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


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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.

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 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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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 BWPl7 (12). Deletion of the entire protein coding regions of both alleles of VPS15 (orf19.130) was accomplished by successive transformation with ARG4 and HIS11 deletion cassettes that were generated by PCR using the oligonucleotides VPS15-XhoI-f and VPS15-XhoI-r (see Table S1) as the template. The resulting strain was successively transformed with plasmid pCIp10-URA3 (13) to reintegrate URA3 at the RP10 locus. To construct the VPS15 complemented strain (VPS15/Δ + VPS15), a 5.95-kb fragment containing VPS15 was generated by high-fidelity PCR with the primers vps15-HindIII-f and vps15-KpnI-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 Stul to direct integration to the RP10 locus of a Ura+ strain (12). 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 plk795 (14) with the primers chr11-k0-f and chr11-k0-r or utr2-k0-f and utr2-k0-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 CHR11 in the vps51Δ/Δ and vps15Δ/Δ mutant strains, a 1.5-kb fragment containing the CHR11 protein coding region was generated by PCR with primers Chr11-hindIII-f and Chr11-hindII-xho1-r (see Table S1 in the supplemental material), using genomic DNA from C. albicans SC5314 as the template. The resulting CHR11 fragment was cloned downstream of the pTD3 promoter of pCIp10-TD3H3. This plasmid was constructed by PCR amplifying the entire TD3 promoter region with the primers pTD3-hgll-f and pTD3-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 CHR11 overexpression plasmid was linearized with Stul 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-TD3H3.

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 aliquots containing 30 μg of total protein were analyzed by SDS-PAGE, transferred to nitrocellulose filters, and probed with rabbit polyclonal antibodies specific for Kar2 (Santa Cruz Biotechnology).
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 NH4HCO3) for 1 h at 55°C followed by alkylay (65 mM iodoacetamide in 100 mM NH4HCO3) for 45 min at room temperature in the dark and subsequent quenching of the reaction by addition of 35 mM dithiothreitol–100 mM NH4HCO3 for 5 min, the cells were thoroughly washed with NH4HCO3 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 μg 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 μl 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-μm 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 (plk) was generated using the MaxEnt3 algorithm in the Biolynx and Masslynx Pepseq software. Proteins were identified by submitting the plk 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 misalignments 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^{−ΔΔC_t}) 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 10^7 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 51Cr release assay as described previously (19, 20). The FaDu cells were incubated overnight with Na2SO4 (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 well. To measure the spontaneous release of 51Cr, uninfected host cells were exposed to medium alone. After a 3-h incubation, the amounts of 51Cr released into the medium and remaining in the cells were determined by gamma counting. The amount of 51Cr released by epithelial cells infected with the various *C. albicans* strains was compared with the amount of 51Cr released by uninfected host cells to calculate the infection-specific release of 51Cr. 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 Hank’s 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 vps15Δ/Δ* and *vps51Δ/Δ* mutants exhibit abnormal vacuolar morphology and impaired retrograde trafficking. In *Saccharomyces cerevisiae*, the vacuum 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, *vps15Δ/Δ* and *vps51Δ/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 *vps15Δ/Δ* cells had very large, single vacuoles (Fig. 1A).
In contrast, the vacuoles of the vps51Δ/Δ mutant were small and fragmented, as reported previously (10). These vacuolar defects were rescued when the vps15Δ/Δ and vps51Δ/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 vps15Δ/Δ and vps51Δ/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 vps15Δ/Δ and vps51Δ/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 vps15Δ/Δ and vps51Δ/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 vps15Δ/Δ and vps51Δ/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 vps51Δ/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 vps51Δ/Δ mutant. Four cell wall proteins had at least a 2-fold increase in abundance in the vps51Δ/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 vps51Δ/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 vps51Δ/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 homodimer, Cna1 and Cnb1, the Crz1 transcription factor, and the Crh11 and Utr2 transglycosylases in the vps15Δ/Δ and vps51Δ/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 vps15Δ/Δ and vps51Δ/Δ mutants. Although FK506 had little effect on the growth of the wild-type strain, it completely inhibited the growth of the vps15Δ/Δ and vps51Δ/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 vps15Δ/Δ and vps51Δ/Δ mutants, we attempted to delete both alleles of CRH11 and UTR2 in these strains. Despite numerous attempts, we were unable to delete both copies of CRH11 and UTR2 in the vps15Δ/Δ mutant, suggesting that either deletion of these genes in the vps15Δ/Δ mutant is synthetically lethal, or the resulting mutants cannot grow under the conditions used to select for transformants. Therefore, we analyzed a vps15Δ/Δ mutant that lacked a single copy of either CRH11 or UTR2. We found that the vps15Δ/Δ chr11Δ and vps15Δ/Δ utr2Δ mutants had even greater susceptibility to Congo red, H2O2, SDS, Mn2+, and protamine than did the vps15Δ/Δ
mutant (Fig. 4A). In contrast, we were successful in deleting both alleles of CHR11 and UTR2 in the vps51Δ/Δ mutant. The resulting vps15Δ/Δ chr11Δ/Δ and vps15Δ/Δ utr2Δ/Δ double mutants were more susceptible to Congo red, H$_2$O$_2$, and SDS than the vps51Δ/Δ mutant (Fig. 4B). However, these double mutants were not more susceptible to Mn$^{2+}$ 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 vps51Δ/Δ and vps15Δ/Δ mutants and tested the capacity of these strains to resist different stressors. Overexpression of CRH11 and UTR2 partially restored resistance to Congo red, H$_2$O$_2$, SDS, Mn$^{2+}$, 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 vps15Δ/Δ and vps51Δ/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 vps15Δ/Δ and vps51Δ/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 vps15Δ/Δ and vps51Δ/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 vps15Δ/Δ and vps51Δ/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 vps15Δ/Δ or vps51Δ/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* vps15Δ/Δ mutant had a large single vacuole, and *S. cerevisiae* mu-
tants that lack members of the Vps15/30/34/38 complex also have enlarged vacuoles (8). In addition, the fragmented vacuole of *C. albicans vps51/H9004* mutant cells is similar to the fragmented vacuole seen in an *S. cerevisiae vps51/H9004* mutant (28). Furthermore, the *C. albicans vps15/H9004* and *vps51/H9004* 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 vps15ΔΔ* and *vps51ΔΔ* mutants had increased susceptibility to fluconazole. In contrast, only the *S. cerevisiae vps15ΔΔ* mutant and not the *vps15ΔΔ* 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 vps15ΔΔ* and *vps51ΔΔ* 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 vps15ΔΔ* and *vps51ΔΔ* 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,

