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Endosomal Trafficking Defects Can Induce Calcium-Dependent Azole Tolerance in *Candida albicans*

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The azole antifungals arrest fungal growth through inhibition of ergosterol biosynthesis. We recently reported that a *Candida albicans vps21*Δ/Δ mutant, deficient in membrane trafficking through the late endosome/prevacuolar compartment (PVC), continues to grow in the presence of the azoles despite the depletion of cellular ergosterol. Here, we report that the *vps21*Δ/Δ mutant exhibits less plasma membrane damage upon azole treatment than the wild type, as measured by the release of a cytoplasmic luciferase reporter into the culture supernatant. Our results also reveal that the *vps21*Δ/Δ mutant has abnormal levels of intracellular Ca^{2+} and, in the presence of fluconazole, enhanced expression of a calcineurin-responsive *RTA2-GFP* reporter. Furthermore, the azole tolerance phenotype of the *vps21*Δ/Δ mutant is dependent upon both extracellular calcium levels and calcineurin activity. These findings underscore the importance of endosomal trafficking in determining the cellular consequences of azole treatment and indicate that this may occur through modulation of calcium- and calcineurin-dependent responses.

Several important antifungal drugs inhibit the synthesis of ergosterol, a lipid that modulates the thickness, fluidity, and permeability of fungal cell membranes (1). These drugs include the azoles, which inhibit lanosterol demethylase (Erg11p). Both the depletion of cellular ergosterol and the accumulation of intermediate sterol species are thought to cause plasma membrane dysfunction and, ultimately, growth arrest following azole treatment (2). The emergence of azole-resistant fungal isolates is a problem of increasing medical significance (3). Several specific mechanisms that contribute to the development of azole resistance in the prevalent human fungal pathogen *Candida albicans* have been described. These mechanisms include increased expression of the target enzyme, point mutations that alter the target enzyme's affinity for the azoles (4, 5), and increased expression of efflux pumps, such as Cdr1p and Mdr1p, that export the azoles out of the fungal cell (6–8). However, the azole resistance of many fungal isolates is not fully accounted for by these well-characterized mechanisms. We recently reported that membrane trafficking through the late endosome/prevacuolar compartment (PVC) has a significant impact upon *C. albicans* azole tolerance (9). Specifically, we found that a deletion of *VPS21*, which encodes a Rab GTPase required for PVC trafficking, substantially enhanced *C. albicans* growth in the presence of the azoles. The azole tolerance of the *vps21*Δ/Δ mutant does not depend upon established mechanisms of azole resistance such as the activity of the well-characterized drug efflux pumps Mdr1p and Cdr1p, or increased target protein expression, but occurs despite the depletion of cellular ergosterol (9). This phenotype resembles an exaggerated form of "trailing growth," a phenomenon that is observed using standard CLSI antifungal susceptibility testing protocols (10), in which a subset of *C. albicans* isolates appear to be azole susceptible at early time points but display significant growth at later time points (10). The objective of this study was to define the mechanism(s) underlying the azole tolerance of the *C. albicans vps21*Δ/Δ mutant.

MATERIALS AND METHODS

Growth conditions. *C. albicans* was routinely grown at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) 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 previously for *Saccharomyces cerevisiae* (11), or 50 μg/ml uridine.

Plasmid construction. Plasmid pLUX (12) was kindly provided by William Fonzi (Georgetown University). Plasmids pLUXVPS21 (13), pKE1 (14), and pKE1-NLUC (15) were described previously. All oligonucleotides used in this study are listed in Table S1 in the supplemental material. The *VPH1* open reading frame (ORF) with 5′- and 3′-untranslated region (UTR) sequences was amplified from SC5314 genomic DNA (gDNA) with HiFi Platinum *Taq* (Invitrogen) and primer set VPH1AMPF and VPH1AMPR-KpnI and cloned between the SacI and KpnI restriction sites of pLUX to produce plasmid pLUXVPH1.

For the construction of reporter plasmid pRTA2prGFPγ, 1,000 bp of the *RTA2* promoter was amplified from SC5314 gDNA with primer pair RTA2prF-KpnI and RTA2prR-SalI and cloned between the KpnI and SalI sites of pKE1 in place of the *ACT1* promoter. The GFPγ (green fluorescent protein) coding sequence was then amplified by using primers GFPAMPF-SalI and GFPAMPR-MluI and cloned downstream of the *RTA2* promoter between the SalI and MluI sites.

***C. albicans* strains.** All strains used in this study are listed in Table S2 in the supplemental material. The *vps21*Δ/Δ, *aps3*Δ/Δ, and *vps21*Δ/Δ

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*aps3*Δ/Δ mutants were constructed in previous studies (13, 14, 16, 17). Control strain YJB6284 (18) was kindly provided by Judith Berman (Tel Aviv University). Transformation of *C. albicans* with DNA constructs was performed by using the lithium acetate method (19). Gene deletion strains were constructed by a PCR-based approach described previously by Wilson et al. (20), using *ura3*Δ/Δ *his1*Δ/Δ *arg4*Δ/Δ strain BWP17 (kindly provided by Aaron Mitchell, Carnegie Mellon University). Strain CAI4 was kindly provided by William Fonzi (Georgetown University).

