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***Candida albicans* VPS1 contributes to protease secretion, filamentation, and biofilm formation**

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Abstract

To investigate the pre-vacuolar secretory pathway in *Candida albicans*, we cloned and analyzed the *C. albicans* homolog of the *Saccharomyces cerevisiae* vacuolar protein sorting gene *VPS1*. *C. albicans* *VPS1* encodes a predicted 694-aa dynamin-like GTPase that is 73.3% similar to *S. cerevisiae* Vps1p. Plasmids bearing *C. albicans* *VPS1* complemented the temperature-sensitive growth, abnormal class F vacuolar morphology, and carboxypeptidase missorting of a *S. cerevisiae* *vps1* null mutant. To study *VPS1* function in *C. albicans*, a conditional mutant strain (tetR-*VPS1*) was generated by deleting the first allele of *VPS1* and placing the second allele under control of a tetracycline-regulatable promoter. With doxycycline, the tetR-*VPS1* mutant was hyper-susceptible to sub-inhibitory concentrations of fluconazole, but not amphotericin B, 5-fluorocytosine, or nonspecific osmotic stresses. The repressed tetR-*VPS1* mutant was defective in filamentation, and secreted less extracellular protease activity. Biofilm production and filamentation within the biofilm were markedly reduced. These results suggest that *C. albicans* *VPS1* has a key role in several important virulence-related phenotypes.

Keywords

Candida albicans; biofilm; protein secretion; vacuole; virulence; *VPS1*

INTRODUCTION

The fungus *Candida albicans* has emerged as an important opportunistic pathogen (Jarvis, 1995; Pfaller and Diekema, 2007). Despite its clinical significance, available methods for the diagnosis and treatment of invasive candidiasis remain suboptimal (Lee and Wong, 2004). A range of *C. albicans* virulence-related proteins have been identified and many of these are cell

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surface or secreted proteins (Haynes, 2001; Ruiz-Herrera et al., 2006; Schaller et al., 2005), but little is known about the intracellular pathways and sorting processes by which these proteins reach their final destinations. In *Saccharomyces cerevisiae*, distinct secreted marker proteins are trafficked differentially through a pre-vacuolar compartment (PVC) prior to exocytosis (Harsay and Bretscher, 1995). Mutants blocked in this pre-vacuolar pathway missort several proteins found in high density post-Golgi vesicles into low density vesicles (Gurunathan et al., 2002; Harsay and Schekman, 2002). To study the pre-vacuolar branch of exocytosis in *C. albicans*, we identified close structural homologs of several *S. cerevisiae* pre-vacuolar secretory genes, including the late-Golgi vacuolar protein sorting gene *VPS1*. The function(s) of this gene's homolog in *C. albicans* have not been previously studied.

S. cerevisiae VPS1 was originally identified in a screen for mutants that missort the vacuolar protein carboxypeptidase Y (CPY) extracellularly (Vater et al., 1992). *S. cerevisiae Vps1p* is an 80 kDa member of the dynamin-GTPase family. Vps1p mediates budding of clathrin-coated vesicles from the late Golgi that are diverted from the general secretory pathway to the vacuole (Bryant and Stevens, 1998). Structurally, Vps1p and dynamin are 45% identical and share a two-domain organization: the N-terminal domain is a GTPase and the C-terminal domain is required for membrane association. In the absence of Vps1p, Golgi and vacuolar membrane proteins are not transported to the PVC, but are instead missorted to the plasma membrane (Nothwehr et al., 1995; Vater et al., 1992; Wilsbach and Payne, 1993). *S. cerevisiae vps1* mutants display a temperature-sensitive growth defect and are characterized by a wild-type vacuole surrounded by abnormal vacuolar fragments, defined as a class "F" vacuolar morphology (Raymond et al., 1992).

A search of the *C. albicans* genome database identified a close structural homolog of *S. cerevisiae VPS1*. Thus, the experiments described below were designed to confirm whether this *C. albicans VPS1* homolog is functionally homologous to *S. cerevisiae VPS1* and to determine whether it contributes to virulence-related phenotypes in *C. albicans*.

MATERIALS AND METHODS

Strains and media

The *S. cerevisiae vps1* null mutant strain YKR001C was from American Type Culture Collection (Manassas, VA). *C. albicans* strains used in this study are indicated in Table 1. Strains were grown at 30 °C in YPD (1% yeast extract, 2% peptone, 2% glucose) supplemented with uridine (80 µg ml⁻¹), or in minimal glucose (0.67% yeast nitrogen base without amino acids [YNB], 2% glucose) supplemented with appropriate amino acids according to auxotrophic requirements. Uracil auxotrophs were selected on 5-fluoro-orotic acid (FOA) medium (minimal glucose, 0.1 mg uridine ml⁻¹, and 0.7 mg FOA ml⁻¹). Complete synthetic medium supplemented with uridine and buffered to pH 4.0 with 150 mM HEPES was used for growth in acidic medium. YPD medium supplemented with uridine and buffered to pH 8.0 with 50 mM sodium succinate and 50 mM NaH₂PO₄ was used for growth in alkaline medium. Doxycycline was added to a final concentration of 20 µg ml⁻¹ where required. Solid media were prepared by adding 2% (w/v) agar.

Preparation of plasmid and genomic DNA

Plasmids were expanded in *Escherichia coli* DH5α or TOP10F' competent cells (Invitrogen) grown in LB medium with ampicillin (100 µg ml⁻¹) at 37 °C. Plasmid DNA was prepared from *E. coli* strains using the Fast Plasmid Mini Kit™ (Eppendorf) following the manufacturer's instructions. Genomic DNA was extracted from yeast cells using the Masterpure™ Yeast DNA Purification Kit (Epicentre Biotechnologies) according to manufacturer's instructions with the

exception of an additional incubation step (1 hr on ice) performed after the addition of the MPC Protein Precipitation Reagent.

Preparation of RNA and transcriptional analysis

RNA was prepared from yeast cells grown to mid-exponential phase in the presence or absence of doxycycline following the hot acidic phenol extraction method (Ausubel et al., 1993). Equal amounts of total RNA were run on a denaturing agarose gel (1% (w/v) agarose, 10 mM sodium orthophosphate buffer, pH 7.0, 7.6% (v/v) formaldehyde) and transferred for 4 hr to a positively charged nylon membrane (Roche) with 50 mM sodium hydroxide. The membrane was probed with a ³²P-labeled DNA fragment of the *VPS1* gene (nt 737-1678) amplified from *C. albicans* strain SC5314 genomic DNA using primers VPS1-5NB and VPS1-3NB. Prehybridization and hybridization steps were performed in UltraHyb™ (Ambion) at 37 °C for 4 and 18 hr, respectively. The membrane was then washed twice in 2x SSC, 0.1% SDS for 5 minutes at 65 °C, followed by two 15-minute washes with 0.1x SSC, 0.1% SDS at 65 °C. Detection was performed by autoradiography (Kodak Biomax MR, Kodak) for 24 hr.

mRNA was prepared from total RNA using the PolyAtract mRNA Isolation System (Promega). RT-PCR was performed using the Access RT-PCR System (Promega) according to the manufacturer's instructions using primers VPS1-5NB and VPS1-3NB and 50 ng mRNA as template. The absence of contaminating DNA was tested in parallel reactions in which the reverse transcriptase enzyme was excluded.

