissue (13, 15, 16). Furthermore, even partially suppressing the expression of either of two Pmc1p orthologues that localize to intracellular acidocalcisomes is sufficient to cause gross morphological abnormalities and severely inhibit the growth of \textit{Trypanosoma brucei} (29), the causative agent of African sleeping sickness. Collectively, these data underscore the critical importance of vacuolar Ca\textsuperscript{2+} uptake by the high-affinity Pmc1p transporter in promoting the survival of infectious eukaryotes within the mammalian host. Given the severity of the pathogenesis defects that result from loss of Pmc1p activity in each of these human pathogens, and in particular, the consequences upon fungal colonization of mammalian tissue, Pmc1p could provide a potentially efficacious target for antifungal therapy. Alternative approaches that prevent the sequestration of Ca\textsuperscript{2+} within the fungal vacuole, or else mobilize intravacuolar calcium, leading to toxicity, may also be viable therapeutic strategies. Whether Pmc1p itself is vulnerable to small-molecule inhibition and if inhibitors with sufficient fungal selective activity can be derived to provide the basis of a viable antimicrobial pharmacotherapy remain to be determined.

Pmc1p is most closely related to the PMCA-type Ca\textsuperscript{2+} pumps of mammals but has several important distinctions (11, 30). First, fungal Pmc1p proteins localize to the vacuolar membrane rather than the plasma membrane, and thus sequester excess calcium into an intracellular store rather than out of the cell (11, 30). Second, while fungal Pmc1p has been classified as a type 2B P-type ATPase, which are typically characterized by a cytoplasmic autoregulatory domain, fungal Pmc1p proteins completely lack this domain. In mammals, the autoinhibitory domain of all four PMCA pumps is found within an extended cytoplasmic C-terminal domain, shown by crystallography to wrap around and block the catalytic core of the protein (31, 32). Intracellular Ca\textsuperscript{2+} fluxes activate PMCA following Ca\textsuperscript{2+}-calmodulin binding to specific sequences in its C terminus that release autoinhibition. Curiously, fungal Pmc proteins completely lack extended N or C termini or the calmodulin binding sites that form the basis of the autoregulatory domains found in higher eukaryotes (11, 31–34). Thus, key structural determinants, as well as the molecular mechanisms by which the activity of these pumps are regulated in infectious fungi, are fundamentally different from those found in their mammalian host.

Previous reports, which were confirmed in this study, found that \textit{C. albicans} \textit{pmc1\textDelta/\textDelta} mutants are resistant to fluconazole on agar plates (18), while their susceptibility is not detectably different from that of the wild type when the standard CLSI broth microdilution protocol is used (20). Here, we determined that the \textit{pmc1\textDelta/\textDelta} mutant’s reduced susceptibility to fluconazole was temperature dependent and therefore may relate to a form of azole tolerance known as “trailing growth” (35) rather than outright azole resistance. Trailing growth is observed with a significant proportion of \textit{C. albicans} isolates and manifests as significant residual growth in the presence of the azole that becomes apparent after 48 h of incubation. In its extreme form, trailing growth can be mistaken for true azole resistance; however, patients and experimental animals infected with trailing isolates generally respond well to treatment with the azoles (36, 37). The effect of temperature on azole tolerance was also observed previously in an endosomal trafficking mutant lacking the Rab GTPase Vps21p. A \textit{C. albicans} \textit{vps21\textDelta/\textDelta} mutant displayed enhanced growth in the presence of fluconazole at
35°C but not at 30°C (38). This enhanced growth in the presence of fluconazole also resembled the trailing growth phenomenon and appeared to be dependent on elevated Ca²⁺ signaling (20, 38). Together, these findings further emphasize the importance of temperature and exogenous Ca²⁺ levels, as well as intracellular Ca²⁺ homeostasis, as determinants of the capacity of C. albicans to survive following exposure to theazole antifungals.

MATERIALS AND METHODS

Growth conditions. C. albicans was routinely grown on YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C, supplemented with uridine (50 µg/ml) when necessary. Transformant selection was carried out on minimal YNB medium (6.75 g/liter yeast nitrogen base without amino acids, 2% dextrose, 2% Bacto agar), supplemented with the appropriate auxotrophic requirements as described for Saccharomyces cerevisiae (39) or 50 µg/ml uridine.

