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Authors

Liu, Yaoping
Solis, Norma V
Heilmann, Clemens J
et al.

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Role of Retrograde Trafficking in Stress Response, Host Cell Interactions, and Virulence of *Candida albicans*

Yaoping Liu,^a Norma V. Solis,^a Clemens J. Heilmann,^b Quynh T. Phan,^a Aaron P. Mitchell,^c Frans M. Klis,^b Scott G. Filler^{a,d}

Division of Infectious Diseases, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, California, USA^a; Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands^b; Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania, USA^c; David Geffen School of Medicine at UCLA, Los Angeles, California, USA^d

In *Saccharomyces cerevisiae*, the vacuolar protein sorting complexes Vps51/52/53/54 and Vps15/30/34/38 are essential for efficient endosome-to-Golgi complex retrograde transport. Here we investigated the function of Vps15 and Vps51, representative members of these complexes, in the stress resistance, host cell interactions, and virulence of *Candida albicans*. We found that *C. albicans vps15Δ/Δ* and *vps51Δ/Δ* mutants had abnormal vacuolar morphology, impaired retrograde protein trafficking, and dramatically increased susceptibility to a variety of stressors. These mutants also had reduced capacity to invade and damage oral epithelial cells *in vitro* and attenuated virulence in the mouse model of oropharyngeal candidiasis. Proteomic analysis of the cell wall of the *vps51Δ/Δ* mutant revealed increased levels of the Crh11 and Utr2 transglycosylases, which are targets of the calcineurin signaling pathway. The transcript levels of the calcineurin pathway members *CHR11*, *UTR2*, *CRZ1*, *CNA1*, and *CNA2* were elevated in the *vps15Δ/Δ* and *vps51Δ/Δ* mutants. Furthermore, these strains were highly sensitive to the calcineurin-specific inhibitor FK506. Also, deletion of *CHR11* and *UTR2* further increased the stress susceptibility of these mutants. In contrast, overexpression of *CRH11* and *UTR2* partially rescued their defects in stress resistance, but not host cell interactions. Therefore, intact retrograde trafficking in *C. albicans* is essential for stress resistance, host cell interactions, and virulence. Aberrant retrograde trafficking stimulates the calcineurin signaling pathway, leading to the increased expression of Chr11 and Utr2, which enables *C. albicans* to withstand environmental stress.

Candida albicans is a major fungal pathogen that causes both mucosal and hematogenously disseminated infections. In order for *C. albicans* to cause disease, it must be able to withstand host-induced stress and nutrient deprivation and invade and damage host tissues. Studies of *Saccharomyces cerevisiae* have demonstrated that proper endosomal trafficking is critical for diverse cellular functions, including signaling, nutrient uptake, and development. Proteins and lipids can be transported anterograde, from the Golgi complex to the endosome, and retrograde, from the endosome back to the Golgi complex (1, 2). In *C. albicans*, the function of anterograde trafficking pathways in host cell interactions, stress resistance, and pathogenicity has been studied in some detail (3–5). In contrast, less is known about how retrograde protein trafficking influences these processes. In *S. cerevisiae*, retrograde transport is mediated by the vacuolar protein sorting complexes Vps15/30/34/38 and Vps51/52/53/54 (6, 7). The Vps15/30/34/38 complex produces a specific pool of phosphatidylinositol-3-phosphate (PtdIns3P) that is necessary for the recruitment and assembly of the retromer complex, which in turn is required for endosome-to-Golgi complex retrograde transport (8). In contrast, the Vps51/52/53/54 complex interacts directly with small GTPases that localize to the *trans*-Golgi network and mediate retrograde trafficking (9).

Previously, we found that a *C. albicans vps51/vps51* insertion mutant had increased susceptibility to a variety of stressors, including Congo red, SDS, H₂O₂, and the antimicrobial peptide protamine (10). Subsequently, we determined that *vps51Δ/Δ* and *vps53Δ/Δ* mutant strains of *C. albicans* had attenuated virulence in a mouse model of hematogenously disseminated candidiasis (11). Paradoxically, these mutants had increased trafficking to the brain, which was due to increased surface expression of the Als3 invasin (11).

In the present study, we investigated the roles of *C. albicans* Vps15 and Vps51 in response to environmental stress, host cell interactions, and virulence during oropharyngeal infection. We found that retrograde trafficking plays a crucial role in enabling the organism to withstand stress, invade and damage host cells, and cause oropharyngeal candidiasis in mice. Furthermore, impaired endosome-to-Golgi complex retrograde trafficking results in constitutive activation of the calcineurin signaling pathway, which leads to enhanced expression of the Chr11 and Utr2 transglycosylases, a response that is essential for survival and stress resistance.

MATERIALS AND METHODS

Growth conditions. All strains were maintained on YPD agar (1% yeast extract [Difco], 2% peptone [Difco], and 2% glucose plus 1.2% Bacto agar). *C. albicans* transformants were selected on synthetic complete medium (2% dextrose and 0.67% yeast nitrogen base [YNB] with ammonium sulfate and synthetic auxotrophic supplements). The FaDu oral epithelial cell line was obtained from the American Type Culture Collection and maintained in Eagle's minimum essential medium with Earle's balanced salt solution (Irvine Scientific) supplemented with 10% fetal bovine serum, 1 mM pyruvic acid, 2 mM L-glutamine, and 0.1 mM non-essential amino acids, as well as penicillin and streptomycin.

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Address correspondence to Scott G. Filler, sfiller@ucla.edu.

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TABLE 1 *C. albicans* strains used in this study