Isolation and analysis of *C. albicans* VPS1

A *C. albicans* gene homologous to *S. cerevisiae* *VPS1* was identified by searching the Candida Genome Database (<http://www.candidagenome.org>) and CandidaDB (<http://genolist.pasteur.fr/CandidaDB>). The coding sequence and approximately 300 bp of upstream and downstream flanking sequence was amplified from *C. albicans* SC5314 genomic DNA using Platinum *Taq*HiFi DNA polymerase (Invitrogen) and primers VPS1-CP5 and VPS1-CP3 (Table 2). PCR reactions were typically heated to 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 2 min 30 sec, with a 10 min final extension at 72 °C. DNA sequencing of the forward and reverse strands confirmed the accuracy of the *C. albicans* *VPS1* open reading frame. Standard methods were used for restriction mapping, subcloning, DNA sequencing, and lithium acetate transformation of *S. cerevisiae* mutants (Ausubel et al., 1993).

Attempted disruption of *C. albicans* VPS1

To disrupt both chromosomal alleles of *C. albicans* *VPS1*, we used a PCR-based gene disruption strategy which employs *cis*-recombination to recycle a *URA3* marker thus preserving uracil auxotrophy (Wilson et al., 2000). PCR amplicons were generated using primers VPS1-5DR and VPS1-3DR (Table 2) and plasmid pDDB57 bearing the *dpl200-URA3-dpl200* gene disruption cassette (from A. Mitchell, Columbia University) as the template. *C. albicans* BWP17 was transformed directly with the PCR reaction mixtures using the lithium acetate method (Ausubel et al., 1993). Uridine prototrophs were selected and purified on synthetic media lacking uracil and uridine, and genomic DNA was extracted by vortexing the transformants with glass beads in phenol-chloroform. Homologous integration of each gene targeting cassette was first verified by allele-specific PCR, using primers flanking the open reading frame and outside of the targeting region of the disruption cassette (Table 2). Next, *C. albicans* genomic DNA was digested with *Bgl*III and *Pst*II and transferred to nylon membranes. The membranes were hybridized in 4x SSC (1x SSC is 0.15M NaCl plus 0.015 M sodium citrate) at 65 °C with a 417 bp digoxigenin-labeled PCR product generated with primers VPS1-5SOU and VPS1-3SOU using SC5314 genomic DNA as a template, after which the

membranes were washed in 0.2x SSC-0.1% sodium dodecyl sulfate (SDS) at 65 °C and analyzed using the DIG DNA detection kit (Roche) according to manufacturer's instructions.

To disrupt the second *VPS1* allele, selected *C. albicans* *VPS1/vps1Δ::dpl200-URA3-dpl200* mutants were expanded in YPD with uridine to permit loss of *URA3* by *cis*-recombination between the flanking *dpl200* repeats, uracil auxotrophs were selected on FOA medium, and the genotype was determined by PCR and by Southern hybridization as described above. The resulting *C. albicans* *VPS1/vps1Δ::dpl200* strains were transformed again with the PCR-generated gene disruption cassette, uracil prototrophs were selected, and their genotypes were analyzed by PCR and by Southern hybridization.

Additional approaches for attempted disruption of *C. albicans* *VPS1*

PCR-based gene disruption using primers *VPS1*-5DRB and *VPS1*-3DRB was also performed as described above, except that the deletion of the second allele was attempted using the *ARG4* marker in plasmid pRS-*ARG4Δ*SpeI (from A. Mitchell, Columbia University) (Wilson et al., 1999). The genotype of the resulting mutants and their wild-type parents were verified by allele-specific PCR using genomic DNA as a template and primers *VPS1*-5DetA and *VPS1*-3DetC (Table 2). Additional primer pairs bearing differing regions of homology (Table 2) were also used for PCR-mediated gene disruption. Finally, transformants recovered after incubation at 25 °C were also analyzed using identical methods.

Construction of a tetracycline-regulated *VPS1* gene in *C. albicans*

One allele of the *C. albicans* *VPS1* gene was deleted in strain THE1 (Nakayama et al., 2000), using primers *VPS1*-5DR and *VPS1*-3DR, according to the PCR-based gene disruption method described by Wilson et al. (2000) to generate strain SMB1 (Table 1). To regenerate *ura3* auxotrophy, strain SMB-1 was first plated onto solid FOA medium. Next, FOA-resistant strains were screened by PCR for the *VPS1/vps1Δ::dpl200* genotype (strain SMB-1F). The regulatable tetracycline promoter was inserted upstream of the remaining *VPS1* allele in strain SMB-1F using a strategy described by Bates et al. (2006) and primers Tet*VPS1*-5DR and Tet*VPS1*-3DR. Transformants carrying the tetR-*VPS1* allele were screened using primers Tet*VPS1*-5Det and Tet*VPS1*-3Det. All other transformants were screened for the genotype of interest using primers *VPS1*-5DetA and *VPS1*-3DetA. The correct genotype of these strains was subsequently confirmed by Southern blot analysis following standard protocols (Ausubel et al., 1993). In brief, genomic DNA prepared from candidate strains was digested with *Cla* I (New England Biolabs) and run on a 0.8% (w/v) agarose gel. A digoxigenin-labeled probe was prepared from genomic DNA isolated from strain SC5314 with primers *VPS1*-5SOU and *VPS1*-3SOU and reagents supplied in the PCR DIG Probe Synthesis Kit (Roche). Table 2 lists primers used in this study.

Analysis of growth and stress tolerance

Growth was assessed in liquid media by measuring OD₆₀₀ at fixed intervals, after the strains were washed and transferred to fresh complete synthetic media with uridine (80 µg ml⁻¹) and diluted to a starting OD₆₀₀ of 0.1. 400 µl cultures of each strain were grown in triplicate in a microtiter plate at 30 or 37 °C for 30 h in an automated Bioscreen C Analyzer (Thermo Labsystems). Shaking of the micro-cultures was performed at high intensity with irregular rotation every 3 min for 20 s and optical densities were measured every half hour. Growth curves were generated automatically using BioLINK software (Thermo Labsystems). Strains were analyzed in conditions of high osmolar stress (2.5 M glycerol or 1 M NaCl), in acidic (pH 4.0) or alkaline (pH 8.0) growth conditions, and different temperatures (30 or 37 °C). Strains grown in complete synthetic media supplemented with uridine were also exposed to sub-inhibitory concentrations of fluconazole, amphotericin B, and 5-fluorocytosine. Pilot growth curves using the *URA3*-complemented wild-type strain, THE1-CIp10, were first

generated to determine the working concentration of each antifungal reagent to be used. After analysis of the growth of strain THE1-Clp10 in varying concentrations of antifungals, the final concentrations of fluconazole, amphotericin B, and 5-fluorocytosine used for phenotypic analysis were 10, 0.5, and 10 $\mu\text{g ml}^{-1}$, respectively.

