**Candida albicans** VPS4 contributes differentially to epithelial and mucosal pathogenesis

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**Abbreviations:** LDH, lactate dehydrogenase; OEMS, oral epithelial model system; RHE, reconstituted human epithelium; SAP, secreted aspartyl protease

We have previously demonstrated that the *C. albicans* pre-vacuolar protein sorting gene VPS4 is required for extracellular secretion of the secreted aspartyl proteases Sap2p and Saps4–6p. Furthermore, the vps4Δ null mutant has been shown to be markedly hypovirulent in a murine tail vein model of disseminated candidiasis. In these experiments, we sought to further define the role of the pre-vacuolar secretion pathway mediated by the pre-vacuolar sorting gene VPS4 in the pathogenesis of epithelial and mucosal infection using a broad range of virulence models. The *C. albicans* vps4Δ mutant demonstrates reduced tolerance of cell wall stresses compared to its isogenic, complemented control strain. In an *in vitro* oral epithelial model (OEM) of tissue invasion, the vps4Δ mutant caused reduced tissue damage compared to controls. Further, the vps4Δ mutant was defective in macrophage killing *in vitro*, and was attenuated in virulence in an *in vivo* Caenorhabditis elegans model representative of intestinal epithelial infection. In contrast, the vps4Δ mutant caused a similar degree of tissue damage in an *in vivo* uroepithelial model of *Candida* infection compared with controls. Furthermore, in an *in vivo* murine model of vaginal candidiasis there was no reduction in fungal colony burden and no differences in vaginal histopathology compared to wild-type and complemented controls. These results suggest that VPS4 contributes to several key aspects of oral epithelial but not uroepithelial infection, and in contrast to systemic infection, plays no major role in the pathogenesis of *Candida* vaginitis. By using a wide range of virulence models, we demonstrate that *C. albicans* VPS4 contributes to virulence according to the specific tissue that is infected. Thus, in order to gain a full understanding of *C. albicans* virulence in relation to a particular gene or pathway of interest, a selected range of infection models may need to be utilized.

**Introduction**

*C. albicans* secretes aspartyl proteases (Saps)¹–³ extracellular phospholipases⁴ and secreted lipases⁵ in order to assist in pathogenesis. *In vivo*, *C. albicans* sap1Δ, sap2Δ, and sap3Δ null mutants are attenuated in virulence, and a triple sap4Δ, sap5Δ, and sap6Δ null mutant is greatly attenuated in virulence in a mouse model of disseminated candidiasis.⁶ In early studies, it was demonstrated that Sap2p secretion in *C. albicans* requires the rab-like GTPases YPT1 and SEC4, which regulate trafficking in the pre- and post-Golgi general secretory pathway, respectively.⁷,⁸ We subsequently demonstrated that the *C. albicans* pre-vacuolar secretory pathway gene VPS4 is required for secretion of Sap2p and Saps4–6p *in vitro*, thus suggesting that these Sap enzymes may be trafficked via a pre-vacuolar branch of exocytosis.⁹ Also supporting this hypothesis is evidence that Sap2p secretion is reduced in a *C. albicans* tet-regulated VPS1 mutant,¹⁰ since Vps1p is a late-Golgi protein mediating pre-vacuolar trafficking. Similarly, we have demonstrated that Sap2p secretion is reduced in the pre-vacuolar pep12Δ mutant, which lacks the t-SNARE proteins required for docking of vesicles to the pre-vacuolar compartment.¹¹ In addition, the vps4Δ mutant is defective in phospholipase and lipase secretion. Furthermore, the vps4Δ mutant also displays differential global secretion of a wide range of extracellularly secreted proteins.¹² *In vivo*, we have previously shown that the *C. albicans* vps4Δ null mutant is markedly attenuated in virulence in a standard mouse tail vein of disseminated candidiasis, thus demonstrating a clear contribution of the pre-vacuolar secretory pathway to pathogenesis.¹³