**TABLE 2** Changes in the cell wall proteome of the *vps51ΔΔ* mutant versus the wild-type strain

<table>
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<th>Parameter and protein</th>
<th>Avg % peptide in:</th>
<th>Fold change</th>
<th>Function(s)</th>
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<tr>
<td></td>
<td>Wild type</td>
<td><em>vps51ΔΔ</em> mutant</td>
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<td>≥2-fold increase in <em>vps51ΔΔ</em> mutant vs wild type</td>
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Exclusively detected in:

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

*b* ND, not detected.
these results suggest that impaired retrograde protein trafficking induces cell wall and/or cell membrane stress in *C. albicans*.

The transglycosylases Chr11 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 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 ± 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.

**FIG 4** Deletion of *CHR11* 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.
Previously, we found that a \( \text{vps51/vps51} \) insertion mutant has decreased capacity to damage an oral epithelial cell line in vitro \(^1\). In the present study, we verified this result with a \( \text{vps51/H9004} \) deletion mutant and also determined that the \( \text{vps15/H9004} \) mutant had a similar host cell damage defect. In addition, we found that the \( \text{vps15/H9004} \) and \( \text{vps51/H9004} \) 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 \( \text{CHR11} \) or \( \text{UTR2} \) partially reversed the hypersusceptibility of the \( \text{vps15/H9004} \) and \( \text{vps51/H9004} \) 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 \( \text{vps15/H9004} \) and \( \text{vps51/H9004} \) mutants had severely attenuated virulence in the mouse model of oropharyngeal candidiasis. Previously, we found that the \( \text{vps51/H9004} \) mutant had reduced virulence in the mouse model of disseminated candidiasis, although it did have increased trafficking to the brain \(^1\). Collectively, these data demonstrate that proper 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 ± SD of 3 experiments, each performed in triplicate. *, \( P < 0.001 \) compared to control strains.
trafficking is essential for the maximal virulence of *C. albicans* during both mucosal and disseminated infections.

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