Visualization of vacuolar morphology

The fluorescent dye FM4-64 (Molecular Probes) was used to visualize endocytic and vacuolar membranes, as previously described (Vida and Emr, 1995). Following incubation with FM4-64, the cells were harvested and viewed directly using differential interference contrast (DIC) and fluorescence microscopy with an Olympus BH50 epifluorescence microscope equipped with a Retiga digital camera (QImaging, Canada), and a 60 x PlanApo oil immersion objective. Red fluorescence filters (excitation filter 533-588 nm, barrier 608-683 nm) (Chroma) were used to visualize structures stained with FM4-64. Digitized images were processed with Adobe Photoshop 5.0.

Enzyme assays

Carboxypeptidase secretion in *S. cerevisiae* strains was detected using the colony immunoblot method, as described by Conibear and Stevens (2002). In brief, colonies from overnight liquid cultures were spotted onto agar plates, and after 24 hours of growth, the colonies were overlaid with a nylon filter. Next, Western blotting was performed using anti-carboxypeptidase Y monoclonal antibody (from Abcam, UK), and detection was performed using chemiluminescence (ECL Detection Kit, Amersham Biosciences) and autoradiography. Extracellular protease secretion was assayed on BSA plates (Crandall and Edwards, 1987). Phospholipase activity was assessed on egg-yolk agar medium, and lipase activity was visualized on YNB agar containing 2.5% (v/v) Tween 80 (Fu et al., 1997). BSA degradation in liquid media was assessed by SDS-PAGE, followed by analysis of Sap2p secretion by Western blot analysis as described (Lee et al., 2007), except that the medium used was 1.18 % Yeast Carbon Base (YCB, Difco™), 0.01 % yeast extract, and 0.1 % BSA.

Filamentation assays

Filamentation was assayed at 37 °C in the following media, in the presence or absence of doxycycline: Medium 199 containing Earle's salts (Invitrogen) supplemented with L-glutamine and buffered with 150 mM HEPES to pH 7.5; 10% (v/v) fetal calf serum in YPD; RPMI-1640 supplemented with L-glutamine (US Biological) and buffered with 165mM MOPS to pH 7.0; and Spider agar medium (Liu et al., 1994). Liquid medium was inoculated with cells from overnight cultures to achieve a starting density of 5×10^6 cells/ml, followed by incubation with shaking at 200 rpm, and visualization by light microscopy at specified time intervals.

Analysis of fungal biofilms

Formation of *C. albicans* biofilms and the XTT-reduction assay were performed as previously described (Ramage and Lopez-Ribot, 2005). Scanning electron microscopy was performed on biofilm samples formed on a coverslip (Thermanox, Nalge Nunc International) after 24 h incubation of a 0.5 ml inoculum containing 1×10^6 cells ml^{-1} according to previously described methods (Ramage et al., 2002).

RESULTS

Properties of *Candida albicans* VPS1

We first identified the *C. albicans* homolog of *S. cerevisiae* VPS1 from a BLAST search of CandidaDB and the Candida Genome Database. *C. albicans* VPS1 contains a 2082 bp intronless open reading frame whose deduced protein product is 73.3% similar to *S. cerevisiae* Vps1p

and includes GTP-binding regions that are conserved in members of the dynamin-like GTPase family of proteins. Next, we cloned *VPS1* by PCR using *C. albicans* strain SC5314 genomic DNA as the template. Sequencing of the cloned PCR product confirmed that it was identical to the published *VPS1* open reading frame.

Complementation of a *Saccharomyces cerevisiae* *vps1* mutant

To determine if the *C. albicans* *VPS1* homolog is functionally homologous to *S. cerevisiae* *VPS1*, *C. albicans* *VPS1* was sub-cloned into the low-copy number yeast shuttle vector pRS316, to generate pCaVPS1. Since *S. cerevisiae* *vps1* mutants display a temperature-sensitive growth phenotype, we next transformed a *S. cerevisiae* *vps1* null mutant with pCaVPS1 or with a plasmid bearing *S. cerevisiae* *VPS1* and assayed growth at permissive (30 °C) and restrictive (37 °C) temperatures. Strains bearing *C. albicans* *VPS1* or wild-type *S. cerevisiae* *VPS1* grew at the restrictive temperature, in contrast to strains bearing empty vector alone which did not (Fig. 1A).

We next examined vacuolar morphology in these strains. *S. cerevisiae* *vps1* mutants bearing either *C. albicans* or *S. cerevisiae* *VPS1* displayed wild-type vacuolar morphology. In contrast, *S. cerevisiae* *vps1* null mutants transformed with empty vector exhibited a class “F” vacuolar morphology, characterized by a fragmented vacuole visible with light microscopy (Fig. 1B).

S. cerevisiae *vps1* mutants mis-sort the vacuolar enzyme carboxypeptidase (CPY) to the extracellular space (Vater et al., 1992), hence we assayed for CPY mis-sorting using the colony immunoblot method. *S. cerevisiae* *vps1* null mutants transformed with plasmids bearing *C. albicans* or *S. cerevisiae* *VPS1* mis-sorted much less CPY extracellularly compared to *S. cerevisiae* *vps1* null mutants transformed with empty vector (Fig. 1C). Taken together, these results indicate that *C. albicans* *VPS1* complements the *S. cerevisiae* *vps1* mutant and therefore is functionally homologous to the corresponding *S. cerevisiae* gene.

Construction of a *C. albicans* *VPS1* conditional mutant

We next sought to study *VPS1* directly in *C. albicans* by generating a *C. albicans* *vps1Δ* null mutant, but our efforts were not successful. The first allele of *C. albicans* *VPS1* was readily deleted in BWP17 using the PCR-based “mini-Urablaster” cassette (Wilson et al., 2000) to generate a *VPS1/vps1Δ::dpl200-ura3-dpl200* strain. Strains were next plated on FOA, and intrachromosomal recombination was confirmed by Southern analysis. However, efforts to generate a strain lacking the remaining wild-type *VPS1* allele were unsuccessful, despite genotypic analysis of 80 independent transformants. Next, we attempted to disrupt the second allele using an alternative PCR-based gene disruption approach, using an *ARG4* selection marker. Genotypic analysis of greater than 120 independent colonies again failed to identify any homozygous null mutants. Additionally, use of gene disruption primers bearing differing regions of homology, and analysis of transformants recovered at lower temperatures (i.e. 25 °C) failed to reveal a *vps1Δ/vps1Δ* null mutant.

Thus, in order to study *C. albicans* *VPS1*, we generated a tetracycline-regulated conditional mutant, strain tetR-VPS1 (Table 1). The correct genotype of all strains was confirmed using PCR and Southern blot analysis (Supplemental Figures 1 and 2). RT-PCR and Northern analysis indicated that in the presence of doxycycline, the *VPS1* transcript was absent in all tetR-VPS1 isolates tested (Fig. 2). In non-repressing conditions, we observed higher levels of *VPS1* transcript in the tetR-VPS1 strains compared to transcript levels in the wild-type strain (Fig. 2), since the Tet-promoter cassette in p99CAU1 provides strong induction of gene expression (Nakayama et al., 2000).