Plasmid construction. All oligonucleotides used in this study are listed in Table S1 in the supplemental material.

The PMC1 open reading frame (ORF) with 761 bp of 5'- and 434 nt of 3'-untranslated-region (UTR) sequences was amplified from SC5314 genomic DNA (gDNA) with primer pair PMC1AMPF-KpnI and PMC1AMPR-SacI and then cloned between the KpnI and SacI restriction sites of pLUX (43) to produce plasmid pLUXPMC1.

For construction of the plasmid pLUXVCX1, the VCX1 ORF with 747 bp of 5'- and 324 bp of 3'-UTR sequences was amplified using primer pair VCX1AMPF-KpnI and VCX1AMPR-SacI and cloned between the KpnI and SacI restriction sites of pLUX.

Candida albicans strains. All strains used in this study are listed in Table S2. Transformation of C. albicans with DNA constructs was performed using the lithium acetate method (40). Gene deletion strains were constructed by the PCR-based approach described by Wilson et al. (19), using the ura3Δ/-his1Δ/-ΔlongΔ/Δ strain BW217, kindly provided by Aaron Mitchell (Carnegie Mellon University).

The pmc1Δ/Δ strain was constructed as follows. PMC1 deletion cassettes were amplified by PCR with primers PMC1DISF and PMC1DISR using pRS-ARG4ΔSpeI, pGEM-HIS1, or pDDB57 (containing a recyclable URA3-d3p200 marker) (20, 21) as the template. Each PMC1 allele was then sequentially deleted from BW217 using HIS1 and ARG4 markers to generate pmc1Δ/Δ ura3Δ/Δ gene deletion mutants. The correct integration of the deletion cassettes was confirmed at each step by PCR with the following primers sets: ARG4INTF2/PMC1AMPR-SacI and ARG4INTR2/PMC1AMPR-KpnI for ARG4 integration or HIS1INTR2/PMC1AMPR-SacI and HIS1INTF2/PMC1AMPR-KpnI for HIS1 integration. The absence of an intact PMC1 allele was confirmed using primer pair PMC1DETF and PMC1DETR. Isogenic mutant and PMC1-reconstituted strains were produced by transforming the pmc1Δ/Δ ura3Δ/Δ mutant with either pLUX (vector alone) or pLUXPMC1 after digestion with NheI. The correct integration of the pLUX vector fully restores URA3 and adjacent IRO1 loci, and this was confirmed by PCR using primer pair LUXINTDETF/LUXINTDETR.

VCX1 deletion cassettes were amplified by PCR with primers VCX1DISF and VCX1DISR using pRS-ARG4ΔSpeI, pGEM-HIS1, or pDDB57 as the templates. The vcx1Δ/Δ ura3Δ/Δ gene deletion mutants were produced by sequential deletion of each VCX1 allele using HIS1 and ARG4 markers. Correct integration of deletion cassettes was confirmed at each step by PCR with primers pairs ARG4INTF2/VCX1AMPR-SacI and ARG4INTR2/VCX1AMPR-KpnI (ARG4 integration), or HIS1INTR2/VCX1AMPR-SacI and HIS1INTF2/VCX1AMPR-KpnI (HIS1 integration). Lack of an intact VCX1 allele was confirmed by using primer pair VCX1DETF/VCX1DETR. Isogenic vcx1Δ/Δ mutant and VCX1-reconstituted strains were produced by transforming the vcx1Δ/Δ ura3Δ/Δ mutant with either NheI-digested pLUX (vector alone) or pLUXVCX1. Correct integration of either plasmid was confirmed by PCR using primer pair LUXINTDETF/LUXINTDETR.

Stress resistance and hyphal growth assays. Each C. albicans strain was grown overnight in YPD at 30°C. The cells were washed in sterile deionized water, the cell density was adjusted to 10⁷ cells/ml, and 1.5 serial dilutions were performed in a 96-well plate. Each cell suspension was then applied to agar plates using a sterile multipronged applicator. Resistance to different stresses was determined on YPD agar containing 5 µg/ml of fluconazole, 0.05% SDS, 1.5 M NaCl, 300 mM LiCl, 5 mM CdSO₄, or 500 mM CaCl₂, with incubation at 30°C for 48 to 96 h. To induce hyphal growth, for each strain, 2.5 µl of a 10⁷ cells/ml cell suspension was spotted on M199 or 10% fetal bovine serum (FBS) agar plates, and incubated for 96 h at 37°C.