VPH1 deletion cassettes were amplified by PCR with primers VPH1DISF and VPH1DISR, using pRS-ARG4ΔSpeI, pGEM-HIS1, or pDDB57 (containing a recyclable *URA3-dpl200* marker) (20, 21) as the template. Each *VPH1* allele was sequentially deleted by using *HIS1* and *ARG4* markers to generate *vph1*Δ/Δ *ura3*Δ/Δ gene deletion mutants. The correct integration of the deletion cassettes was confirmed at each step by PCR with the following primers sets: ARG4INTF2 plus VPH1DETR2 and ARG4INTR2 plus VPH1DETF2 for *ARG4* integration or HIS1INTR2 plus VPH1DETF2 for *HIS1* integration. The absence of an intact *VPH1* allele was confirmed by using primer pair VPH1DETF and VPH1DETR. Isogenic mutant and *VPH1*-reconstituted strains were produced by transforming the *vph1*Δ/Δ *ura3*Δ/Δ mutant with either pLUX (vector alone) or pLUXVPH1 after linearization 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 and LUXINTDETR.

A previously described *vps21*Δ/Δ *ura3*Δ/Δ *arg4*Δ/Δ strain (13) was used to construct the *vps21*Δ/Δ *vph1*Δ/Δ double mutant. The first allele of *VPH1* was deleted with the *URA3-dpl200* selection marker, and the second allele was deleted with the *ARG4* marker. Replacement of the first *VPH1* allele with the *URA3-dpl200* selection marker was confirmed by using primer pairs URA3INTF2 plus VPH1DETR2 and URA3INTR2 plus VPH1DETF2. The resulting *vps21*Δ/Δ *vph1*Δ/Δ double mutant was then selected on YNB agar plates supplemented with uridine and 5-fluoroorotic acid (5-FOA) (22) to select for *ura3*$^-$ segregants that had excised the *URA3* marker gene. The correct excision of the *URA3* marker from the *vph1*Δ-*URA3-dpl200* loci was confirmed by using the flanking primer pair VPH1AMPF and VPH1AMPR-KpnI. Prototrophic and isogenic mutant and reconstituted control strains were then made by transforming the *vps21*Δ/Δ *vph1*Δ/Δ double mutant with NheI-digested pLUX, pLUX-VPS21, or pLUXVPH1.

C. albicans strains expressing cytoplasmic nanoluciferase (Nluc) (23) (produced with permission from Promega Corporation) were produced by using the previously described plasmid pKE1-NLUC (15), which was linearized by using NheI and transformed into *ura3*$^-$ recipient strains. Strains carrying the *RTA2-GFP*γ reporter were made by transforming CAI4 and *vps21*Δ/Δ *ura3*$^-$ strains with NheI-digested pRTA2prGFPγ. The correct integration of these pLUX-based vectors fully restores *URA3* and adjacent *IRO1* loci, and this was confirmed by PCR using primer pair LUXINTDETF and LUXINTDETR.

Luciferase-based membrane integrity assay. *C. albicans* strains expressing Nluc were cultured overnight in YPD at 30°C. Two hundred microliters of each culture was then transferred to an Eppendorf tube, and the cells were pelleted by using a microcentrifuge. The culture supernatant was then removed, and the cells were resuspended in 1 ml YNB and further diluted 1:100 in YNB medium. Two hundred microliters of each cell suspension was then dispensed into the wells of a round-bottomed 96-well plate. After a 24-h incubation in a 30°C or 35°C standing incubator, the cells in each well were resuspended by mixing using a multichannel pipette. The plates were then centrifuged at 1,200 rpm in a benchtop centrifuge to pellet the cells. Fifty microliters of the culture supernatant was then transferred to a white, flat-bottomed, 96-well plate, and Nluc activity was determined by using the Nano-Glo luciferase assay reagent (Promega Corporation), according to the manufacturer's instructions. Luminescence was then measured by using a Cytation 5 plate reader (BioTek Instruments, Inc.). Growth in each well after incubation overnight was also determined by measuring the optical density at 600 nm (OD_{600}) from samples diluted 1:10 in distilled water using a separate flat-bottomed 96-well plate, and each luminescence reading was normalized to the corresponding OD_{600} reading.

Biomass measurement and determination of cell viability. *C. albicans* strains were grown overnight in YNB at 30°C, washed and subcultured in 50 ml of fresh YNB at 2×10^6 cells/ml in the presence of 5 μg/ml of fluconazole or 0.5% dimethyl sulfoxide (DMSO), and incubated at 30°C or 35°C for 24 h with shaking. Cell viability was then determined as CFU from culture samples following appropriate dilution in YNB and plating onto YPD agar plates. Forty milliliters of each culture was also pelleted at 3,500 rpm, washed by resuspension in 5 ml of deionized H_2O, and harvested by filtration using a preweighed 0.22-μm GS filter membrane (Millipore). Cell pellets were dehydrated at 65°C for 24 h, and dry weight was determined.