Growth and response to osmotic stress in vitro

Lack of *VPS1* gene expression did not affect growth in complete synthetic medium at either 30 or 37 °C (Fig. 3A). No difference in growth was observed between the wild-type and tetR-*VPS1* mutant strains, with or without doxycycline, when grown under alkaline (pH 8.0) or acidic (pH 4.0) conditions, conditions of osmotic stress (in media containing high concentrations of salt and glycerol) or at temperatures of 30 and 37 °C (data not shown).

Growth and response to antifungal agents in vitro

Next we tested growth in the presence of sub-inhibitory concentrations of several antifungal agents at 30 and 37 °C. No growth difference between the wild-type and tetR-*VPS1* strain was seen when either amphotericin B or 5-fluorocytosine was used, in the presence or absence of doxycycline (data not shown). However, a clear growth difference occurred when these strains were grown in medium containing fluconazole (Fig. 3B). The heterozygous strain SLZK1 (*VPS1/vps1Δ*) grew less well compared to the parental strain BWP17 (data not shown). Similarly, the tetR-*VPS1* strain expressing a single copy of *VPS1* grew less well than the wild-type strain THE1-CIp10 (Fig. 3B). This growth defect increased even more when *VPS1* expression was repressed in the presence of doxycycline, at both 30 and 37 °C (Fig. 3B).

Characterization of vacuolar morphology

The *S. cerevisiae vps1* mutant exhibits a class F vacuolar morphology which is described as having a large central vacuole surrounded by smaller vacuole-like fragments (Raymond et al., 1992). Vacuolar staining of the tetR-*VPS1* mutant strain with FM4-64 showed a single large vacuole (Fig. 4A) similar to the vacuole seen in the *S. cerevisiae vps1* mutant described by Mullins and Bonifacino (2001). We also visualized the vacuolar morphology of the tetR-*VPS1* strain using thin-section electron microscopy. Unexpectedly, no major differences in vacuolar morphology were apparent between strain THE1-CIp10 and the tetR-*VPS1* mutant strain, in either the presence or absence of doxycycline (Fig. 4B).

Secretion of extracellular proteases in the tetR-*VPS1* mutant

Since *S. cerevisiae VPS1* plays an important role in pre-vacuolar secretion (Gurunathan et al., 2002; Rothman et al., 1990), we sought to determine the effect of the loss of function of *C. albicans VPS1* on secretion of extracellular proteases. When inoculated on medium containing BSA as the sole nitrogen source, wild-type *C. albicans* secretes aspartyl proteinases which result in a halo surrounding the colony due to extracellular proteolysis. The strains carrying a single copy of the *VPS1* gene (SMB1 and SMB1F) produced a similar amount of extracellular BSA degradation as the wild-type strain (data not shown). Also, the addition of doxycycline did not affect the levels of BSA degradation in these strains (data not shown). Under non-repressing conditions, the tetR-*VPS1* mutant strain produced the same amount of BSA degradation as the wild-type strain (Fig. 5A). However, when transcription of the *VPS1* gene was repressed in the tetR-*VPS1* strain by addition of doxycycline, a clear decrease in the amount of proteolysis was observed.

Further analysis of protease secretion using liquid BSA degradation and SDS-PAGE also produced similar results as the plate assay (Fig. 5B). When analyzed by Western blotting using anti-Sap2p polyclonal antibodies (gift of B. Hube, International Leibniz Research School), the tetR-*VPS1* strain with doxycycline produced much less extracellular Sap2p than the tetR-*VPS1* strain without doxycycline (Fig. 5C).

We next assayed for phospholipase secretion using egg yolk agar plates, however, no difference was seen among these strains with or without doxycycline (data not shown). In contrast, on YNB-Tween 80 plates, which have been used to assay for lipase secretion, the tet-*VPS1* strain

with doxycycline produced much less lipolysis than the tet-VPS1 strain without doxycycline (Fig. 5D). Taken together, these results suggest that lack of *VPS1* expression reduces secretion of extracellular proteases and lipases.

Filamentation in the tetR-VPS1 mutant

Another important virulence attribute in *C. albicans* is the ability to switch between yeast, pseudohyphal, and filamentous forms (Braun and Johnson, 2000; Brown and Gow, 1999). We observed that wild-type *C. albicans* colonies inoculated onto BSA plates begin to produce filamentous structures on day 3. Interestingly, when expression of the *VPS1* gene was repressed, the tetR-VPS1 strain did not produce filaments on BSA, even after 8 days (Fig. 6A). Similar results were also observed on conventional filamentation medium, i.e. Spider agar, M199 agar, and YPD agar with 10% fetal calf serum (Fig. 6A and 6B). On these agar plates, in derepressing conditions the tetR-VPS1 strain produced the same filamentous morphology as the wild-type parental strain. In repressing conditions (with doxycycline), the tetR-VPS1 mutant strain produced much less filamentation compared to the wild-type or derepressed tetR-VPS1 strains. A clear lag in filamentation was also seen when the tetR-VPS1 strain was grown in liquid RPMI-1640 and doxycycline at 37 °C (Fig. 6C). When assayed in liquid 10% fetal calf serum, similar results were also noted, although the difference in filamentation was less pronounced (Fig. 6D). These results suggest that *VPS1* expression is required for normal filamentation.

Biofilm formation in the tetR-VPS1 mutant

We next hypothesized that the pre-vacuolar secretory defect of the tetR-VPS1 strain could affect fungal biofilm formation, since both secretion and filamentation were abnormal. We first compared the structure of *Candida* biofilms formed after 24 h using light microscopy. Biofilm morphology of the wild-type strains (in the absence or presence of doxycycline) and the derepressed tetR-VPS1 mutant was similar. However, in the absence of a functional *VPS1* expression, the biofilm formed was markedly less dense (Fig. 7A). Scanning electron microscopy demonstrated that the tetR-VPS1 strain produced a sparse biofilm that was composed predominantly of yeast cells and pseudohyphal structures when doxycycline was present (Fig. 7B). In contrast, without doxycycline, the biofilm structure formed by the tetR-VPS1 strain was composed of a dense population of predominantly mature filamentous forms.

Biofilm mass measured by the XTT reduction assay was similar for the derepressed tetR-VPS1 mutant strain and the wild-type control (Fig. 7A). A modest reduction in metabolic activity was seen in the wild-type strain when doxycycline was present. However, a significantly greater reduction in metabolic activity was measured in the tetR-VPS1 strain when doxycycline was added (p-value < 0.001). Thus, these results suggest that *VPS1* contributes to normal biofilm formation in *C. albicans*.