Biofilm formation assay. Biofilm formation was assessed using a protocol based on that described by O'Toole (41). Each C. albicans strain was grown overnight in YPD broth at 30°C. Each culture was then washed twice in sterile phosphate-buffered saline (PBS), the cell density was adjusted to 1 × 10⁶ per ml in RPMI medium (pH 7.0), and 200 µl was dispensed into the wells of a flat-bottomed 96-well plate. After incubation at 37°C for 24 h, each well was rinsed 3 times with sterile PBS and then stained with 0.01% crystal violet for 15 min. Each well was then rinsed 3 times with sterile water, and the dye was eluted with 95% ethanol (200 µl/well); 150 µl of resolubilized dye from each well was then transferred to a new flat-bottomed microtiter plate, and the optical density at 570 nm (OD570) was measured using a microplate reader.

Antifungal susceptibility testing. Antifungal susceptibility testing of all the strains included in this study was performed using the broth microdilution method described in the CLSI document M27-A3 (42) in a 96-well plate format. All drugs for susceptibility testing used in this study were diluted in dimethyl sulfoxide (DMSO) to 200 times the final concentration. RPMI 1640 medium (Sigma-Aldrich) was prepared according to the CLSI document; the medium was buffered with morpholinepropanesulfonic acid.
(MOPS) and pH adjusted using NaOH and HCl. Plates were incubated at 25°C, 35°C, or 42°C without shaking for 24 or 48 h. The content of each well was carefully resuspended by pipetting up and down before the OD600 was measured using a Biotek Cytation 5 plate reader.

Susceptibility testing using fluconazole Etest strips was performed on agar plates with MOPS-buffered RPMI medium at pH 7. Cell suspensions were streaked onto the RPMI plates using sterile cotton applicators. Etest strips were applied on the surface of the agar, and the plates were incubated at 35°C for 24 to 48 h.

**Ethics statement.** The animals used in this study were housed in American Association for Accreditation of Laboratory Animal Care (AAALAC)-approved facilities at the University of Tennessee Health Science Center (UTHSC). The Institutional Animal Care and Use Committee (IACUC) at UTHSC approved the use of all animals and procedures (IACUC protocol numbers 15-081 and 16-156). Mice were given standard rodent chow and water *ad libitum*. Mice were monitored daily for signs of distress, including noticeable weight loss and lethargy, and for the body condition score. The IACUC at UTHSC uses the Public Health Policy on Humane Care and Use of Laboratory Animals (PHS) and the Guide for the Care and Use of Laboratory Animals as a basis for establishing and maintaining an institutional program for activities involving animals. To ensure high standards for animal welfare, the IACUC at UTHSC remains compliant with all applicable provisions of the Animal Welfare Act (AWAR), guidance from the Office of Laboratory Animal Welfare (OLAW), and the American Veterinary Medical Association Guidelines on Euthanasia.

Mouse model of disseminated candidiasis. *C. albicans* strains were grown overnight in YPD broth at 30°C with shaking. Stationary-phase cultures of *C. albicans* strains were washed twice in sterile, endotoxin-free phosphate-buffered saline (PBS) and resuspended in PBS at 5 × 10⁶ cells/ml. Groups of 6 BALB/c mice per *C. albicans* strain were then inoculated via tail vein injections with 100 µl of the desired cell suspension ( ~ 5 × 10⁶ cells). Viable cell counts of each inoculum were confirmed by plating appropriate dilutions on YPD agar plates and counting the colonies formed after 48 h of incubation at 30°C. Mice were then monitored for 14 days postinfection, and those showing signs of distress were humanely euthanized. Animals surviving to the end of the experiment (day 14) were euthanized, and their kidneys were extracted, weighed, and homogenized in PBS. Serial dilutions of kidney homogenates were plated on YPD agar plates containing 50 µg/ml of chloramphenicol. The CFU/g of kidney tissue was then determined from the number of colonies formed on the plates after 48 h of incubation at 30°C.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00715-18.