Work in the model yeast *Saccharomyces cerevisiae* has demonstrated that exocytic cargo is sorted and transported by at least 2 different routes, namely the general secretory pathway, and a pre-vacuolar sorting pathway.¹⁴,¹⁵ *C. albicans* VPS4 encodes a key AAA-type ATPase that mediates vesicle budding from the pre-vacuolar compartment for trafficking to the vacuole, or alternatively for exocytosis via a pre-vacuolar secretory pathway.
C. albicans vps4Δ mutants accumulate a distinctive, aberrant pre-vacuolar compartment and enlarged vacuole, termed a class "E" vacuolar compartment. In S. cerevisiae, some cell wall maintenance enzymes are sorted via the general secretory pathway, whereas inducible, environmentally-regulated proteins are sorted via a pre-vacuolar pathway. Analogously, in the pathogenic yeast C. albicans, it appears that environmentally-regulated virulence proteins such as the Saps may also follow a pre-vacuolar route of exocytosis.

In order to further define additional contributions of pre-vacuolar secretion to virulence-related attributes, we first investigated the role of VPS4 in tolerance of cell wall and antifungal stresses, and in macrophage killing in an in vitro model. We further characterized the role of VPS4 in virulence by utilizing a Caenorhabditis elegans intestinal model of infection. Next, we sought to determine the specific role of VPS4 in epithelial and mucosal infection by utilizing in vitro models of oral epithelial and uroepithelial infection, and in an in vivo model of murine vaginal candidiasis. Thus, we surveyed the contribution of pre-vacuolar secretion to virulence mediated by C. albicans VPS4, using a wide range of virulence models.

Results

The vps4Δ null mutant is hyper-susceptible to cell wall stress and has reduced adherence

In order to successfully survive in a wide range of host environments, including colonization or infection of mucosal sites, invasion of kidneys and other organs, and infection of blood, C. albicans must adapt to a wide range of host temperatures, pH, and osmotic stresses. Thus, in order to gain a more thorough understanding of the role of C. albicans VPS4 in stress tolerance, we assayed the ability of the vps4Δ null mutant to tolerate cell wall stresses and antifungal agents in vitro, which we had not previously studied (Fig. 1). Although there was only a modest reduction in growth on media containing the detergent SDS, there was a substantial reduction in growth on media containing the cell wall perturbing agents Congo Red or Calcofluor White compared to controls. The vps4Δ null mutant was also much more susceptible than control strains to sub-inhibitory concentrations of the cell wall active agent caspofungin, and to a modest extent the ergosterol synthesis inhibitor, fluconazole.

The vps4Δ null mutant is defective in macrophage killing and has attenuated virulence in a C. elegans model of infection

We then assayed the ability of the vps4Δ mutant to kill macrophages in an in vitro macrophage killing assay (Fig. 2). Compared to both DAY185 and the VPS4 reintegrant, the vps4Δ null mutant displayed a significant reduction in its ability to kill macrophages following phagocytosis. The VPS4 reintegrant showed slightly reduced macrophage killing compared to DAY185 (Fig. 2A); however, this difference was not statistically significant.

Next, we studied the role of VPS4 in virulence in a C. elegans intestinal model of infection (Fig. 3). C. elegans is a widely-studied soil-dwelling nematode that has been utilized as a model host for a variety of different pathogens, including C. albicans, and C. elegans infection by C. albicans has been shown to involve both C. albicans hyphal formation and a specific C. elegans immune response, highlighting the robust utility of this model. In the C. elegans model system, which has been used to study virulence in a broad range of pathogenic yeast and bacterial species, the normal laboratory food source is replaced with the pathogen of interest as the food source. Upon ingestion of C. albicans cells, the nematodes develop a persistent intestinal infection cumulating in C. albicans hyphal cells penetrating the worm cuticle. Therefore, we infected C. elegans nematodes with C. albicans DAY185, the vps4Δ mutant, or the VPS4 reintegrant strain and compared them with nematodes fed non-pathogenic E. coli OP50 as a negative control (Fig. 3A). Median survival of worms infected with DAY185 was 42 h. Virulence of the vps4Δ mutant against C. elegans nematodes was attenuated, with a median survival of 66 h. The VPS4 reintegrant had an intermediate phenotype, with a median survival of 45 h. All survival curves were
significantly different from one another as determined by the Mantel-Cox test and the log-rank test for trend (GraphPad Prism v. 6.0). Worms scored as dead were further analyzed via light microscopy (Fig. 3B). Nematodes infected with C. albicans vps4Δ had less hyphae visible protruding from the worm cuticle.