DISCUSSION

The major goals of this study were to determine if the pathogenic fungus *C. albicans* has a pre-vacuolar exocytic pathway analogous to the pathway described in *S. cerevisiae* (Harsay and Schekman, 2002), and to determine the contribution of *C. albicans VPS1* to key virulence-related phenotypes *in vitro*. When a search of the *C. albicans* genome database revealed a close structural homolog of the *S. cerevisiae* vacuolar protein sorting gene *VPS1*, we used a complementation approach to study gene function. First, the temperature-sensitivity, vacuolar morphology, and protein sorting phenotypes of a *S. cerevisiae vps1* null mutant were corrected by plasmids bearing *C. albicans VPS1*, but not by empty vector. Taken together, these observations confirmed that the gene designated *C. albicans VPS1* is both a structural and functional homolog of *S. cerevisiae VPS1*.

Our next objective was to study *VPS1* directly in *C. albicans*. However, despite screening a large number of transformants, our efforts to generate a *C. albicans vps1Δ* null mutant were unsuccessful. Although we initially hypothesized that *C. albicans VPS1* may be an essential gene, subsequent phenotypic analysis of the conditional tet-regulated *vps1* mutant has indicated otherwise. Other possibilities that could account for our inability to generate a *vps1Δ* null mutant include an allele-specific reduction in the efficiency of homologous recombination, or reduction in the overall fitness of a potential null mutant.

Unexpectedly, the repressed *C. albicans* tetR-*VPS1* mutant did not display a characteristic class “F” vacuolar morphology. It should be noted that the *S. cerevisiae vps1* mutant has a vacuolar morphology that is intermediate between wild-type strains and that of class “B” vacuolar mutants (Raymond et al., 1992). The class “B” vacuole typically consists of a large central vacuole, which we also observed in the *C. albicans* tetR-*VPS1* mutant (with doxycycline), often surrounded by smaller vacuole-like compartments, which we were not able to discern in the tetR-*VPS1* mutant. Thus, it is possible that our thin-section electron microscopic approach was not ideally suited for visualizing these vacuolar fragments, or perhaps that *C. albicans VPS1* does not perform an identical role as *S. cerevisiae VPS1* in vacuolar biogenesis.

Proteins encoded by a number of vacuolar protein sorting (*VPS*) genes have been shown to play a role in several virulence-related properties in *C. albicans in vitro*. These proteins include the phosphatidylinositol 3-kinase Vps34p (Bruckmann et al., 2000), the vacuolar sorting protein Vps11p (Palmer et al., 2003), components of the endosomal sorting complex required for transport (ESCRT complexes) Vps28p and Vps32p (Cornet et al., 2005), and the AAA-ATPase Vps4p (Lee et al., 2007). Attenuated virulence in the *vps4Δ*, *vps28Δ*, *vps32Δ*, and *vps34Δ* null mutants has also been demonstrated *in vivo* (Bruckmann et al., 2000; Cornet et al., 2005; Lee et al., unpublished data). We thus sought to characterize virulence-related phenotypes in our tetR-*VPS1* conditional mutant strain.

In the presence of doxycycline, transcription of the *VPS1* gene is tightly repressed in the tetR-*VPS1* mutant strain. We have demonstrated that loss of function of *C. albicans VPS1* does not affect growth in complete synthetic or rich medium. Hence any phenotypic differences from the wild-type or derepressed tetR-*VPS1* can be attributed to the loss of function of the *VPS1* gene. Next, we have shown that the tetR-*VPS1* mutant strain was hyper-susceptible to sub-inhibitory concentrations of fluconazole. Strains expressing only one copy of the *VPS1* gene, i.e. heterozygous strain SLZK1 (*VPS1/vps1Δ*) and the tetR-*VPS1* (*tetR-VPS1/vps1Δ*) strain, were sensitive to sub-inhibitory concentrations of fluconazole. In the repressed tetR-*VPS1* strain (i.e., with the addition of doxycycline), the sensitivity to fluconazole was even greater. Thus we have demonstrated a gene dosage effect of *VPS1* on the tolerance of *C. albicans* to sub-inhibitory concentrations of fluconazole.

Whereas growth of the tetR-*VPS1* mutant strain was inhibited by fluconazole, it was not affected by either amphotericin B or 5-fluorocytosine. This phenotype is similar to the *vps28Δ* and *vps32Δ* homozygous knockout mutants which are hyper-susceptible to azoles, but not amphotericin B and 5-fluorocytosine, in a *RIM101*-dependent manner (Cornet et al., 2006). Vps28p and Vps32p regulate the expression of *RIM101*, *PHR1*, and *PHR2* (Cornet et al., 2005), which are genes involved in the alkaline response (Davis et al., 2000). Cornet *et al.* (2006) showed that in the presence of a functional Rim101 protein, *vps28Δ* and *vps32Δ* null mutants were no longer hyper-susceptible to either fluconazole or voriconazole. In contrast to the *vps28Δ* and *vps32Δ* null mutants, growth of the tetR-*VPS1* strain was not affected when grown under repressing conditions in alkaline medium (data not shown) suggesting that the pH-sensing mechanism in this strain is intact. However, it should be noted that a *RIM101*-independent pH-sensing mechanism exists in *C. albicans*, which allows the organism to survive

when grown in alkaline conditions through induction of the alkaline-repressed *PHR2* gene (Davis et al., 2000).

Secreted hydrolytic enzymes of *C. albicans*, such as the family of secreted aspartyl proteases (Saps), lipases, and phospholipase B have been shown to play a role in tissue invasion and evasion of the host-immune response (Ghannoum, 2000; Naglik et al., 2003; Schaller et al., 2005). Deletion of the *VPS11* gene results in a strain that is deficient in secretion of Saps and lipases (Palmer et al., 2003). The class E type of vacuolar mutants *vps34Δ* and *vps4Δ* are also unable to secrete Sap2 protein in *C. albicans* (Kitanovic et al., 2005; Lee et al., 2007). Interestingly, the *C. albicans vps4Δ* null mutant secretes an increased amount of extracellular proteolytic activity on BSA plates, which has been identified as missorted carboxypeptidase. However, specific Western blotting reveals a complete absence of extracellular Sap2p (Lee et al., 2007). Here we report a role for the late-Golgi vacuolar protein sorting gene *VPS1* in the secretion of proteases and lipases. We have demonstrated that the *C. albicans* tetR-*VPS1* mutant secretes less extracellular proteolytic activity when grown on BSA agar and reduced lipolytic activity on YNB-Tween agar in repressing conditions. Western blotting of culture supernatants has indicated a reduction in extracellular Sap2p, suggesting that reduced Sap2p secretion may be the origin of this reduced extracellular proteolytic activity. This reduced proteolytic phenotype is similar to that seen with the *C. albicans vps11Δ* null mutant. *VPS11* is thought to act not only at a trafficking step immediately prior to vacuolar fusion, but in the late-Golgi as well (Rieder and Emr, 1997; Robinson et al., 1991; Sato et al., 2000). Perhaps interruption of the pre-vacuolar secretory pathway at an immediate post-Golgi step reduces the overall amount of secreted proteases, whereas at a later pre-vacuolar step, Sap2p secretion is interrupted, but not secretion of missorted vacuolar enzymes such as carboxypeptidase. It will be interesting to examine the phenotypes of late-vacuolar *vps* mutants, such as class “D” vacuolar mutants, in order to determine if Saps and other secreted hydrolytic enzymes are trafficked through a pre-vacuolar pathway, or if some other post-translational regulatory mechanism exists.