Strain	Genotype	Source or reference
BWP17	<i>ura3Δ::λimm434/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	12
DAY185	<i>ura3Δ::λimm434/ura3Δ::λimm434 arg4::hisG::ARG4::URA3/arg4::hisG his1::hisG::HIS1/his1::hisG</i>	31
CAI4 mutant	<i>ura3::λimm434/ura3</i>	32
<i>vps15Δ/Δ</i> mutant	<i>vps15Δ::HIS1/vps15Δ::ARG4 RP10::URA3 ura3Δ::λimm434/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	This study
<i>vps15Δ/Δ</i> -complement strain	<i>vps15Δ::HIS1/vps15Δ::ARG4 RP10::URA3::VPS15 ura3Δ::λimm434/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	This study
<i>vps51Δ/Δ</i> mutant	<i>vps51Δ::HIS1/vps51Δ::ARG4 ura3Δ::λimm434::URA3/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	11
<i>vps51Δ/Δ</i> -complement strain	<i>vps51Δ::HIS1/vps51Δ::ARG4 ura3Δ::λimm434::URA3::VPS51/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	11
<i>vps15Δ/Δ-chr11Δ</i> mutant	<i>vps15Δ::HIS1/vps15Δ::ARG4 chr11Δ::URA3 ura3Δ::λimm434::URA3/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	This study
<i>vps15Δ/Δ-utr2Δ</i> mutant	<i>vps15Δ::HIS1/vps15Δ::ARG4 utr2Δ::URA3 ura3Δ::λimm434::URA3/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	This study
<i>vps51Δ/Δ-chr11Δ/Δ</i> mutant	<i>vps51Δ::HIS1/vps51Δ::ARG4 chr11Δ::URA3/chr11Δ::NAT1 ura3Δ::λimm434::URA3/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	This study
<i>vps51Δ/Δ-utr2Δ/Δ</i> mutant	<i>vps51Δ::HIS1/vps51Δ::ARG4 utr2Δ::URA3/utr2Δ::NAT1 ura3Δ::λimm434::URA3/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	This study
<i>vps15Δ/Δ-pTDH3-CRH11</i> mutant	<i>vps15Δ::HIS1/vps15Δ::ARG4 RP10::URA3::pTDH3-CRH11 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	This study
<i>vps15Δ/Δ-pTDH3-UTR2</i> mutant	<i>vps15Δ::HIS1/vps15Δ::ARG4 RP10::URA3::pTDH3-UTR2 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	This study
<i>vps51Δ/Δ-pTDH3-CRH11</i> mutant	<i>vps51Δ::HIS1/vps51Δ::ARG4 RP10::URA3::pTDH3-CRH11 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	This study
<i>vps51Δ/Δ-pTDH3-UTR2</i> mutant	<i>vps51Δ::HIS1/vps51Δ::ARG4 RP10::URA3::pTDH3-UTR2 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	This study

Strain construction. The *C. albicans* strains used in this study are listed in Table 1. All *C. albicans* mutant strains constructed for this study were derived from strain BWP17 (12). Deletion of the entire protein coding regions of both alleles of *VPS15* (orf19.130) was accomplished by successive transformation with *ARG4* and *HIS1* deletion cassettes that were generated by PCR using the oligonucleotides *vps15-ko-f* and *vps15-r* (see Table S1 in the supplemental material). The resulting strain was subsequently transformed with pCIP10-*URA3* (13) to reintegrate *URA3* at the *RP10* locus. To construct the *VPS15* complemented strain (*vps15Δ/Δ*+*VPS15*), a 5.95-kb fragment containing *VPS15* was generated by high-fidelity PCR with the primers *v15-hind-rev-f* and *vps15-kpn-r* (see Table S1), using genomic DNA from *C. albicans* SC5314 as the template. This PCR product was digested with *HindIII* and *KpnI* and then subcloned into pCIP10, which had been linearized with *HindIII* and *KpnI*. The resulting construct was linearized with *StuI* to direct integration to the *RP10* locus of a *ura⁻vps15Δ/Δ* mutant.

To delete the entire protein coding regions of *CHR11* and *UTR2* in the *vps15Δ/Δ* and *vps51Δ/Δ* mutants, *URA3* and *NAT1* deletion cassettes were generated by PCR using the templates pGEM-*URA3* (12) and pJk795 (14) with the primers *crh11-ko-f* and *crh11-ko-r* or *utr2-ko-f* and *utr2-ko-r* (see Table S1 in the supplemental material). The resulting deletion cassettes were used to transform *ura⁻vps51Δ/Δ* and *vps15Δ/Δ* mutants.

To overexpress *CRH11* in the *vps51Δ/Δ* and *vps15Δ/Δ* mutant strains, a 1.5-kb fragment containing the *CRH11* protein coding region was generated by PCR with primers *Crh11-hindIII-f* and *Crh11-xho1-r* (see Table S1 in the supplemental material), using genomic DNA from *C. albicans* SC5314 as the template. The resulting *CRH11* fragment was cloned downstream of the *pTDH3* promoter of pCIP10-TDH3. This plasmid was constructed by PCR amplifying the entire *pTDH3* promoter region with the primers *pTDH3-bglII-f* and *pTDH3-hind-xho-r* (see Table S1), using genomic DNA from *Saccharomyces cerevisiae* as a template, digesting the resulting fragment with *BglII* and *XhoI*, and subcloning it into pCIP10, which had been linearized with *BglII* and *XhoI*. The *CRH11* overexpression plasmid was linearized with *StuI* to direct integration to the *RP10* locus of the *ura⁻vps51Δ/Δ* and *vps15Δ/Δ* mutant strains.

Overexpression of *UTR2* in the *vps51Δ/Δ* and *vps15Δ/Δ* mutant

strains was generated similarly, except that primers *Utr2-hindIII-f* and *Utr2-xho1-r* (see Table S1 in the supplemental material) were used to PCR amplify a 1.6-kb DNA fragment containing *UTR2* for ligation into pCIP10-TDH3.

Vacuolar staining. The vacuolar morphology of the *C. albicans* strains was visualized by pulse-chase staining with FM4-64 as described previously (10). Briefly, each strain was grown to the log phase in YPD broth at 30°C. The cells were harvested by centrifugation and resuspended in YPD broth, after which FM4-64 (Invitrogen) was added to a final concentration of 25 μM. The cells were incubated at 30°C for 30 min and then harvested by centrifugation. They were resuspended in fresh YPD broth and incubated for an additional 90 min. During the last 60 min of incubation, a polyclonal anti-*C. albicans* antibody (Biodesign International) conjugated with Alexa Fluor 488 (Molecular Probes) was added to the medium to label the cell surface of the organisms. Next the cells were rinsed once in phosphate-buffered saline, resuspended in YNB broth (0.17% YNB, 2% glucose), and imaged by confocal microscopy.

Kar2 secretion assays. To determine if there was impaired retrograde protein trafficking in the various mutants, their secretion of Kar2 was determined (15). Cells were grown overnight at 30°C in 50 ml YNB-S medium: 6.7 g/liter yeast nitrogen base, 20 g/liter sucrose, and 75 mM MOPS [3-(*N*-morpholino)-2-hydroxypropanesulfonic acid] set to pH 7.4. The culture supernatants were collected by centrifugation, and equivalency in the growth of the different *C. albicans* strains was verified by the size of the pellets. The proteins in supernatants were concentrated to 50 μl using an Amicon centrifugal filter (Millipore). The concentrated proteins were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and analyzed by immunoblotting using an anti-Kar2 antibody (Santa Cruz Biotechnology).