The vps4Δ null mutant causes less tissue damage in an oral epithelial invasion model

We next sought to assess the role of VPS4 in the ability to cause invasion and tissue damage in a commercial reconstituted oral epithelial model (SkinEthic, Lyon, France) (Fig. 4). After 10 h, the vps4Δ null mutant exhibited a significant reduction in its ability to cause tissue damage, as measured by LDH release, compared to its controls (data not shown). After 24 h, the vps4Δ null mutant exhibited significantly reduced tissue damage compared to DAY185 or the VPS4 reintegrant strain (Fig. 4).

Effects of the vps4Δ mutation on tissue damage in an uroepithelial invasion model

We next sought to assess the role of VPS4 in the pathogenesis of urinary epithelial invasion. Therefore, we infected an uroepithelial cell line with C. albicans DAY185, vps4Δ, and the VPS4 reintegrant cells for 24 h, and performed colony counts of adherent C. albicans cells after 2 h and 24 h of contact with uroepithelial cells (Fig. 5A). There was no difference in the adherence of C. albicans DAY185, vps4Δ, or the VPS4 reintegrant strains after 2 h. After 24 h, there was a modest but statistically significant reduction in adherence by the vps4Δ strain compared to both DAY185 and the VPS4 reintegrant. Next, we assessed the role of
VPS4 in uroepithelial cell invasion and tissue damage, as measured by LDH release, after 2 and 24 h of infection (Fig. 5B). After 2 h, the amount of LDH released by uninfected uroepithelial cells was 17.3 ± 4.6, and the amount of LDH released by uroepithelial cells infected with *C. albicans* DAY185, *vps4Δ*, or the VPS4 reintegrant was not significantly different from the uninfected control. After 24 h, the amount of LDH released by uninfected uroepithelial cells was 14.46 ± 8.8. At this timepoint, uroepithelial cells infected with each *C. albicans* strain exhibited a statistically significant increase in LDH release as compared to the uninfected control. These results indicate that infection with *C. albicans* results in significant uroepithelial cell tissue damage after 24 h. However, the *vps4Δ* null mutant did not cause a significant difference in tissue damage as measured by LDH release compared to either DAY185 or the VPS4 reintegrant at either timepoint.

**Effects of the vps4Δ mutation on virulence in a mouse model of vaginal candidiasis**

Previously, we had shown that pre-vacuolar secretion mediated by VPS4 is a key contributor to virulence in a disseminated mouse model of infection. The current *in vitro* studies indicated reduced ability to kill macrophages and decreased tissue damage in an oral epithelial model of *C. albicans* infection. Furthermore, since Sap2p is expressed during mucosal forms of *Candida* infection, we next sought to directly determine the role of VPS4 in *C. albicans* vaginitis in an *in vivo* standard murine model of vaginal candidiasis. After establishment of vaginal infection, we assessed vaginal fungal colony burden at pre-specified time points (Fig. 6A). At early time points, *C. albicans vps4Δ* vaginal fungal burden was reduced compared to DAY185 or the VPS4 reintegrant, although not significantly. However, by day 14, total vaginal fungal burden was similar in all 3 strains. Histopathological examination of fungal invasion showed no major differences in filamentation or tissue damage (Fig. 6B).