**FIG S1**, DO CX file, 0.2 MB.

**FIG S2**, DO CX file, 2.2 MB.

**FIG S3**, DO CX file, 0.6 MB.

**FIG S4**, DO CX file, 0.1 MB.

**TABLE S1**, PDF file, 0.1 MB.

**TABLE S2**, PDF file, 0.1 MB.

**ACKNOWLEDGMENTS**

This work was funded by the National Institute of Allergy And Infectious Diseases of the National Institutes of Health under award number R01AI099080.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

**REFERENCES**

1. Klionsky DJ, Herman PK, Emr SD. 1990. The fungal vacuole: composition, function, and biogenesis. Microbiol Rev 54:266–292.

2. Veses V, Richards A, Gow NA. 2008. Vacuoles and fungal biology. Curr Opin Microbiol 11:503–510. https://doi.org/10.1016/j.mib.2008.09.017.

3. Cyert MS, Philpott CC. 2013. Regulation of cation balance in *Saccharomyces cerevisiae*. Genetics 193:677–713. https://doi.org/10.1534/genetics.112.147207.

4. Dunn T, Gable K, Beeler T. 1994. Regulation of cellular Ca²⁺ by yeast vacuoles. J Biol Chem 269:7273–7278.

5. Bonilla M, Nastase KK, Cunningham KW. 2002. Essential role of calcineurin in response to endoplasmic reticulum stress. EMBO J 21:2343–2353. https://doi.org/10.1093/emboj/21.10.2343.

6. Kraus PR, Heitman J. 2003. Coping with stress: calmodulin and calcineurin in model and pathogenic fungi. Biochem Biophys Res Commun 301:1151–1157.

7. Marchi S, Paterngrani S, Missiroli S, Morciano G, Rimessi A, Wieckowski MR, Giorgi C, Pinton P. 2018. Mitochondrial and endoplasmic reticulum calcium homeostasis and cell death. Cell Calcium 69:62–72. https://doi.org/10.1016/j.ceca.2017.05.003.

8. Pinton P, Giorgi C, Siviero R, Zecchini E, Rizzuto R. 2008. Calcium and apoptosis: ER-mitochondria Ca²⁺ transfer in the control of apoptosis. Oncogene 27:6407–6418. https://doi.org/10.1038/onc.2008.308.

9. Miseta A, Kellermayer R, Aiello DP, Fu L, Bedwell DM. 1999. The vacuolar Ca²⁺/H⁺ exchanger Vcx1p/Hum1p tightly controls cytosolic Ca²⁺ levels in *S. cerevisiae*. FEBS Lett 451:132–136.

10. Cunningham KW, Fink GR. 1996. Calcineurin inhibits Vcx1-dependent H⁺/Ca²⁺ exchange and induces Ca²⁺ levels in *S. cerevisiae*. Mol Cell Biol 16:2226–2237.

11. Pittman JK. 2011. Vacular Ca²⁺ uptake. Cell Calcium 50:139–146. https://doi.org/10.1016/j.ceca.2011.01.004.

12. Cunningham KW, Fink GR. 1994. Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking PMC1, a homolog of plasma membrane Ca²⁺ ATPases. J Cell Biol 124:351–363.

13. Squizani ED, Oliveira NK, Reuwsaat JCV, Marques BM, Lopes W, Gerber
Stathopoulos AM, Cyert MS. 1997. Calcineurin acts through the CRZ1 pathway in yeast. Eukaryot Cell 6:1100–1110.

Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR. 2013. TCN1-encoded transcription factor to regulate gene expression in yeast. Genetics 193:1043–1054.

Yu Q, Wang F, Zhao Q, Chen J, Zhang B, Ding X, Wang H, Yang B, Lu G, Zhang B, Li M. 2014. A novel role of the vacuolar calcium channel Vcx1 in stress response, morphogenesis and pathogenicity of Candida albicans. Int J Med Microbiol 304:339–350. https://doi.org/10.1016/j.ijmm.2013.11.022

Blankenship JR, Wormley FL, Boyce MK, Schell WA, Filler SG, Perfect JR, Heitman J. 2003. The vacuolar calcium channel Vcx1 is required for Candida albicans survival in serum and virulence. Eukaryot Cell 2:422–430.