Antifungal susceptibility testing. Antifungal susceptibility testing of all the strains included in this study was performed by using the broth microdilution method described in CLSI document M27-A3 (24), in a 96-well-plate format. All drugs for susceptibility testing used in this study were diluted in DMSO in 2-fold dilutions at 200 times the final concentration. Fluconazole was obtained from Sigma-Aldrich. RPMI 1640 medium (Sigma-Aldrich) was prepared according to CLSI standards (24), the medium was buffered with morpholinepropanesulfonic acid (MOPS), and the pH was adjusted to 7.0 using NaOH. Plates were incubated without shaking at 35°C for 24 or 48 h unless otherwise stated. The content of each well was carefully resuspended by pipetting up and down before the OD_{600} was measured using a Cytation 5 plate reader (BioTek Instruments, Inc.). Checkerboard assays with fluconazole and the calcineurin inhibitor cyclosporine or FK506 (Tacrolimus) were performed in a 96-well plate in MOPS-buffered RPMI 1640 (pH 7.0) according to CLSI standards.

Determination of intracellular Ca^{2+} concentrations. The relative levels of intracellular free Ca^{2+} were determined by using the Ca^{2+}-sensitive dye Fura 2-AM (Fura-2-acetoxymethyl ester; EMD Millipore Corporation). *C. albicans* strains were grown overnight in YPD at 30°C and washed in phosphate-buffered saline (PBS), and ~10^7 cells were resuspended in 200 μl PBS (pH 7.2) (without $CaCl_2$ and $MgCl_2$) plus 10 μM Fura 2-AM. Each cell suspension was then dispensed into the wells of a round-bottomed 96-well plate. Following incubation at 37°C for 1 h, cells were pelleted and washed three times with PBS. Fluorescence was determined by dual excitation at 340 nm and 380 nm with a single emission at 505 nm in a Cytation 5 plate reader (BioTek Instruments, Inc.), with relative Ca^{2+} levels being expressed as the ratio of the fluorescence intensity upon excitation at 380 nm (F_{380}) to the F_{340} (25).

GFPγ reporter assays. *C. albicans* strains expressing GFPγ from the *RTA2* promoter were grown overnight in YPD at 30°C. Cells were washed and resuspended in YNB, and ~1.3×10^6 cells per well were dispensed into a round-bottomed 96-well plate. Cells were treated with either 1 μg/ml fluconazole or 0.5% DMSO and incubated at 35°C for 24 h. GFPγ fluorescence intensity was then quantified by using a Cytation 5 plate reader (BioTek Instruments, Inc.) with excitation at 488 nm and emission at 507 nm. Growth was determined by measuring the OD_{600} for normalization of the fluorescence signal.

RESULTS

The *C. albicans vps21*Δ/Δ mutant is less susceptible than wild type to azole-induced membrane damage. We previously reported that the *vps21*Δ/Δ mutant exhibits enhanced growth in the presence of the azole antifungals versus wild-type (WT) controls, despite ergosterol depletion (9). We therefore examined plasma membrane integrity following azole treatment using a luciferase-based assay (15) to determine if the *vps21*Δ/Δ mutant exhibited signs of membrane damage. A cytoplasmic version of nanoluciferase (Nluc; Promega Corp.) (23) was expressed in mutant and control strains (see Fig. S1 in the supplemental material), and the amount of Nluc released into the culture supernatant was com-