**Discussion**

It has been increasingly recognized that there are multiple aspects of pathogenesis required for the different types of
infection by the opportunistic fungal pathogen \textit{Candida albicans},\textsuperscript{24} which has led to the concomitant development and use of a wide range of infection models.\textsuperscript{25} In prior studies, we have demonstrated that the pre-vacuolar secretory pathway mediated by the vacuolar protein sorting gene \textit{VPS4} is required for secretion of Sap2 and Saps4–6 \textit{in vitro},\textsuperscript{9} and that mutants lacking \textit{VPS4} are dramatically reduced in virulence in a mouse tail vein model of disseminated infection.\textsuperscript{13} Thus, the major goals of this study were to determine if the pre-vacuolar branch of the secretory pathway mediated by \textit{VPS4} is required for superficial (i.e., epithelial and mucosal) infection. We therefore sought to further define the role of pre-vacuolar secretion in epithelial and mucosal virulence using a series of \textit{in vitro} assays, and an \textit{in vivo} model of \textit{Candida vaginitis}.

More recently, the contribution of Saps to virulence has come into question, as the influence of the \textit{URA3} positional effect on virulence may have served as a confounder of these earlier studies. For example, in reconstituted epithelial models\textsuperscript{26,27} and a systemic mouse model,\textsuperscript{28} Sap1–6 mutants were of similar virulence to wild-type \textit{C. albicans}. However, Saps4–6 have recently been shown to induce apoptosis of oral epithelial cells.\textsuperscript{29} Nonetheless, in addition to deficient Sap2p and Sap4–6p secretion, we had previously demonstrated that the \textit{vps4} \textit{D} mutant was deficient in lipase and phospholipase secretion \textit{in vitro}, despite having an intact general secretory pathway.\textsuperscript{9,13} Furthermore, we also determined that global secretion of a wide range of extracellularly secreted proteins was altered in the \textit{vps4} \textit{D} mutant.\textsuperscript{12} Thus, we hypothesized that the \textit{vps4} \textit{D} mutant would be defective in epithelial virulence, given these demonstrated alterations in secretion of virulence-related and other canonically secreted proteins. The oro-epithelial, but not the uroepithelial, model of infection showed reduced tissue damage caused by the \textit{vps4} \textit{D} mutant during experimental \textit{in vitro} infection. The \textit{vps4} \textit{D} mutant also was less able to tolerate cell wall stressors and antifungal agents, particularly the cell wall active agent caspofungin. It should also be noted that the \textit{VPS4} re-integrant strain (with a single copy of \textit{VPS4}) tended to display an intermediate phenotype between the diploid wild-type strain DAY185 and the null mutant;\textsuperscript{13} this gene dosage effect is not an uncommon finding in \textit{C. albicans} genetics.\textsuperscript{11,30} Thus, these findings suggest that pre-vacuolar secretion is required for some, but not all, epithelial forms of infection. Recent elegant work on \textit{Candida} – epithelial interactions has underlined the complexity of the epithelial response to \textit{C. albicans} infection.\textsuperscript{29,31–34} However, immuno-pathologic differences between oral and uroepithelial cells upon infection with \textit{C. albicans} have yet to be elucidated. Because the \textit{vps4} \textit{D} mutant is impaired in trafficking, we hypothesize that \textit{C. albicans} \textit{vps4} \textit{D} mislocalizes a protein (or proteins) required for virulence in oral epithelial, but not uroepithelial, infection. Furthermore, we have

\textbf{Figure 6.} The \textit{C. albicans} \textit{vps4} \textit{D} mutant is virulent in a mouse model of vaginal candidiasis. \textit{C. albicans} DAY185, \textit{vps4} \textit{D}, and the \textit{VPS4} re-integrant were tested for virulence in a mouse model of vaginal candidiasis. Mice were infected with \textit{5} \textsuperscript{\times} \textit{10}\textsuperscript{5} \textit{C. albicans} cells or with a PBS-only control. (A) Fungi burden after 3, 7 and 10 days of \textit{C. albicans} infection. Fungal burden is indicated by CFUs per mL. (B) Vaginal histopathology comparing tissue invasion and damage caused by (a) wild-type \textit{SC5314}, (b) wild-type \textit{DAY185}, (c) \textit{vps4} \textit{D} and (d) the \textit{VPS4} re-integrant strain. Paraffin sections of vaginas from randomly selected mice were prepared and stained with hematoxylin.
previously identified global alterations in extracellularly secreted proteins in the vps4Δ null mutant; most notably, the extracellular proteins Chs3p, Pra1p, Mp65p and Sun41p are reduced.12 Given the differences in environmental pH (pH 7.4 of the RPMI-1640 used in the oral epithelial model, and pH 5.8 of the artificial urine used in the uroepithelial model), it is possible that the ability of the vps4Δ mutant to cause infection is affected by environmental pH. Specifically, Pra1p is a pH-sensitive zinc scavenger protein; a pra1Δ null mutant causes reduced damage in a HUVEC endothelial monolayer infection model at alkaline pH.35 We conjecture that at the higher pH of the oral epithelial model used in these studies, diminished expression of Pra1p may reduce virulence further in the vps4Δ mutant compared to its controls.