Stathopoulos AM, Cyert MS. 1997. Calcineurin acts through the CRZ1 pathway in yeast. Genes Dev 11:3432–3444.

Luo S, Rohloff P, Cox J, Uyemura SA, Decampo R. 2004. Trypsinosoma brucei plasma membrane-type Ca^{2+}-ATPase 1 (TbPMC1) and 2 (Tb-PMC2) genes encode functional Ca^{2+}-ATPases localized to the acidic calciosomes and plasma membrane, and essential for Ca^{2+} homeostasis and growth. J Biol Chem 279:14427–14439. https://doi.org/10.1074/jbc.M309978200.

Cunningham KW. 2011. Acidic calcium stores of Saccharomyces cerevisiae. Cell Calcium 50:129–138. https://doi.org/10.1016/j.ceca.2011.01.010

Di Leva F, Domi T, Fedrizzi L, Lim D, Carafoli E. 2008. The plasma membrane Ca^{2+}-ATPase of animal cells: structure, function and regulation. Arch Biochem Biophys 476:65–74. https://doi.org/10.1016/j.abb.2008.02.026

Enyedi A, Vorherr T, James P, McCormick DJ, Filoteo AG, Carafoli E, Penniston JT. 1989. The calmodulin binding domain of the plasma membrane Ca^{2+} pump interacts both with calmodulin and with another part of the pump. J Biol Chem 264:12313–12321.

Palmgren MG, Axelsen KB. 1998. Evolution of P-type ATPases. Biochim Biophys Acta 1365:37–45.

Hofmann F, James P, Vorherr T, Carafoli E. 1993. The C-terminal domain of the plasma membrane Ca^{2+} pump contains three high affinity Ca^{2+} binding sites. J Biol Chem 268:10252–10259.

Arrington-Skaggs BA, Lee-Yang W, Ciblak MA, Frade JP, Brandt ME, Hajijeh RA, Harrison LH, Sofair AN, Warnock DW, Candidemia Active Surveillance Group. 2002. Comparison of visual and spectrophotometric methods of broth microdilution MIC end point determination and evaluation of a sterol quantitation method for in vitro susceptibility testing of fluconazole and itraconazole against trailing and nontrailing Candida isolates. Antimicrob Agents Chemother 46:2477–2481.

Peters BM, Luna-Tapia A, Tournu H, Rybak JM, Rogers PD, Palmer GE. 2017. An azole-tolerant endosomal trafficking mutant of Candida albicans is susceptible to azole treatment in a mouse model of vaginal candidiasis. Antimicrob Agents Chemother 61:e00084-17. https://doi.org/10.1128/AAC.00084-17.

Rex JH, Nelson PW, Paetznick VL, Lozano-Chiu M, Espinell-Ingroff A, Annaise JJ. 1998. Optimizing the correlation between results of testing in vitro and therapeutic outcome in vivo for fluconazole by testing critical isolates in a murine model of invasive candidiasis. Antimicrob Agents Chemother 42:129–134. https://doi.org/10.1128/AAC.42.1.129.

Luna-Tapia A, Kerns ME, Eberle KE, Jursic BS, Palmer GE. 2015. TrAFFicking through the late endosome significantly impacts Candida albicans tolerance of the azole antifungals. Antimicrob Agents Chemother 59:2410–2420. https://doi.org/10.1128/AAC.04239-14.

Burke D, Dawson D, Stearns T, Laboratory CSH. 2000. Methods in yeast genetics: a cold spring harbor laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Gietz D, St Jean A, Woods RA, Schiestl RH. 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 20:1425.

O’Toole GA. 2011. Microtiter dish biofilm formation assay. J Vis Exp 60:e3472. https://doi.org/10.3791/3472.

Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard, 3rd ed. CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA.

Ramon AM, Fonzi WA. 2003. Diverged binding specificity of Rim101p, the Candida albicans ortholog of PcsC. Eukaryot Cell 2:718–728.