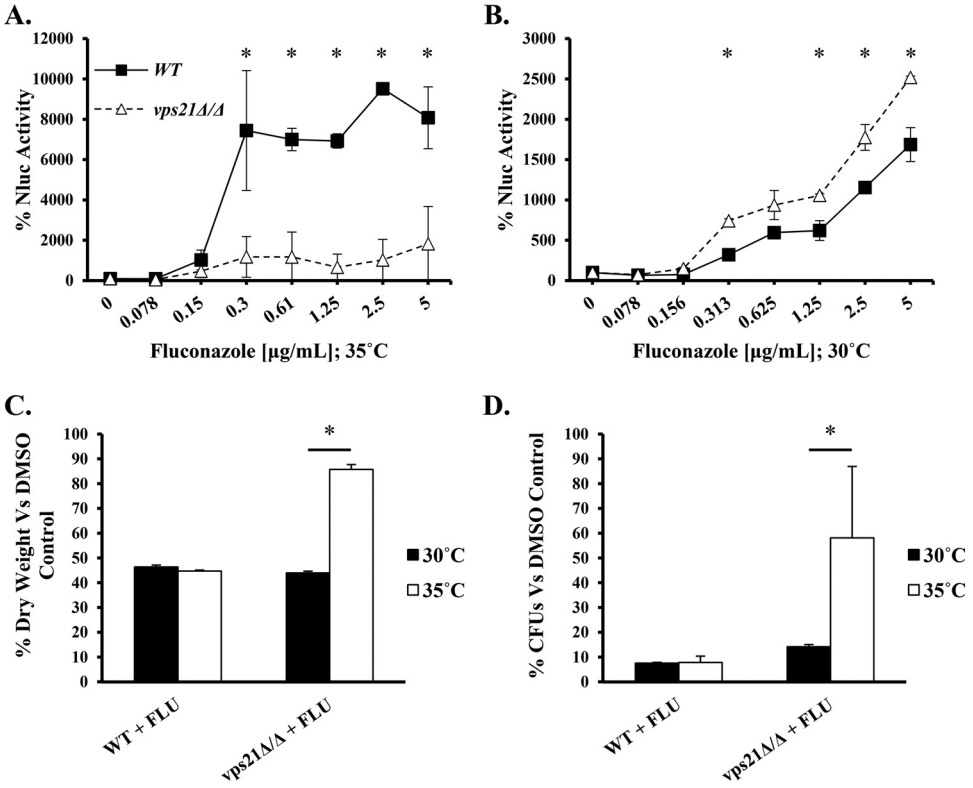

FIG 1 The *C. albicans vps21*Δ/Δ mutant exhibits less membrane damage following azole treatment. (A and B) The cytoplasmic Nluc expression vector pKE1-Nluc was introduced into the *vps21*Δ/Δ mutant and wild-type control (CAI4) strains. Each strain was then grown in 96-well plates in YNB medium with various concentrations of fluconazole or DMSO solvent (minus-drug control). After 24 h of incubation at 35°C (A) or 30°C (B), the culture supernatant was removed from each well, and Nluc activity was assayed by using a luminescent substrate. Nluc activity measured in relative light units was normalized for fungal growth (OD_{600}) and expressed as a percentage of the value for the minus-drug controls. The means and standard deviations of data from three biological replicates are shown for each drug concentration. (C and D) *vps21*Δ/Δ mutant and WT strains of *C. albicans* were introduced into YNB at 2×10^6 cells/ml in the presence of 0.5% DMSO or 5 μg/ml of fluconazole (FLU) and grown at 30°C or 35°C. After 24 h, biomass was measured as dry weight (C), and cell viability was determined as CFU (D). Dry weight and CFU are expressed as percentages of the values for the DMSO control for each strain. The means ± standard deviations of data from two biological replicates are shown for panels C and D. The means for each group were compared by using a two-tailed *t* test. *, $P < 0.05$.

pared following treatment with fluconazole. Fluconazole treatment at 35°C caused significant Nluc release from the WT strain, indicating a loss of plasma membrane integrity (Fig. 1A). However, at this temperature, the *vps21*Δ/Δ mutant released significantly less Nluc upon fluconazole treatment than the WT, indicating less membrane damage. In contrast, at 30°C, the mutant released levels of Nluc that were similar to, or even slightly higher than, those released by the WT (Fig. 1B), indicating that fluconazole induces similar levels of membrane permeabilization in either strain at the lower temperature. This is consistent with our previously reported observation that the continued growth of the *vps21*Δ/Δ mutant in the presence of the azoles (as measured by the OD_{600}) occurs at 35°C but not at 30°C (9). To further confirm the temperature dependence of the azole tolerance of the *vps21*Δ/Δ mutant, we compared its growth with and without fluconazole using two additional measures, dry weight and CFU (Fig. 1C and D). By both measures, at 35°C, the *vps21*Δ/Δ mutant was more tolerant of fluconazole than the WT, while at 30°C, the growth of both strains was severely inhibited. As such, it appears that at 35°C, the azole antifungals cause less membrane damage to the *vps21*Δ/Δ mutant than to the WT, and this likely accounts for its continued growth.

Azole tolerance of the *C. albicans vps21*Δ/Δ mutant does not depend upon Vph1p function. A recent study reported that inhibition of the V-ATPase proton pump that is responsible for the acidification of the vacuole may in part underlie the antifungal activity of the azoles (1). In *S. cerevisiae*, a *vps21*Δ mutant is deficient in the delivery of the V-ATPase to the fungal vacuole and thus vacuolar acidification, as evidenced by reduced quinacrine accumulation (26). In contrast, we observed that the *C. albicans vps21*Δ/Δ mutant accumulates more quinacrine than the control strains, consistent with increased vacuolar acidification (see Fig. S2 in the supplemental material). To determine if the azole tolerance of the *vps21*Δ/Δ mutant depends upon V-ATPase activity, we deleted both alleles of *VPH1*, a gene encoding a vacuole-specific subunit of the V-ATPase (27). However, the loss of *VPH1* did not affect the susceptibility of the wild-type control strain to fluconazole or the trailing growth of the *vps21*Δ/Δ mutant (Fig. 2). These data demonstrate that the azole tolerance of the *vps21*Δ/Δ mutant does not depend upon V-ATPase activity.