In our animal model of Candida vaginitis, we did not detect any differences in tissue damage. We did observe a modest decrease in fungal burden at 3 and 7 days post-infection; however, by day 14, fungal burden in mice infected with C. albicans vps4Δ was statistically indistinguishable from mice infected with wild-type C. albicans. Given the clear in vitro findings regarding reduced cell wall integrity and decreased tissue damage in the oral epithelial model of infection, as well as decreased macrophage killing and attenuated virulence in the C. elegans intestinal epithelial model of infection, the minimal contribution of VPS4 to Candida vaginitis infection in this model was unexpected. It is quite possible that more dramatic in vivo findings could be seen with an oral model of Candida infection. Nonetheless, there are clear examples of genes for which null mutations lead to attenuated virulence in systemic infection but not in vaginitis. Examples of these genes include the filamentation-related transcription factors EFG1, CPH1, and NRG1.36 These results indicate that the contribution of VPS4 to pathogenesis is a complex phenomenon, where abnormal vacuolar structure and function differentially impact virulence according to the specific tissue that is infected. Importantly, in order to fully understand the contribution of a particular gene or pathway of interest to virulence, use of a wide range of pathogenesis models is required.

**Methods**

**Strains and media**

C. albicans strains SC5314 (from W. Fonzi, Georgetown University), DAY185 (from A. Mitchell, Carnegie Mellon University), vps4Δ null mutant, and vps4Δ+VPS4 reintegrant12 were grown at 30°C in YPD (1% yeast extract, 2% peptone, 2% glucose), or in minimal glucose (0.67% yeast nitrogen base without amino acids [YNB], 2% glucose). Solid media were prepared by adding 2% agar. Cell wall stress was assayed on complete synthetic media (CSM) (0.67% YNB, 0.079% complete synthetic mixture, 2% glucose, 2% agar) containing either 0.02% SDS, 200 μg/mL Congo red (Sigma-Aldrich, #860956), or 50 μg/mL calcofluor white (Sigma-Aldrich, #18909). The ability of strains to respond to challenge with antifungals was tested on YPD with 0.1 μg/mL caspofungin (Merck) or 4 μg/mL fluconazole (Sigma-Aldrich, #F8929) added. For cell wall stress and antifungal tolerance assays, serial dilutions of overnight cultures (1 × 10^6, 2 × 10^6, 4 × 10^6, and 8 × 10^6 cells per mL) were spotted onto agar media and incubated 48 h at 30°C.

**Statistical analyses**

For all experiments, results were analyzed for statistical differences using one-way ANOVA, followed by the Tukey’s multiple comparison test (GraphPad Prism 5.01). A result was considered significant when P < 0.05 compared to all other treatments.

**Macrophage killing assay**

The macrophage killing assay was performed as previously described.37 Briefly, the murine macrophage cell line J774A.1 (ATCC) was grown for 18 h in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) +5% fetal calf serum (FCS) at 37°C, 10% CO2. Next, macrophages cells were co-incubated with the C. albicans control strain DAY185, the vps4Δ null, and the isogenic vps4Δ+VPS4 reintegrant at a multiplicity of infection (MOI) of 2 for 24 h in high-glucose DMEM + 5% FCS at 37°C, 10% CO2. Macrophage killing was then assessed using the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, L-3224) which fluorescently stains live and dead macrophage cells green and red, respectively. Cells were visualized via fluorescence microscopy. Live macrophage cells were counted for 5 to 6 fields per treatment. The experiment was completed 4 times independently, and the average of all 4 experiments is presented.