In addition to the altered levels of protease secretion, some of the previously characterized *vps* mutants have also been shown to be defective in filamentation. There are several regulatory pathways mediating yeast-to-hyphal morphogenesis in *C. albicans* [reviewed in Liu (2001), Dhillon et al., (2003), and Monge et al (2006)]. These include a cAMP-protein kinase A pathway in which Efg1p is involved (Stoldt et al., 1997), a MAP-kinase pathway in which Cph1p plays a key role (Liu et al., 1994), the Tup1p-mediated pathway (Braun and Johnson, 1997), a Rim101p-dependent pH-sensing pathway (Davis et al., 2000), and Czf1p-mediated filamentation which is described when *C. albicans* is embedded in solid medium (Brown et al., 1999). *C. albicans vps28Δ* and *vps34Δ* null mutants exhibit defects in filamentation that are dependent on the *RIM101* pathway (Cornet et al., 2005). The *C. albicans vps34Δ* null mutant produces more hyphae than the wild-type control when embedded in solid agar matrix at 23 °C (Kitanovic et al., 2005), but when grown in liquid hyphae-inducing medium at 37 °C, filamentation is considerably delayed in the mutant strain (Bruckmann et al., 2000). The *C. albicans vps11Δ* null mutant is unable to produce filaments and it has been suggested that *VPS11* may play a role in vacuolar expansion required for yeast to hyphal morphogenesis (Palmer et al., 2003; Palmer et al., 2005). We have shown here that the loss of function of *C. albicans VPS1* resulted in delayed filamentation when grown on strong or weak filamentation-inducing medium.

We hypothesize that the filamentation defect of the repressed tetR-*VPS1* strain may result from mislocalization of Kex2p from the Golgi complex, where it is required for processing of proteins contributing to hyphal formation. In *S. cerevisiae*, Vps1p mediates retention of the endoprotease Kex2p in the Golgi membrane (Wilsbach and Payne, 1993). Mutations in *VPS1* result in mislocalization of Kex2p to the vacuole where it is degraded by Pep4p (Wilsbach

and Payne, 1993). Studies of the *KEX2* homolog in *C. albicans* demonstrate that disruption of *KEX2* results in a strain that is unable to produce hyphae when grown in filamentation-inducing conditions (Newport and Agabian, 1997). The authors hypothesized that Kex2p may be required for post-translational processing of components of the cell wall and other proteins. This hypothesis was supported by a subsequent study in which a genome-wide search identified a number of hydrolases, adhesins, cell wall components, and outer membrane proteins that contained putative Kex2p processing sites (Newport et al., 2003). Thus, a potential mechanism for the filamentation defect in the repressed tetR-*VPS1* strain is that in the absence of Vps1p, Kex2p is mislocalized to the vacuole and degraded, resulting in abnormal processing of proteins required for filamentation.

Candida biofilms are composed of a mixed community of differentiated cells and extracellular matrix. The basic stages of biofilm formation include the (1) adhesion of cells to a surface conducive for biofilm formation, (2) formation of a basal layer that serves as an anchor, followed by (3) pseudohyphal and hyphal growth and the formation of the extracellular matrix (Nobile and Mitchell, 2006). We have shown that when transcription of *VPS1* is repressed, the tetR-*VPS1* strain formed a sparse biofilm composed predominantly of pseudohyphal and yeast cells. Interestingly, Marchais et al. (2005) observed increased expression of *C. albicans VPS1* in germ tubes adherent to polystyrene, suggesting that *C. albicans VPS1* may play a role in adhesion. Furthermore, *VPS1* may also play a role in later stages of biofilm formation that are characterized by extensive differentiation and filamentation of cells. *VPS1* may also contribute to biofilm formation in response to amino acid starvation. In genome-wide profiling studies, Tournu et al. (2005) showed increased expression of *C. albicans VPS1* in a *GCN2*- and *GCN4*-dependent manner during amino acid starvation. *GCN4* encodes a transcriptional activator within the general amino acid control response that is over-expressed during biofilm formation, which is in turn associated with amino acid starvation (Garcia-Sanchez et al., 2004).

Thus, we have shown that *C. albicans VPS1* is involved in tolerance of azoles, protease secretion, filamentation, and biofilm formation, suggesting an important role of the pre-vacuolar secretory pathway in *C. albicans* virulence. We are currently undertaking further investigations on the mechanism by which *VPS1* effects control on secretion, filamentation, and biofilm formation *in vitro*, and studies of virulence *in vivo* using fly and murine models of invasive candidiasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Ausubel, FM.; Brent, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K. Current protocols in molecular biology. Wiley; New York: 1993.
- Bates S, Hughes HB, Munro CA, Thomas WP, MacCallum DM, Bertram G, Atrih A, Ferguson MA, Brown AJ, Odds FC, Gow NA. Outer chain N-glycans are required for cell wall integrity and virulence of *Candida albicans*. J Biol Chem 2006;281:90–98. [PubMed: 16263704]
- Braun BR, Johnson AD. Control of filament formation in *Candida albicans* by the transcriptional repressor *TUP1*. Science 1997;277:105–109. [PubMed: 9204892]
- Braun BR, Johnson AD. *TUP1*, *CPH1* and *EFG1* make independent contributions to filamentation in *Candida albicans*. Genetics 2000;155:57–67. [PubMed: 10790384]
- Brown AJ, Gow NA. Regulatory networks controlling *Candida albicans* morphogenesis. Trends Microbiol 1999;7:333–338. [PubMed: 10431207]
- Brown DH Jr, Giusani AD, Chen X, Kumamoto CA. Filamentous growth of *Candida albicans* in response to physical environmental cues and its regulation by the unique *CZF1* gene. Mol Microbiol 1999;34:651–662. [PubMed: 10564506]
- Bruckmann A, Kunkel W, Hartl A, Wetzker R, Eck R. A phosphatidylinositol 3-kinase of *Candida albicans* influences adhesion, filamentous growth and virulence. Microbiology 2000;146:2755–2764. [PubMed: 11065354]
- Bryant NJ, Stevens TH. Vacuole biogenesis in *Saccharomyces cerevisiae*: Protein transport pathways to the yeast vacuole. Microbiol Mol Biol Rev 1998;62:230–247. [PubMed: 9529893]
- Conibear E, Stevens TH. Studying yeast vacuoles. Methods Enzymol 2002;351:408–432. [PubMed: 12073360]
- Cornet M, Bidard F, Schwarz P, Da Costa G, Blanchin-Roland S, Dromer F, Gaillardin C. Deletions of endocytic components *VPS28* and *VPS32* affect growth at alkaline pH and virulence through both *RIM101*-dependent and *RIM101*-independent pathways in *Candida albicans*. Infect Immun 2005;73:7977–7987. [PubMed: 16299290]
- Cornet M, Gaillardin C, Richard ML. Deletions of the endocytic components *VPS28* and *VPS32* in *Candida albicans* lead to echinocandin and azole hypersensitivity. Antimicrob Agents Chemother 2006;50:3492–3495. [PubMed: 17005841]
- Crandall M, Edwards JE Jr. Segregation of proteinase-negative mutants from heterozygous *Candida albicans*. J Gen Microbiol 1987;133:2817–2824. [PubMed: 3329678]
- Davis D, Wilson RB, Mitchell AP. *RIM101*-dependent and -independent pathways govern pH responses in *Candida albicans*. Mol Cell Biol 2000;20:971–978. [PubMed: 10629054]
- Dhillon NK, Sharma S, Khuller GK. Signaling through protein kinases and transcriptional regulators in *Candida albicans*. Crit Rev Microbiol 2003;29:259–275. [PubMed: 14582620]
- Fu Y, Ibrahim AS, Fonzi W, Zhou X, Ramos CF, Ghannoum MA. Cloning and characterization of a gene (*LIP1*) which encodes a lipase from the pathogenic yeast *Candida albicans*. Microbiol 1997;143:331–340.
- Garcia-Sanchez S, Aubert S, Iraqui I, Janbon G, Ghigo JM, d'Enfert C. *Candida albicans* biofilms: A developmental state associated with specific and stable gene expression patterns. Eukaryot Cell 2004;3:536–545. [PubMed: 15075282]
- Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. Clin Microbiol Rev 2000;13:122–143. [PubMed: 10627494]
- Gurunathan S, David D, Gerst JE. Dynamin and clathrin are required for the biogenesis of a distinct class of secretory vesicles in yeast. EMBO J 2002;21:602–614. [PubMed: 11847108]