The *vps21*Δ/Δ mutant has abnormal intracellular calcium levels. Ca^{2+}-based signaling has been shown to induce adaptive responses that promote *C. albicans* survival following azole treatment (28). The fungal vacuole is the major storage site for intra-

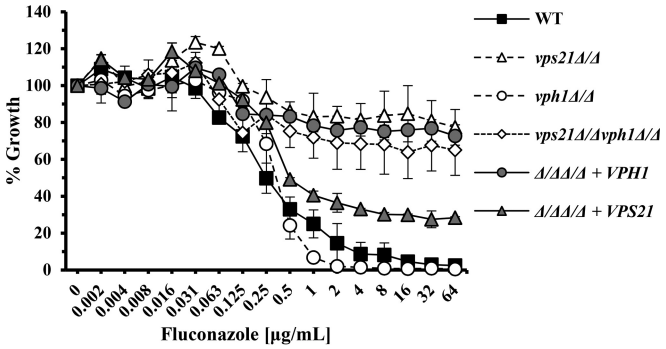

FIG 2 Deletion of *VPH1* from the *vps21*Δ/Δ mutant does not restore azole sensitivity. *VPH1*, encoding a vacuole-specific subunit of the V-ATPase proton pump, was deleted from either the wild type or the *C. albicans vps21*Δ/Δ mutant, and the fluconazole susceptibility of each strain was tested by using standard CLSI broth microdilution protocols. Growth was measured after 48 h of incubation as the OD_{600} and expressed as a percentage of the values for the minus-drug (DMSO alone) control wells. The WT is strain YJB6284. The means ± standard deviations of data from two biological replicates are shown.

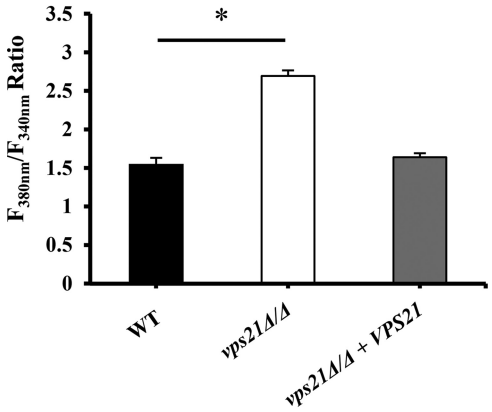

FIG 3 The *C. albicans vps21*Δ/Δ mutant has abnormal intracellular Ca^{2+} levels. Each strain was grown in YPD broth at 30°C, labeled with the Ca^{2+}-responsive dye Fura 2-AM, and washed before intracellular free Ca^{2+} levels were measured spectroscopically with excitation at 340 nm (F_{340}) or 380 nm (F_{380}) and emission at 505 nm. Intracellular Ca^{2+} levels are expressed as F_{380}/F_{340} ratios, and for each strain, the means ± standard deviations of data from four biological replicates are shown. The means for each group were compared by using a two-tailed *t* test. *, $P < 0.0001$.

cellular Ca^{2+} (29). Therefore, to determine if the vacuolar trafficking defects of the *vps21*Δ/Δ mutant impact cellular Ca^{2+} homeostasis, we compared the levels of intracellular free calcium using the calcium-responsive dye Fura 2-AM (30). The wild-type strain treated with the antiarrhythmic drug amiodarone (AMD), which causes cytoplasmic Ca^{2+} surges in *S. cerevisiae* (31), was used as a positive control and induced a dose-dependent increase in the F_{380}/F_{340} ratio (see Fig. S3 in the supplemental material). Interestingly, the F_{380}/F_{340} ratio of Fura 2-AM-stained *vps21*Δ/Δ mutant cells was also significantly higher than that of WT control cells, indicating that the mutant has abnormally high intracellular Ca^{2+} levels (Fig. 3).

The azole tolerance of the *vps21*Δ/Δ mutant is dependent upon extracellular calcium availability. We next examined if the azole tolerance phenotype of the *vps21*Δ/Δ mutant was affected by the availability of extracellular Ca^{2+}. The addition of 1 mM $CaCl_2$ to the medium had little effect upon the growth of either the *vps21*Δ/Δ mutant or control strains in the presence of fluconazole (Fig. 4A). However, the addition of the calcium chelator EGTA reduced the mutant's growth in the presence of fluconazole (Fig. 4B and C), establishing a requirement for extracellular calcium for its azole tolerance. This conclusion is further supported by the fact that the calcium channel blocker nisoldipine, belonging to the dihydropyridine class of Ca^{2+} channel blockers, also prevented the growth of the *vps21*Δ/Δ mutant in the presence of fluconazole (Fig. 4D and E). Dihydropyridines were previously shown to inhibit the major plasma membrane Ca^{2+} channel Cch1p-Mid1p of yeast (32), which facilitates the influx of external Ca^{2+} into the cytoplasm.