Susceptibility to stressors. The susceptibilities of the various *C. albicans* strains to Congo red (300 mg/ml), H₂O₂ (7.5 mM), SDS (0.01%), MnCl₂ (7.5 mM), CuSO₄ (5 mM), protamine (2 mg/ml), fluconazole (12 μg/ml), and FK506 (25 ng/ml) were tested by spot assays. In the experiment using the double deletion mutants (e.g., *vps15Δ/Δ chr11Δ* and *vps51Δ/Δ chr11Δ/Δ* strains), we used slightly lower concentrations of Congo red (200 mg/ml) and protamine (1.8 mg/ml) to detect differences

in susceptibilities between the single and double mutants. In all experiments, the organisms were grown overnight in YPD broth at 30°C, after which serial 10-fold dilutions were spotted onto YPD agar plates containing the various stressors. The plates were incubated for 64 h at 30°C and then imaged. Each experiment was performed in duplicate.

Cell wall purification. The cell wall isolation, protein extraction, and proteomic analysis with liquid chromatography-tandem mass spectrometry (LC-MS/MS) were performed as described previously (16). Briefly, cells were grown in 50 ml YNB-S medium buffered at pH 7.4 at 30°C for 18 h to the early stationary phase from a starting optical density at 600 nm (OD_{600}) of 0.05. After the cells were harvested by centrifugation, the *C. albicans* cells were disrupted in a BeadBeater (BioSpec Products) using 0.25- to 0.50-mm glass beads in the presence of a protease inhibitor cocktail. To remove noncovalently linked proteins and intracellular contaminants, isolated cell walls were washed extensively with 1 M NaCl followed by washing with Milli-Q water. The resulting pellets were boiled four times for 10 min in fresh SDS extraction buffer, washed several times with Milli-Q water, and lyophilized overnight. After treatment of the wall pellets with reducing solution (10 mM dithiothreitol in 100 mM NH_4HCO_3) for 1 h at 55°C followed by alkylation (65 mM iodoacetamide in 100 mM NH_4HCO_3) for 45 min at room temperature in the dark and subsequent quenching of the reaction by addition of 55 mM dithiothreitol–100 mM NH_4HCO_3 for 5 min, the cells were thoroughly washed with NH_4HCO_3 and either stored at –80°C or directly trypsinized for mass spectrometry.

Mass spectrometric analysis of the cell wall proteome. Sample preparation for MS analysis was performed as outlined previously (16, 17). After treatment of 4 mg of freeze-dried cell wall pellet with 2 μ g Trypsin Gold (Promega) for 18 h, the resulting peptide mix was desalted using a C_{18} tip column (Varian). Next, 250 ng of the desalted peptides in 10 μ l 0.1% trifluoroacetic acid (TFA) was injected into an Ultimate 2000 nano-high-performance liquid chromatography (nano-HPLC) system (LC Packings) equipped with a PepMap100 C_{18} reversed-phase column (75- μ m inner diameter, 25-cm length; Dionex). The peptides were separated and eluted along a linear acetonitrile gradient, directly ionized via electrospray, and then introduced into a quadrupole time of flight (Q-TOF) mass spectrometer (Micromass). After acquisition of all mass spectra, a peak list (pkl) was generated using the MaxEnt3 algorithm in the Biolynx and Masslynx Peptide software. Proteins were identified by submitting the pkl files to an internally licensed version of MASCOT (Matrix Science), searching against a complete open reading frame (ORF) translation of the *C. albicans* genome. In the MASCOT analysis, two miscleavages and a tolerance of 0.5 Da for peptides and MS/MS were allowed. Based on probabilistic MASCOT scoring, a *P* value of <0.05 was considered significant for peptide identification. Three independently obtained biological samples (the biological replicates) were analyzed for each strain. Each biological sample was subjected to two MS/MS runs (the technical replicates). For a semiquantitative analysis of our data, we calculated for each growth condition the percent peptide identifications. For each biological replicate, the number of peptide identifications per protein was divided by the total number of identified peptides in the complete run. The percent peptide counts were averaged for each strain. The fold changes for the respective proteins were then calculated.

mRNA analyses. The mRNA levels of calcineurin-related genes (*CNA1*, *CNB1*, *CRZ1*, *UTR2*, and *CRH11*) were measured by real-time PCR using the primers listed in Table S1 in the supplemental material. Samples of exponentially growing *C. albicans* cells (OD_{600} , 0.5) were collected by centrifugation at 4°C. The fungal RNA was extracted by the hot phenol method (10). For real-time PCR, cDNA was prepared using RETROscript kit (Life Technologies) following the manufacturer's protocol. Real-time PCR was performed in duplicate in an optical 96-well plate using the Power SYBRGreen PCR master mix (Life Technologies). The calcineurin target gene mRNA expression levels were analyzed using the cycle threshold ($2^{-\Delta\Delta CT}$) method with *ACT1* as the endogenous control (18).

Endocytosis assay. The endocytosis of the various *C. albicans* mutants by oral epithelial cells was determined by our standard differential fluorescence assay, as described previously (19, 20). Briefly, FaDu cells were grown on fibronectin-coated glass coverslips and infected with 10^5 cells of *C. albicans* in RPMI 1640 medium. After incubation for 90 min, the cells were rinsed twice with phosphate-buffered saline (PBS) and then fixed in 3% paraformaldehyde. The nonendocytosed organisms were stained with an anti-*C. albicans* rabbit serum (Biodesign International) conjugated with Alexa Fluor 568 (Invitrogen). After being rinsed extensively with PBS, the FaDu cells were permeabilized with 0.05% (vol/vol) Triton X-100 in PBS. Next, the cell-associated organisms (the endocytosed plus nonendocytosed organisms) were stained with the anti-*C. albicans* rabbit serum conjugated with Alexa Fluor 488 (Invitrogen). Finally, the coverslips were viewed with an epifluorescence microscope. The number of organisms endocytosed by the FaDu cells was determined by subtracting the number of cell-associated organisms (labeled with Alexa Fluor 568) from the total number of organisms (labeled with Alexa Fluor 488). At least 100 organisms were counted on each coverslip, and all experiments were performed in triplicate.

Damage assay. The extent of damage to FaDu oral epithelial cells caused by the various *C. albicans* strains was measured using a ^{51}Cr release assay as described previously (19, 20). The FaDu cells were incubated overnight with $Na_2^{51}CrO_4$ (MP Biomedicals, Inc.) in a 96-well tissue culture plate. The following day, the unincorporated tracer was removed by rinsing, and the cells were infected with 5×10^5 organisms in RPMI 1640 per well. To measure the spontaneous release of ^{51}Cr , uninfected host cells were exposed to medium alone. After a 3-h incubation, the amounts of ^{51}Cr released into the medium and remaining in the cells were determined by gamma counting. The amount of ^{51}Cr released by epithelial cells infected with the various *C. albicans* strains was compared with the amount of ^{51}Cr released by uninfected host cells to calculate the infection-specific release of ^{51}Cr . Each experiment was performed in triplicate on at least three separate occasions.