- Harsay E, Bretscher A. Parallel secretory pathways to the cell surface in yeast. *J Cell Biol* 1995;131:297–310. [PubMed: 7593160]
- Harsay E, Schekman R. A subset of yeast vacuolar protein sorting mutants is blocked in one branch of the exocytic pathway. *J Cell Biol* 2002;156:271–285. [PubMed: 11807092]
- Haynes K. Virulence in *Candida* species. *Trends Microbiol* 2001;9:591–596. [PubMed: 11728872]
- Jarvis WR. Epidemiology of nosocomial fungal infections, with emphasis on *Candida* species. *Clin Infect Dis* 1995;20:1526–1530. [PubMed: 7548503]
- Kitanovic A, Nguyen M, Vogl G, Hartmann A, Gunther J, Wurzner R, Kunkel W, Wolf S, Eck R. Phosphatidylinositol 3-kinase *VPS34* of *Candida albicans* is involved in filamentous growth, secretion of aspartic proteases, and intracellular detoxification. *FEMS Yeast Res* 2005;5:431–439. [PubMed: 15691748]
- Kurtz MB, Cortelyou MW, Kirsch DR. Integrative transformation of *Candida albicans*, using a cloned *Candida ade2* gene. *Mol Cell Biol* 1986;6:142–149. [PubMed: 3023819]
- Lee SA, Jones J, Khalique Z, Kot J, Alba M, Bernardo S, Seghal A, Wong B. A functional analysis of the *Candida albicans* homolog of *Saccharomyces cerevisiae VPS4*. *FEMS Yeast Res* 2007;7:973–985. [PubMed: 17506830]
- Lee, SA.; Wong, B. Advances in diagnostic methods for invasive *Candida* and *Aspergillus* infections. In: Domer, J.; Kobayashi, G., editors. *The Mycota: A comprehensive treatise on fungi as experimental systems for basic and applied research*. Springer; Berlin, Germany: 2004. p. 97-114.
- Liu H. Transcriptional control of dimorphism in *Candida albicans*. *Curr Opin Microbiol* 2001;4:728–735. [PubMed: 11731326]
- Liu H, Kohler J, Fink GR. Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. *Science* 1994;266:1723–1726. [PubMed: 7992058]
- Marchais V, Kempf M, Licznar P, Lefrancois C, Bouchara JP, Robert R, Cottin J. DNA array analysis of *Candida albicans* gene expression in response to adherence to polystyrene. *FEMS Microbiol Lett* 2005;245:25–32. [PubMed: 15796975]
- Monge RA, Roman E, Nombela C, Pla J. The MAP kinase signal transduction network in *Candida albicans*. *Microbiol* 2006;152:905–912.
- Mullins C, Bonifacino JS. Structural requirements for function of yeast GGAs in vacuolar protein sorting, alpha-factor maturation, and interactions with clathrin. *Mol Cell Biol* 2001;21:7981–7994. [PubMed: 11689690]
- Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev* 2003;67:400–428. [PubMed: 12966142]
- Nakayama H, Mio T, Nagahashi S, Kokado M, Arisawa M, Aoki Y. Tetracycline-regulatable system to tightly control gene expression in the pathogenic fungus *Candida albicans*. *Infect Immun* 2000;68:6712–6719. [PubMed: 11083786]
- Newport G, Agabian N. *KEX2* influences *Candida albicans* proteinase secretion and hyphal formation. *J Biol Chem* 1997;272:28954–28961. [PubMed: 9360967]
- Newport G, Kuo A, Flattery A, Gill C, Blake JJ, Kurtz MB, Abruzzo GK, Agabian N. Inactivation of *Kex2p* diminishes the virulence of *Candida albicans*. *J Biol Chem* 2003;278:1713–1720. [PubMed: 12419804]
- Nobile CJ, Mitchell AP. Genetics and genomics of *Candida albicans* biofilm formation. *Cell Microbiol* 2006;8:1382–1391. [PubMed: 16848788]
- Nothwehr SF, Conibear E, Stevens TH. Golgi and vacuolar membrane proteins reach the vacuole in *vps1* mutant yeast cells via the plasma membrane. *J Cell Biol* 1995;129:35–46. [PubMed: 7698993]
- Palmer GE, Cashmore A, Sturtevant J. *Candida albicans VPS11* is required for vacuole biogenesis and germ tube formation. *Eukaryot Cell* 2003;2:411–421. [PubMed: 12796286]
- Palmer GE, Kelly MN, Sturtevant JE. The *Candida albicans* vacuole is required for differentiation and efficient macrophage killing. *Eukaryot Cell* 2005;4:1677–1686. [PubMed: 16215175]
- Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: A persistent public health problem. *Clin Microbiol Rev* 2007;20:133–163. [PubMed: 17223626]
- Ramage G, Lopez-Ribot JL. Techniques for antifungal susceptibility testing of *Candida albicans* biofilms. *Methods Mol Med* 2005;118:71–79. [PubMed: 15888936]