The azole tolerance of the *vps21*Δ/Δ mutant is dependent upon calcineurin activity. Calcium-dependent activation of the calcineurin serine/threonine protein phosphatase was previously shown to induce azole tolerance in *C. albicans* (28). Upon azole exposure, increased calcineurin activity enhances the transcription of a number of specific genes involved in plasma membrane maintenance and the stress response, including *RTA2* (33, 34). Since the *vps21*Δ/Δ mutant has abnormal levels of intracellular Ca^{2+}, we investigated the possibility that calcineurin-dependent responses contribute to its azole tolerance. To compare calcineurin-responsive gene expression levels in mutant and control strains, we used a reporter construct consisting of the GFPγ coding sequence (35) under the control of the calcineurin-responsive *RTA2* promoter (34, 36). As expected, treatment with fluconazole enhanced GFP expression in the control strain (Fig. 5A). Furthermore, treatment with the calcineurin inhibitor cyclosporine suppressed fluconazole-induced expression of GFP (see Fig. S4 in the supplemental material), establishing the responsiveness of this reporter construct to calcineurin activity. Interestingly, treatment of the *vps21*Δ/Δ mutant with fluconazole induced a significantly larger response from the *RTA2pr-GFP* reporter than that observed for the WT control strain (Fig. 5A). Finally, inhibition of calcineurin activity using either cyclosporine (Fig. 5B to D) or tacrolimus (FK506) (see Fig. S5 in the supplemental material) was sufficient to abolish the growth of the *vps21*Δ/Δ mutant in the presence of fluconazole. Thus, the ability of the *vps21*Δ/Δ mutant to grow in the presence of the azole antifungals depends upon calcineurin activity.

DISCUSSION

In a previous study, we established that the *C. albicans vps21*Δ/Δ vacuolar trafficking mutant, deficient in transport through the PVC, is able to grow in the presence of fluconazole at 35°C despite ergosterol depletion (9). In contrast, a *C. albicans vps15*Δ/Δ mutant, deficient in retrograde trafficking from the PVC back to the Golgi apparatus (37), as well as two mutants unable to form multivesicular bodies within the PVC (38) are sensitive to fluconazole. Collectively, these data indicate that membrane trafficking through the PVC is an important determinant of the cellular consequences of azole treatment. Here, we report that the *vps21*Δ/Δ mutant releases less cytoplasmic content than do WT strains following fluconazole treatment at 35°C, suggesting that it sustains less membrane damage. We also found that the *vps21*Δ/Δ mutant has abnormally high levels of intracellular Ca^{2+} compared to those of the WT under routine culture conditions. Finally, the mutant's continued growth in the presence of the azoles depends upon the availability of extracellular Ca^{2+} and is abrogated by inhibitors of the calcineurin phosphatase.