Mouse model of oropharyngeal candidiasis. The virulence of the different *C. albicans* strains was assessed using the mouse model of oropharyngeal candidiasis as described previously (19, 21, 22). Briefly, eight male BALB/c mice per strain of *C. albicans* were immunosuppressed by subcutaneous injection with 225 mg/kg of cortisone acetate (Sigma-Aldrich) on days –1, +1, and +3 relative to the day of infection. On the day of infection, each mouse was anesthetized and inoculated sublingually for 75 min with a swab saturated with 10^6 *C. albicans* cells per ml of Hanks' balanced salt solution (HBSS). After 5 days of infection, each mouse was sacrificed, the tongue was excised, weighed, and homogenized, and the number of CFU was determined. The animal experiments were approved by the Animal Care and Use Committee at the Los Angeles Biomedical Research Institute.

Statistical analyses. Differences among the interactions of the various strains of *C. albicans* with the FaDu oral epithelial cell line were compared using the Student *t* test. Differences in the fungal burden of mice infected with these strains were analyzed using the Wilcoxon rank sum test. *P* values of ≤ 0.05 were considered significant.

RESULTS

C. albicans vps15 Δ/Δ and vps51 Δ/Δ mutants exhibit abnormal vacuolar morphology and impaired retrograde trafficking. In *Saccharomyces cerevisiae*, the vacuolar protein sorting complexes Vps15/30/34/38 and Vps51/52/53/54 are essential for maintenance of vacuolar morphology and retrograde endosome-to-Golgi complex transport (6–9). To examine how the absence of Vps15 or Vps51 affects vacuolar morphology, *vps15 Δ/Δ* and *vps51 Δ/Δ* mutants were labeled with FM4-64, which specifically stains the vacuolar membranes (23). These two mutants exhibited different defects in vacuolar morphology. Compared to wild-type cells, the *vps15 Δ/Δ* cells had very large, single vacuoles (Fig. 1A).

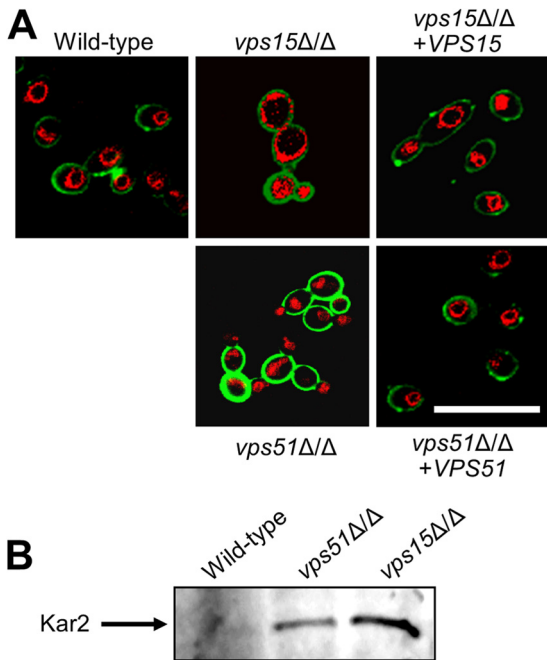


FIG 1 Deletion of either *VPS15* or *VPS51* results in aberrant vacuolar morphology and impaired retrograde endosome to Golgi complex protein trafficking. (A) Vacuoles of the indicated strains were visualized by pulse-chase staining with FM4-64, which fluoresces red. The cell walls were counterstained with an Alexa Fluor-labeled anti-*C. albicans* antibody, which fluoresces green. Scale bar, 5 μ m. (B) Immunoblot of concentrated culture medium in which the indicated *C. albicans* strains were grown. The blot was probed with an anti-Kar2 antibody.

In contrast, the vacuoles of the *vps51* Δ/Δ mutant were small and fragmented, as reported previously (10). These vacuolar defects were rescued when the *vps15* Δ/Δ and *vps51* Δ/Δ mutants were complemented with intact copies of *VPS15* and *VPS51*, respectively. These observations demonstrate that Vps15 and Vps51 are necessary for normal vacuolar morphology in *C. albicans*.

To determine if Vps15 and Vps51 are required for retrograde protein transport in *C. albicans*, we examined whether the *vps15* Δ/Δ and *vps51* Δ/Δ mutants secreted Kar2 (BiP). This endoplasmic reticulum (ER)-resident protein normally chaperones other proteins to the Golgi complex and back. However, when retrograde transport is defective, Kar2 is secreted (24, 25). In immunoblotting experiments, we found that wild-type *C. albicans* did not secrete Kar2 into the medium, whereas both the *vps15* Δ/Δ and *vps51* Δ/Δ mutants secreted this protein (Fig. 1B). The accumulation of Kar2 in the medium was not due to cell lysis, because no *C. albicans* Act1 was detected in the medium (data not shown). These findings indicate that deletion of either *VPS15* or *VPS51* disrupts retrograde protein trafficking in *C. albicans*.

***VPS15* and *VPS51* are necessary for normal stress resistance in *C. albicans*.** Next, to determine if impaired retrograde trafficking alters the ability of *C. albicans* to withstand environmental stress, we tested the susceptibility of the *vps15* Δ/Δ and *vps51* Δ/Δ mutants to a range of stressors. Both mutants had significantly increased susceptibility to Congo red, H₂O₂, SDS, Mn²⁺, Cu²⁺, fluconazole, and the antimicrobial peptide protamine (Fig. 2). This increased susceptibility was reversed when the *vps15* Δ/Δ and *vps51* Δ/Δ mutants were complemented with wild-type copies of

VPS15 and *VPS51*, respectively. Thus, proper retrograde trafficking is necessary for *C. albicans* to resist many different types of stressors.

Impaired retrograde trafficking alters the cell wall proteome. The impaired stress resistance of the *vps51* Δ/Δ mutant and its altered organ tropism during disseminated infection (11) suggested that defective retrograde protein trafficking alters the cell wall proteome. To investigate this possibility, we used mass spectrometry to compare the cell wall proteome of wild-type *C. albicans* with that of the *vps51* Δ/Δ mutant. Four cell wall proteins had at least a 2-fold increase in abundance in the *vps51* Δ/Δ mutant compared to the wild-type strain (Table 2). All of these cell wall proteins, except Als3, are known to be induced by exposure of *C. albicans* to the cell wall-perturbing agent Congo red (16). In addition, Sap9 and Sod5, which have been shown to be increased under a variety of surface stress conditions, were detected exclusively in the cell wall of the mutant strain. This similarity supports the model that impaired retrograde trafficking induces cell wall stress, which in turn stimulates the expression of cell wall proteins to counteract this stress.