**C. elegans model of infection**

The C. elegans infection model was adapted from a previously described assay,21 with one key modification. C. elegans nematodes reproduce rapidly, producing progeny that can interfere with the infection assay and that can cause matricidal killing of worms that is unrelated to infection.18 Rather than genetically or chemically inhibiting reproduction as has been done previously,18-21 we utilized a temperature-dependent inhibition strategy where wild-type C. elegans nematodes were maintained at 30°C rather than 25°C post-infection, as 30°C is a temperature at which worm reproduction but not viability is inhibited.38 Uninfected nematodes were maintained on standard worm growth medium (Nematode growth media [NGM] + Escherichia coli OP50)39 at 30°C as a negative control to ensure that the higher temperature did not affect worm survival. C. elegans N2 nematodes were obtained from the Caenorhabditis Genetics Center.

Standard nematode maintenance was performed at 15°C on NGM + E. coli plates as described previously.39 To prepare C. albicans infection plates, 50 μL cells from overnight cultures C. albicans strains DAY185, vps4Δ null mutant, and vps4Δ+VPS4 reintegrant was spread onto YPD plates in a square lawn, then incubated at 30°C for 18 h. C. elegans nematodes were synchronized to the same life-stage via bleaching as described previously.39 Then, once nematodes reached the L4 life-stage, worms were washed from NGM plates using 7 mL M9 buffer (3 g/L KH₂PO₄, 6 g/L Na₂HPO₄, 5 g/L NaCl, 1 mM MgSO₄)39 and
collected via centrifugation at 6000 rpm for 1 minute. Collected nematodes were added to the center of *C. albicans* lawns on YPD plates and incubated at 30°C for 4 hours to infect nematodes. After 4 hours, worms were washed from infection plates with 7 mL M9 buffer, taking care to avoid the *C. albicans* lawn. Then, worms in M9 were collected via centrifugation and added to the center of unseeded NGM plates to allow the M9 buffer to dry. Then, 80–100 nematodes per treatment were transferred to unseeded NGM plates via picking with a platinum-wire pick. As a negative control, collected nematodes were added to the center of an unseeded YPD plate and incubated at 30°C for 4 hours. After 4 hours, 100–150 uninfected nematodes were transferred via picking with a platinum-wire pick to NGM + *E. coli* OP50 plates. Plates were incubated at 30°C. Nematode survival was monitored at selected intervals up to 120 h post-infection; worms were scored as dead if they did not respond to stimulation with a platinum wire pick. Nematodes that crawled off the plate were excluded from analysis. The experiment was conducted twice independently. Survival curves and statistical analyses were completed using GraphPad Prism v. 6.0.

**Oral epithelial model of infection**

The capacity for oral epithelial tissue invasion of the *C. albicans* strains was assessed in a reconstituted human epithelium (RHE) oral epithelial model system (OEMS) of tissue invasion (SkinEthic Laboratories) as previously described. Briefly, oral epithelial tissue was added to a 6-well plate containing 1 mL of SkinEthic Maintenance Medium and grown at 37°C, 5% CO₂ for 24 h. *C. albicans* cells were synchronized cells to the same growth phase, as described previously. Then, 2 × 10⁶ phase-synchronized *C. albicans* cells were co-incubated with epithelial cells for 10 h–24 h at 37°C with 5% CO₂. 50 μL PBS was added to epithelial cells to serve as an untreated control. At selected time points, culture medium was transferred to 1.5 mL microcentrifuge tubes and centrifuged at 10,000 × g for 5 min at 4°C. The supernatant was transferred to a fresh 1.5 mL microcentrifuge tube. Then, lactate dehydrogenase (LDH) release was assayed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, G1780) according to manufacturer’s instructions. The LDH positive control (CytoTox 96®, Promega) was used to generate a standard curve using known concentrations of LDH; LDH units of experimental samples were calculated by extrapolation from the standard curve. The number of LDH units released by the untreated control was subtracted from the total LDH units released by the oral epithelial cells in each of the experimental samples.