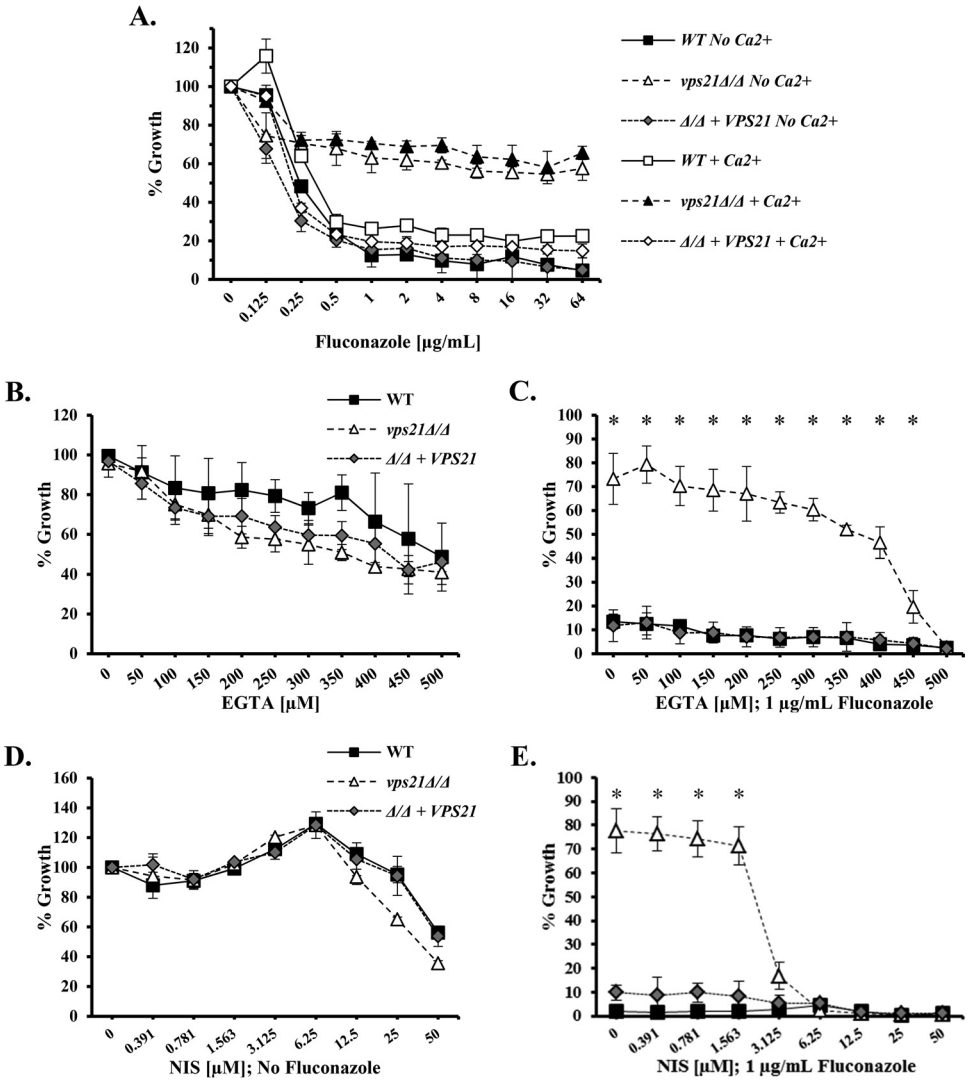

FIG 4 Azole tolerance of the *C. albicans vps21*Δ/Δ mutant depends upon extracellular calcium. (A) The susceptibility of the *C. albicans vps21*Δ/Δ mutant to fluconazole was compared to those of the wild-type (YJB6284) and isogenic reconstituted strains in medium supplemented with 1 mM $CaCl_2$ by using standard CLSI broth microdilution protocols. Growth was measured as the OD_{600} and expressed as a percentage of the values for the minus-drug (DMSO) control wells. (B and C) Comparison of the growth of the *C. albicans vps21*Δ/Δ mutant in the presence of various concentrations of the Ca^{2+} chelator EGTA without (B) and with (C) 1 μg/ml of fluconazole. For each strain, growth is expressed as a percentage of the values for the minus-fluconazole, minus-EGTA control. (D and E) Comparison of the growth of the *vps21*Δ/Δ mutant, wild-type (YJB6284), and reconstituted strains in dose-response experiments with the Ca^{2+} channel blocker nisoldipine (NIS) in the absence (D) or presence (E) of 1 μg/ml fluconazole. The growth of each strain is expressed as a percentage of the growth in the minus-drug (DMSO) control wells. The means ± standard deviations of data from two biological replicates are shown in each panel. The means for each group were compared by using a two-tailed *t* test. *, $P < 0.05$.

The fungal vacuole is the major intracellular storage site of calcium. Accordingly, the transient cytoplasmic Ca^{2+} fluxes that activate calcineurin are regulated by Yvc1p, a channel that releases vacuolar calcium (31), and the Pmc1p and Vcx1p pumps that sequester cytoplasmic calcium into the vacuole (39, 40). As for many integral membrane proteins, Yvc1p, Pmc1p, and Vcx1p are likely delivered from the Golgi apparatus to the vacuole via the PVC (41). Thus, inefficient localization of these calcium transporters to the fungal vacuole in the *vps21*Δ/Δ mutant may underlie the elevated cytoplasmic calcium levels. Abnormal Ca^{2+} homeostasis could potentially increase the basal level of calcineurin activity and/or affect the magnitude or duration of calcineurin signaling in the mutant, in turn enhancing azole tolerance. This is supported by the *RTA2* reporter data, which indicate that the *vps21*Δ/Δ mutant exhibits enhanced expression of calcineurin-responsive genes in the presence of fluconazole. However, in preliminary studies, we have found that the deletion of either the *PMC1* or *VCX1* gene alone is not sufficient to impact the susceptibility of *C. albicans* to fluconazole or induce trailing growth under standard CLSI assay conditions (see Fig. S6 in the supplemental material). Nonetheless, it is clear from our experiments that chemical inhibition of calcineurin is sufficient to abrogate the growth of the *vps21*Δ/Δ mutant in the presence of fluconazole. Interestingly, Liu et al. (42) showed that dihydropyridine treatment suppresses the fluconazole-induced expression of the two calcineurin subunits, sensitizing azole-resistant *C. albicans* isolates to fluconazole. Thus, the