Activation of the calcineurin pathway targets Crh11 and Utr2 is a key compensatory response in *C. albicans* mutants with impaired retrograde trafficking. The cell wall of the *vps51* Δ/Δ mutant was highly enriched for Crh11 and Utr2 compared to the cell wall of the wild-type strain (Table 2). In the proteomic investigation of the *vps51* Δ/Δ mutant, approximately 20% of the total peptides analyzed were from Crh11 and Utr2. In contrast, peptides from these two proteins accounted for only 9% of the total peptides isolated from the wild-type strain. Crh11 and Utr2 are known targets of the calcineurin stress response pathway (26). Thus, we hypothesized that this pathway is activated as a compensatory response to impaired retrograde trafficking. To investigate this hypothesis, we first analyzed the mRNA levels of the subunits of the calcineurin heterodimer, Cna1 and Cnb1, the Crz1 transcription factor, and the Crh11 and Utr2 transglycosylases in the *vps15* Δ/Δ and *vps51* Δ/Δ mutants. All of these calcineurin-related genes, especially *CRZ1*, *CHR11*, and *UTR2*, were upregulated in both mutants (Fig. 3A). Next, we tested the effects of FK506, a specific calcineurin inhibitor (27), on the growth of the *vps15* Δ/Δ and *vps51* Δ/Δ mutants. Although FK506 had little effect on the growth of the wild-type strain, it completely inhibited the growth of the *vps15* Δ/Δ and *vps51* Δ/Δ mutants (Fig. 3B). These results demonstrated that calcineurin activity is critical for the growth of these mutants.

In *C. albicans*, *CRH11*, *CRH12*, and *UTR2* are predicted to specify members of the CRH family of (chitin) transglycosylases. *CRH11* and *UTR2*, but not *CRH12*, are targets of the calcineurin signaling pathway (26). To explore whether downstream components of the calcineurin signaling pathway were required for the growth and stress resistance of the *vps15* Δ/Δ and *vps51* Δ/Δ mutants, we attempted to delete both alleles of *CHR11* and *UTR2* in these strains. Despite numerous attempts, we were unable to delete both copies of *CHR11* and *UTR2* in the *vps15* Δ/Δ mutant, suggesting that either deletion of these genes in the *vps15* Δ/Δ mutant is synthetically lethal, or the resulting mutants cannot grow under the conditions used to select for transformants. Therefore, we analyzed a *vps15* Δ/Δ mutant that lacked a single copy of either *CHR11* or *UTR2*. We found that the *vps15* Δ/Δ *chr11* Δ and *vps15* Δ/Δ *utr2* Δ mutants had even greater susceptibility to Congo red, H₂O₂, SDS, Mn²⁺, and protamine than did the *vps15* Δ/Δ

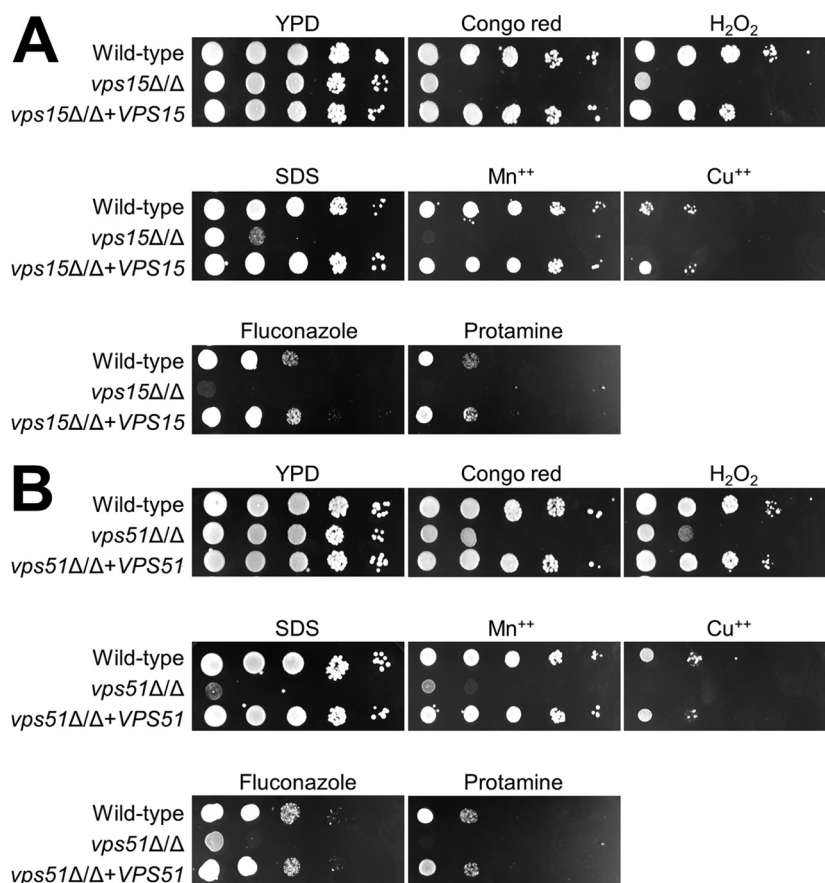


FIG 2 *C. albicans* *vps15* Δ/Δ and *vps51* Δ/Δ mutants have increased susceptibility to a variety of stressors. The *vps15* Δ/Δ (A) and *vps51* Δ/Δ (B) mutants, as well as their respective control strains, were incubated at 30°C on YPD agar plates containing the indicated stressors and imaged after 64 h.

mutant (Fig. 4A). In contrast, we were successful in deleting both alleles of *CHR11* and *UTR2* in the *vps51* Δ/Δ mutant. The resulting *vps15* Δ/Δ *chr11* Δ/Δ and *vps15* Δ/Δ *utr2* Δ/Δ double mutants were more susceptible to Congo red, H₂O₂, and SDS than the *vps51* Δ/Δ mutant (Fig. 4B). However, these double mutants were not more susceptible to Mn²⁺ or protamine.