**Uroepithelial model of infection**

To study the contribution of *VPS4* to uroepithelial infection, we utilized a *Candida*-uroepithelial infection model that has been previously described. Cells from overnight cultures of *C. albicans* DAY185, *vps4Δ* null mutant, and *vps4Δ+ VPS4* reintegrand strains were harvested by centrifugation for 5 min and washed twice with PBS (0.1 mol l⁻¹, pH 7). A human urinary epithelial cell line (TCC-SUP) was cultured at 37°C, 5% CO₂ in DMEM + 10% FCS for 24 h. A suspension of 1 × 10⁷ yeast cells/mL in DMEM+10%FCS was prepared and 500 μl of that suspension was added to the confluent layer of urinary epithelial cells. After 2 h and 24 h of contact between the urinary epithelial cells and yeast cells at 37°C, 5% CO₂, the culture medium was removed and cells were washed with PBS to remove non-adherent cells. 100 μl of Trypsin was added to each well for 15 min at 37°C. After incubation, 900 μl PBS + 10% FCS were added and the adherent cells at the bottom of the plate were scraped. After serial dilutions, 25 μl of the resultant suspension were plated on SDA plates and incubated at 37°C. CFUs were counted after 24 h.

Next, we measured LDH release by uroepithelial cells after 2 h and 24 h of infection with *C. albicans*. TCC-SUP cells were cultured at 37°C, 5% CO₂ in DMEM+10% FCS for 24 h. Cells from overnight cultures of *C. albicans* SC5314, *vps4Δ* null mutant, and *vps4Δ+ VPS4* reintegrand strains were harvested by centrifugation for 5 min and washed twice with PBS (0.1 mol l⁻¹, pH 7). A suspension of 1 × 10⁷ yeast cells/mL in DMEM + 10% FCS was prepared and 500 μl of that suspension was added to the confluent layer of urinary epithelial cells. After 2 h and 24 h, the release of LDH in the medium was measured using the CytoTox-ONE™ kit according to the manufacturer’s instructions. A positive control using a lysis solution was used to ensure a maximum LDH release was performed. The effect of *C. albicans* SC5314, *vps4Δ*, or *vps4Δ+ VPS4* on epithelial cells was expressed as the percentage of LDH released compared to the maximum LDH release control. The experiment was repeated in triplicate in 3 independent assays.

**Murine model of vaginal candidiasis**

Female BALB/c (H-2b) mice, 4 to 6 weeks of age (National Cancer Institute/Charles River Laboratories, Wilmington, MA), were used in these experiments. Mice were housed at The University of Texas at San Antonio Small Animal Laboratory vivarium and handled according to guidelines approved by the institutional animal care and use committee. The virulence of the *vps4Δ* null mutant and controls was assessed utilizing a standard murine model of vaginal candidiasis. In this model, 72 h prior to vaginal initiation of *candidal* infection, mice were treated subcutaneously with 0.5 mg of estradiol valerate and weekly thereafter, since vaginitis in rodents is inducible only under conditions of pseudoestrous. Next, *C. albicans* blastoconidia (5 × 10⁵) from the wild-type, *vps4Δ* null, and *vps4Δ + VPS4* reintegrand strains obtained from a fresh stationary-phase culture in 20 μl of PBS were inoculated into the vaginas of estrogen-treated mice. Control animals were inoculated with 20 μl of PBS only. At weekly intervals, mice were sacrificed, followed immediately by vaginal lavage with 100 μl of PBS and gentle scraping of vaginal tissue. Fungal burden was compared by performing colony counts on serial 1:10 dilutions of the lavage fluid, followed by plating on Sabouraud dextrose agar plates and incubating for 48–72 h at 30°C. Next, vaginal histopathology was assessed in order to compare the degree of tissue invasion and damage. Paraffin sections of vaginas from randomly selected mice were prepared and stained with hematoxylin for histological examination.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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