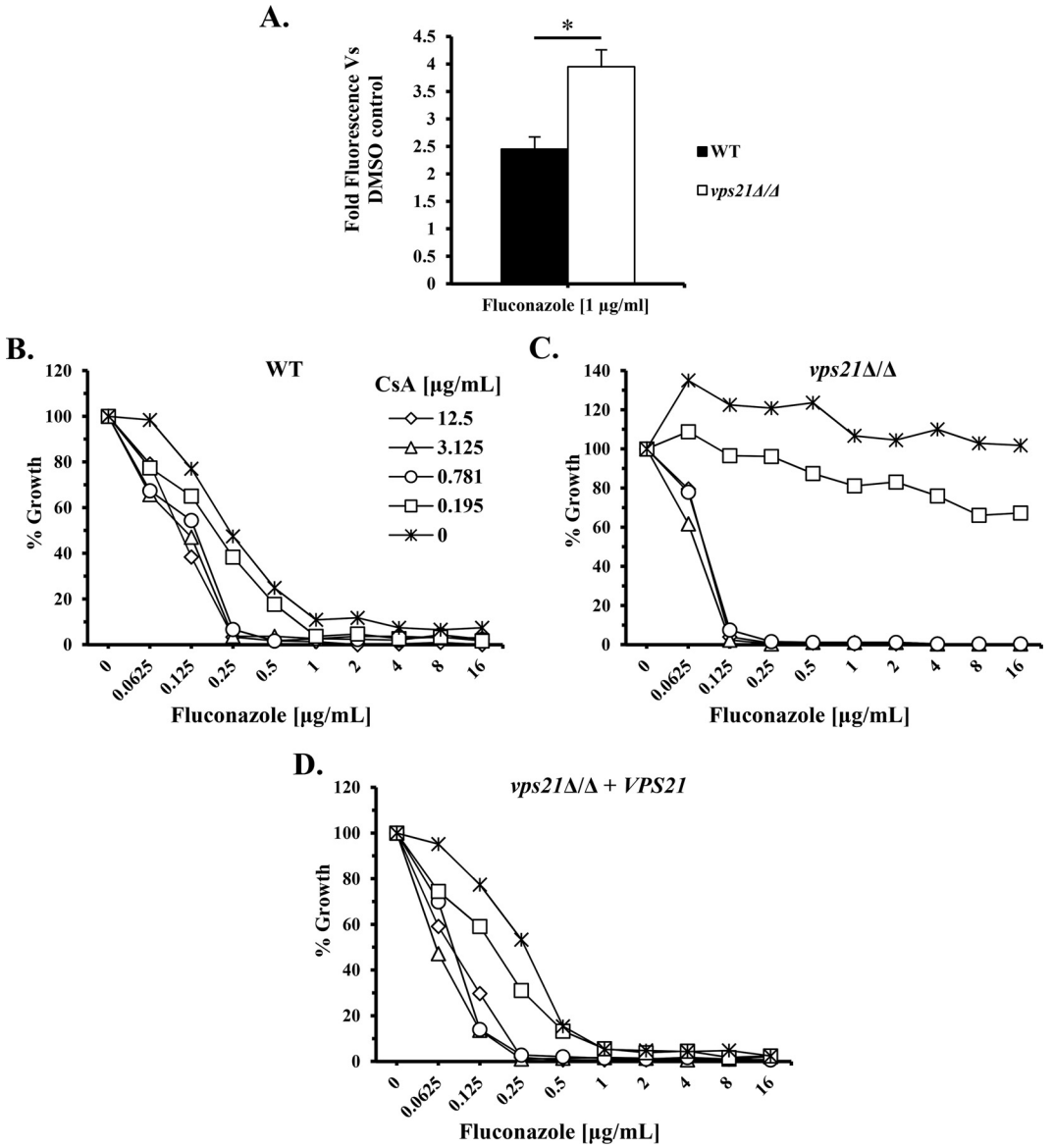

FIG 5 Inhibition of calcineurin signaling suppresses the azole tolerance of the *vps21*Δ/Δ mutant. (A) The *RTA2pr-GFP*γ expression construct was introduced into *C. albicans* strain CAI4 (WT) and the *vps21*Δ/Δ mutant. The resulting strains were grown in YNB with or without 1 μg/ml fluconazole for 24 h before GFP fluorescence was quantified by excitation at 488 nm and emission at 507 nm. The fluorescence intensity was then normalized to the cell density (OD_{600}) for each strain and expressed as fold fluorescence compared to the value for the DMSO control. The means ± standard deviations of data from four biological replicates are shown. The means for each group were compared by using a two-tailed *t* test. *, $P < 0.0004$. (B to D) Comparison of the growth of the *vps21*Δ/Δ mutant, WT (YJB6284), and reconstituted strains in the presence of both the calcineurin activity inhibitor cyclosporine (CsA) and fluconazole using a checkerboard assay. Growth of the WT (B), the *vps21*Δ/Δ mutant (C), and the isogenic control strain (D) was determined as the OD_{600} and expressed as a percentage of the values for the minus-drug (DMSO) control. Data from a representative experiment are shown, but similar results were obtained in a second repeat experiment.

resensitization of the *vps21*Δ/Δ mutant by nisoldipine is also consistent with the mutant's azole tolerance depending upon enhanced calcineurin-dependent responses.

The azole tolerance of the *vps21*Δ/Δ mutant contrasts with data for a previously reported *vps15*Δ/Δ mutant defective in retrograde trafficking from the PVC back to the Golgi apparatus. Despite also exhibiting elevated expression levels of calcineurin-dependent genes, this mutant is highly sensitive to fluconazole (37). The reasons for this are unclear; however, it is apparent that the specific PVC trafficking step affected is a crucial determinant of the physiological impact of the azoles on the fungal cell. In support of this notion, we found that blocking of the AP-3-mediated Golgi apparatus-to-vacuole trafficking route through the deletion of *APS3* (17) is sufficient to eliminate the trailing growth of the *vps21*Δ/Δ mutant (see Fig. S7 in the supplemental material), indicating that its azole tolerance depends upon this pathway. However, the loss of the AP-3-mediated pathway alone has no impact on the azole susceptibility of *C. albicans* (9). Collectively, these findings indicate that complex interactions occur between the PVC membrane-trafficking and ergosterol biosynthetic pathways of *C. albicans*.

Studies performed to date have suggested that the trailing-

growth phenotype is usually associated with *in vivo* susceptibility to azole therapy (43, 44). However, it is unclear how, or if, the azole-tolerant phenotype of the *vps21*Δ/Δ mutant relates to that of trailing clinical isolates or if these clinical isolates have abnormal endosomal trafficking. A first step in establishing the potential clinical relevance of our observations will be to examine the azole susceptibility of the *vps21*Δ/Δ mutant *in vivo* using an appropriate mouse model of infection.

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