To further ascertain the role of Crh11 and Utr2 in the stress resistance of *C. albicans* mutants with impaired retrograde trafficking, we overexpressed *CRH11* and *UTR2* in the *vps51* Δ/Δ and *vps15* Δ/Δ mutants and tested the capacity of these strains to resist different stressors. Overexpression of *CRH11* and *UTR2* partially restored resistance to Congo red, H₂O₂, SDS, Mn²⁺, and protamine (Fig. 5). These results further demonstrate that increased expression of Crh11 and Utr2 can enhance cell wall integrity and increase the stress resistance of the *vps15* Δ/Δ and *vps51* Δ/Δ mutants.

Retrograde trafficking is required for maximal *C. albicans* invasion of and damage to oral epithelial cells. Next, we investigated the capacity of the *vps15* Δ/Δ and *vps51* Δ/Δ mutants to invade and damage the FaDu oral epithelial cell line *in vitro*. Both of these mutants had a least a 50% reduction in epithelial cell invasion, compared to the wild-type strain (Fig. 6A and B). Furthermore, in contrast to the wild-type strain, these mutants caused virtually no damage to the oral epithelial cells (Fig. 6C and D). Overexpression of *CRH11* and *UTR2* in the *vps15* Δ/Δ and *vps51* Δ/Δ mutants did not rescue their epithelial cell invasion and

damage defects (data not shown). Collectively, these results indicate that retrograde trafficking is necessary for normal invasion of and damage to oral epithelial cells *in vitro*. In addition, although Chr11 and Utr2 contribute to stress resistance when retrograde trafficking is impaired, they do not appear to influence host cell invasion or damage.

Retrograde trafficking is essential for full virulence in the murine model of oropharyngeal candidiasis. The impaired capacity of the *vps15* Δ/Δ and *vps51* Δ/Δ mutants to withstand stress and invade and damage oral epithelial cells *in vitro* suggested that these mutants might have impaired virulence during oropharyngeal infection. This hypothesis was tested using a mouse model of oropharyngeal candidiasis. We found that the oral fungal burden of mice infected with either the *vps15* Δ/Δ or *vps51* Δ/Δ mutant was approximately 6,000-fold lower than that of mice infected with the wild-type or complemented strains (Fig. 7). Therefore, normal retrograde trafficking is essential for maximal virulence during oropharyngeal candidiasis.

DISCUSSION

In *S. cerevisiae*, the Vps15/30/34/38 and Vps51/52/53/54 complexes are known to mediate retrograde protein trafficking from the endosome to the Golgi complex (6–9). The present results suggest that *C. albicans* Vps15 and Vps51 function similarly to their *S. cerevisiae* homologs. For example, the *C. albicans* *vps15* Δ/Δ mutant had a large single vacuole, and *S. cerevisiae* mu-

TABLE 2 Changes in the cell wall proteome of the *vps51Δ/Δ* mutant versus the wild-type strain

Parameter and protein	Avg % peptide in:			Function(s) ^a
	Wild type	<i>vps51Δ/Δ</i> mutant	Fold change	
≥2-fold increase in <i>vps51Δ/Δ</i> mutant vs wild type				
Als3	0.7	2.5	3.4	Adhesin, invasins, ferritin binding
Phr2	2.2	6.1	2.8	Glycosyl hydrolase family 7, expressed at acidic pH
Crh11	5.2	11.7	2.3	Predicted chitin transglycosylase, calcineurin regulated
Utr2	3.8	8.4	2.2	Predicted chitin transglycosylase, calcineurin regulated
≥2-fold decrease in <i>vps51Δ/Δ</i> mutant vs wild type				
Sod4	5.3	0.4	12.9	Yeast-associated superoxide dismutase
Cht2	11.5	3.8	3.1	Chitinase
Ssr1	8.2	3.4	2.4	β-Glucan-associated Ser/Thr-rich cell wall protein with role in cell wall structure
Mp65	6.7	3.0	2.2	Cell-wall glucan metabolism, adhesin
Ecm33	20.8	9.5	2.2	Involved in cell wall organization
<2-fold difference				
Rbt5	4.4	2.3	1.9	Heme-binding CFEM protein, adhesin
Als4	6.0	3.4	1.8	Adhesin
Pga7	0.7	1.1	0.6	CFEM protein, biofilm formation
Phr1	8.6	10.7	1.3	Glycosyl hydrolase family 72, expressed at basic to neutral pH
Ywp1	5.2	6.2	1.2	Yeast-associated wall protein, may promote dispersal of yeast-form cells
Pga4	6.6	5.6	1.2	Glycosyl hydrolase family 72
Pir1	2.3	2.3	1.0	Non-GPI cell wall protein, cell wall organization
Exclusively detected in:				
Wild type				
Pra1	1.9	ND ^b		Adhesin, zinc binding
<i>vps51Δ/Δ</i> mutant				
Als family	ND	1.5		Family peptide
Als1	ND	2.6		Adhesin
Als2	ND	0.3		Adhesin
Phr family	ND	2.3		Family peptide
Sap9	ND	9.2		GPI-anchored secreted aspartyl protease
Sod5	ND	2.7		Hyphal-associated superoxide dismutase
Ecm331	ND	0.4		Unknown function, mainly membrane-localized
Yps7	ND	0.4		Aspartic endopeptidase, degrades α pheromone

^a All proteins presented here are glycosylphosphatidylinositol (GPI) proteins, unless otherwise indicated.

^b ND, not detected.

tants that lack members of the Vps15/30/34/38 complex also have enlarged vacuoles (8). In addition, the fragmented vacuole of *C. albicans vps51Δ/Δ* mutant cells is similar to the fragmented vacuole seen in an *S. cerevisiae vps51Δ* mutant (28). Furthermore, the *C. albicans vps15Δ/Δ* and *vps51Δ/Δ* mutants both secreted Kar2 into the medium, demonstrating that Vps15 and Vps51 are required for normal retrograde protein trafficking.

The *C. albicans* mutants with defective retrograde trafficking had a marked increase in susceptibility to a diverse panel of stressors. Deletion of *VPS15* and *VPS51* in *S. cerevisiae* also results in increased susceptibility to many different stressors (www.yeastgenome.org/). However, deletion of *VPS15* and *VPS51* has different effects on the stress susceptibility of *C. albicans* compared to *S. cerevisiae*. For instance, we found that both the *C. albicans vps15Δ/Δ* and *vps51Δ/Δ* mutants had increased susceptibility to fluconazole. In contrast, only the *S. cerevisiae vps15Δ* mu-

tant and not the *vps15Δ/Δ* mutant has increased susceptibility to this drug (29). These differences suggest that defective retrograde trafficking has somewhat different effects in *C. albicans* than in *S. cerevisiae*. Furthermore, the markedly increased susceptibility of the *C. albicans vps15Δ/Δ* and *vps51Δ/Δ* mutants to fluconazole suggests that pharmacologic inhibition of retrograde trafficking may be promising approach for potentiating the activity of azole antifungal agents.

The analysis of the cell wall proteome of the *C. albicans vps15Δ/Δ* and *vps51Δ/Δ* mutants showed a striking enrichment in proteins involved in cell wall organization and remodeling. Many of these proteins are also increased in the cell wall of *C. albicans* cells exposed to Congo red, Calcofluor white (16), or heat stress (17). Interestingly, exposure to fluconazole alters the cell wall proteome similarly to Congo red (16), indicating that plasma membrane stress may cause a cell wall stress response. Collectively,

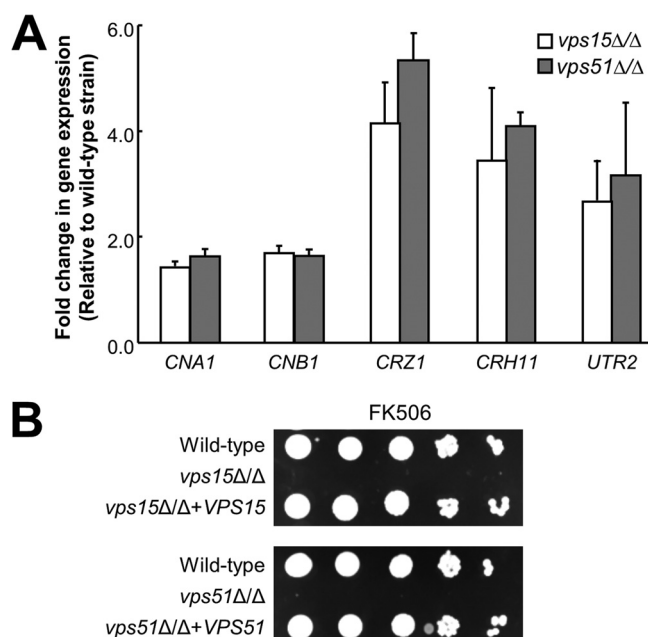


FIG 3 Activation of the calcineurin pathway in *vps15Δ/Δ* and *vps51Δ/Δ* mutants is necessary for viability. (A) Real-time PCR analysis of mRNA levels of the indicated calcineurin-related genes in the *vps15Δ/Δ* and *vps51Δ/Δ* mutants. Results are expressed as the fold change relative to the wild-type strain and are the means \pm standard deviations (SD) of 2 independent experiments. (B) Increased susceptibility of the *vps15Δ/Δ* and *vps51Δ/Δ* mutants to the calcineurin-specific inhibitor FK506. The indicated strains were incubated at 30°C on YPD agar plates containing 25 ng/ml FK506 and imaged after 64 h.

these results suggest that impaired retrograde protein trafficking induces cell wall and/or cell membrane stress in *C. albicans*.

The transglycosylases Crh11 and Utr2, which were enriched in the cell walls of the *vps15Δ/Δ* and *vps51Δ/Δ* mutants, are targets of the calcineurin pathway (26). In *C. albicans*, the calcineurin protein phosphatase governs a number of physiological processes, including cell wall biosynthesis and tolerance to fluconazole (26, 30). Several lines of evidence indicate that not only was the calcineurin pathway activated in the *vps15Δ/Δ* and *vps51Δ/Δ* mutants, but activation of this pathway was required for these mutants to withstand environmental stress. First, transcript levels of multiple genes (*CNA1*, *CNB1*, *CRZ1*, *CHR11*, and *UTR2*) whose products function in the calcineurin pathway were increased in these mutants. Second, Chr11 and Utr2 protein levels were increased in the cell wall of the *vps51Δ/Δ* mutants. Third, both the *vps15Δ/Δ* and *vps51Δ/Δ* mutants were extremely sensitive to the calcineurin inhibitor FK506. Fourth, deletion of either a single copy of *CHR11* or *UTR2* in the *vps15Δ/Δ* mutant or both copies of these genes in the *vps51Δ/Δ* mutant further accentuated the stress resistance defects of these strains. Finally, overexpression of either *CHR11* or *UTR2* in the *vps15Δ/Δ* and *vps51Δ/Δ* mutants partially reversed their hypersusceptibility to stressors. These results suggest the model that impaired retrograde trafficking results in impaired cell wall integrity, leading to activation of the calcineurin signaling pathway. Increased calcineurin signaling enhances the expression of the Chr11 and Utr2 transglycosylases, which results in cell wall remodeling and partially compensates for the cell wall defects induced by impaired retrograde trafficking.

Of note, in *S. cerevisiae*, deletion of *VPS15* does not result in increased susceptibility to FK506 (the *vps51Δ/Δ* deletion mutant has not been tested) (29). Therefore, the calcineurin pathway is not essential to compensate for impaired retrograde trafficking in this yeast, as it is in *C. albicans*.

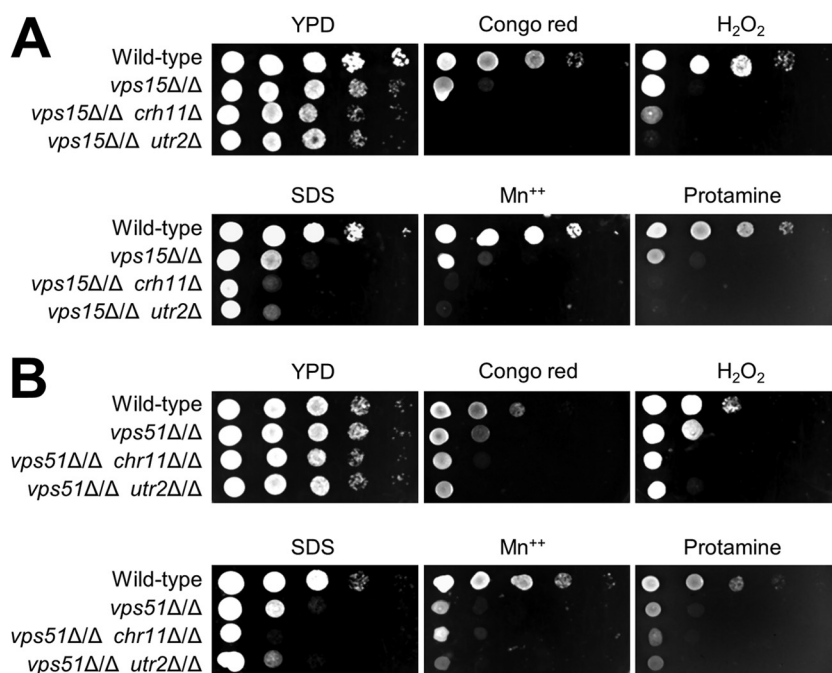


FIG 4 Deletion of *CRH11* and *UTR2* in the *vps15Δ/Δ* (A) and *vps51Δ/Δ* (B) mutants results in increased susceptibility to a variety of stressors. The indicated strains were incubated on YPD plates containing the various stressors at 30°C and imaged after 64 h.

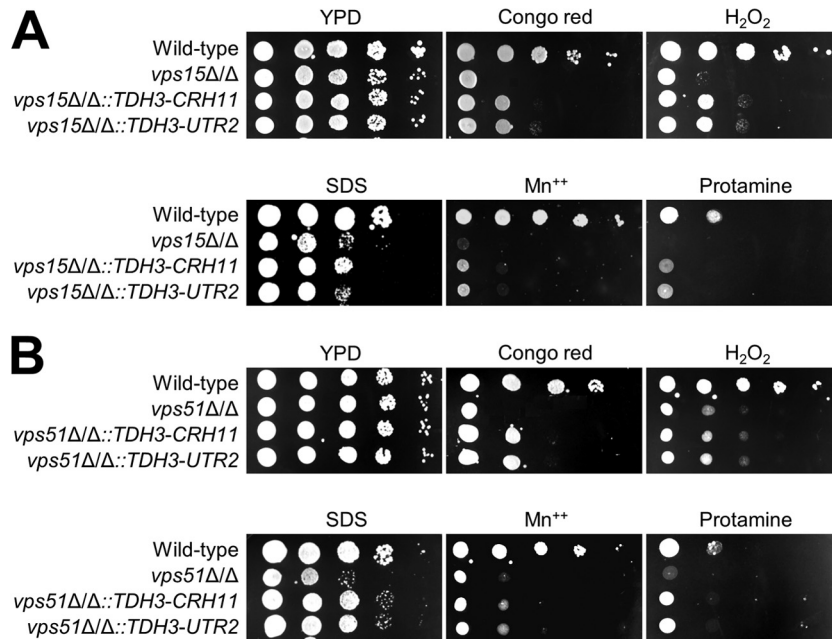


FIG 5 Overexpression of *CRH11* and *UTR2* in the *vps15Δ/Δ* (A) and *vps51Δ/Δ* (B) mutants partially restores resistance to environmental stress. The indicated strains were incubated on YPD plates containing the various stressors at 30°C and imaged after 64 h.

Previously, we found that a *vps51/vps51* insertion mutant has decreased capacity to damage an oral epithelial cell line *in vitro* (10). In the present study, we verified this result with a *vps51Δ/Δ* deletion mutant and also determined that the *vps15Δ/Δ* mutant had a similar host cell damage defect. In addition, we found that the *vps15Δ/Δ* and *vps51Δ/Δ* mutants had significantly reduced invasion of oral epithelial cells, which likely contributed to their

impaired capacity to cause epithelial cell damage. It is probable that the altered cell wall proteome of these mutants caused by aberrant retrograde trafficking accounted for their host interaction defects. Although overexpression of *CHR11* or *UTR2* partially reversed the hypersusceptibility of the *vps15Δ/Δ* and *vps51Δ/Δ* mutants to environmental stressors, it did not rescue their host cell interaction defects. Thus, the compensatory effects of *Chr11* and *Utr2* on cell wall integrity are insufficient to restore the capacity of these mutants to invade and damage epithelial cells.

As predicted by their *in vitro* defects in stress response and host cell interactions, both the *vps15Δ/Δ* and *vps51Δ/Δ* mutants had severely attenuated virulence in the mouse model of oropharyngeal candidiasis. Previously, we found that the *vps51Δ/Δ* mutant had reduced virulence in the mouse model of disseminated candidiasis, although it did have increased trafficking to the brain (11). Collectively, these data demonstrate that proper retrograde

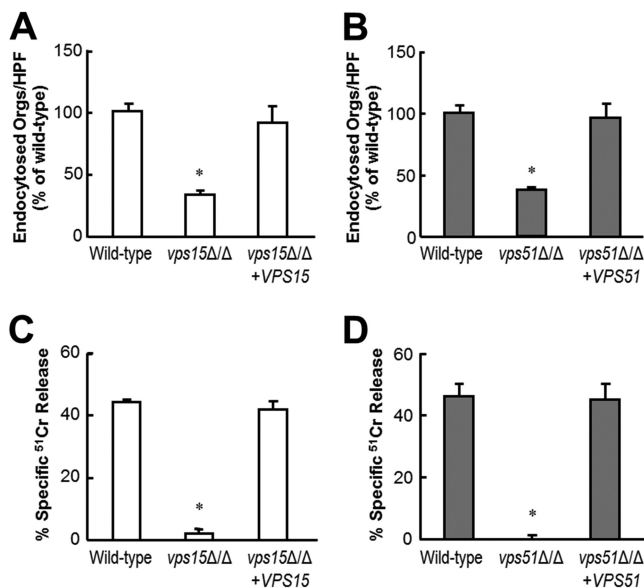


FIG 6 Retrograde trafficking is required for maximal invasion of and damage to oral epithelial cells. (A and B) FaDu epithelial cells were incubated with the indicated strains for 2 h, after which the number of endocytosed organisms was determined. (C and D) Extent of FaDu oral epithelial cell damage caused by the indicated strains after 3 h. Results are means \pm SD of 3 experiments, each performed in triplicate. *, $P < 0.001$ compared to control strains.

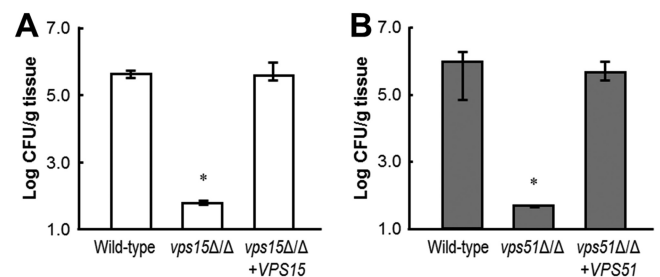


FIG 7 *C. albicans vps15Δ/Δ* and *vps51Δ/Δ* mutants have attenuated virulence in the mouse model of oropharyngeal. The oral fungal burden of mice infected with the *vps15Δ/Δ* (A) and *vps51Δ/Δ* (B) mutants and their respective control strains was measured after 5 days of infection. Results are the medians \pm interquartile ranges of 7 mice per strain. *, $P < 0.01$ compared to control strains.

trafficking is essential for the maximal virulence of *C. albicans* during both mucosal and disseminated infections.

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