- Ramage G, Saville SP, Wickes BL, Lopez-Ribot JL. Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl Environ Microbiol* 2002;68:5459–5463. [PubMed: 12406738]
- Raymond CK, Howald-Stevenson I, Vater CA, Stevens TH. Morphological classification of the yeast vacuolar protein sorting mutants: Evidence for a prevacuolar compartment in class E *vps* mutants. *Mol Biol Cell* 1992;3:1389–1402. [PubMed: 1493335]
- Rieder SE, Emr SD. A novel RING finger protein complex essential for a late step in protein transport to the yeast vacuole. *Mol Biol Cell* 1997;8:2307–2327. [PubMed: 9362071]
- Robinson JS, Graham TR, Emr SD. A putative zinc finger protein, *Saccharomyces cerevisiae* Vps18p, affects late Golgi functions required for vacuolar protein sorting and efficient alpha-factor prohormone maturation. *Mol Cell Biol* 1991;11:5813–5824. [PubMed: 1840635]
- Rothman JH, Raymond CK, Gilbert T, O'Hara PJ, Stevens TH. A putative GTP binding protein homologous to interferon-inducible Mx proteins performs an essential function in yeast protein sorting. *Cell* 1990;61:1063–1074. [PubMed: 2112425]
- Ruiz-Herrera J, Elorza MV, Valentin E, Sentandreu R. Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. *FEMS Yeast Res* 2006;6:14–29. [PubMed: 16423067]
- Sato TK, Rehling P, Peterson MR, Emr SD. Class C Vps protein complex regulates vacuolar SNARE pairing and is required for vesicle docking/fusion. *Mol Cell* 2000;6:661–671. [PubMed: 11030345]
- Schaller M, Borelli C, Korting HC, Hube B. Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses* 2005;48:365–377. [PubMed: 16262871]
- Stoldt VR, Sonneborn A, Leuker CE, Ernst JF. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *EMBO J* 1997;16:1982–1991. [PubMed: 9155024]
- Tournu H, Tripathi G, Bertram G, Macaskill S, Mavor A, Walker L, Odds FC, Gow NA, Brown AJ. Global role of the protein kinase *GCN2* in the human pathogen *Candida albicans*. *Eukaryot Cell* 2005;4:1687–1696. [PubMed: 16215176]
- Vater CA, Raymond CK, Ekena K, Howald-Stevenson I, Stevens TH. The Vps1 protein, a homolog of dynamin required for vacuolar protein sorting in *Saccharomyces cerevisiae*, is a GTPase with two functionally separable domains. *J Cell Biol* 1992;119:773–786. [PubMed: 1429836]
- Vida TA, Emr SD. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J Cell Biol* 1995;128:779–792. [PubMed: 7533169]
- Wilsbach K, Payne GS. Vps1p, a member of the dynamin GTPase family, is necessary for Golgi membrane protein retention in *Saccharomyces cerevisiae*. *EMBO J* 1993;12:3049–3059. [PubMed: 8344247]
- Wilson RB, Davis D, Enloe BM, Mitchell AP. A recyclable *Candida albicans* *URA3* cassette for PCR product-directed gene disruptions. *Yeast* 2000;16:65–70. [PubMed: 10620776]
- Wilson RB, Davis D, Mitchell AP. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. *J Bacteriol* 1999;181:1868–1874. [PubMed: 10074081]

Fig. 1. Complementation of a *S. cerevisiae vps1* null mutant
Over-expression of *C. albicans VPS1* complements the temperature-sensitivity of a *S. cerevisiae vps1* null mutant (A). Low copy number yeast shuttle plasmids (pRS316) containing *C. albicans VPS1* (Ca *VPS1*) and *S. cerevisiae VPS1* (Sc *VPS1*) conferred on a temperature-sensitive *S. cerevisiae vps1* mutant the ability to grow at 37 °C, but empty vector alone did not. **Over-expression of *C. albicans VPS1* complements the *S. cerevisiae vps1* null mutant vacuolar phenotype (B).** Low copy number plasmids (pRS316) containing Sc *VPS1*, Ca *VPS1*, or no insert were introduced into a *S. cerevisiae vps1* null mutant strain. Overnight cultures of strains grown in rich media were shifted to fresh media and examined during late exponential phase and stained with the vacuolar dye FM4-64. Live cells were examined by epifluorescence and DIC microscopy. The strain bearing vector alone displays a characteristic class "F" vacuolar morphology. **Over-expression of *C. albicans VPS1***

complements carboxypeptidase (CPY) missorting of a *S. cerevisiae vps1* null mutant

(C). *S. cerevisiae vps1* mutants misort CPY to the extracellular space, in contrast to wild-type strains, which do not. Therefore, overnight cultures of a *S. cerevisiae* wild-type strain and *S. cerevisiae vps1* null mutant strains transformed with a vector bearing either Ca *VPS1*, Sc *VPS1*, or vector alone were spotted on solid media. Next, a colony immunoblot was performed by overlaying the spotted colonies with a nylon filter, followed by Western blotting using monoclonal antibody to *S. cerevisiae* CPY. A positive signal indicates missorting of CPY to the extracellular space.

Fig. 2. Transcriptional analyses of *VPS1* in the *tetR-VPS1* strains

A Northern blot was probed with a ^{32}P -labeled PCR product amplified from strain SC5314 using primers *VPS1*-5NB and *VPS1*-3NB (Table 2). There is complete repression of the *VPS1* transcript in the *tetR-VPS1* strains when doxycycline is present. RT-PCR on mRNA isolated from the same total RNA used in the Northern blot also showed complete repression of *VPS1* transcription in the *tetR-VPS1* strains in the presence of doxycycline. An ethidium bromide stained gel shows normalized loading of the RNA samples used in the Northern blot.

Fig. 3. Characterization of growth of the tetR-VPS1 strain

Growth of the tetR-VPS1 mutant strain was tested in the presence of sub-inhibitory concentrations of fluconazole. In complete synthetic medium supplemented with uridine at 37 °C, growth of the tetR-VPS1 strain was similar to the parental strain THE1 (A). However, growth of the tetR-VPS1 strain was inhibited in the presence of 10 $\mu\text{g ml}^{-1}$ fluconazole (B). This sensitivity to fluconazole was increased when transcription of *VPS1* was completely repressed with the addition of doxycycline. Growth curves were performed in triplicate, and corresponding error bars are indicated. The differences in growth between strain tetR-VPS1 without doxycycline and with doxycycline is statistically significant (* $p < 0.0001$). Similarly, the differences in growth between strain tetR-VPS1 without doxycycline and THE1-CIp10 is statistically significant (§ $p < 0.0001$). Statistical significance was tested by fitting growth curves to lognormal models which yielded R-squares of 99%.

Fig. 4. Vacuolar morphology of the *tetR-VPS1* strain

Vacuolar morphology was visualized by FM4-64 staining (A). A 10 µm bar is shown in either white or black. Thin-section electron microscopy images are shown in (B). A solid arrowhead is used to designate the vacuole. In either set of images, no major differences in vacuolar morphology were seen between the *URA3*-complemented wild-type strain THE1-Clp10 and the *tetR-VPS1* strain, with or without doxycycline.

Fig. 5. Secretion of proteolytic enzymes

Proteolysis assay on BSA plates (A). Overnight cultures were spotted onto BSA plates and incubated at 30 °C for 24 and 48 hours. The relative amount of extracellular protease activity is indicated by the halo surrounding the fungal colony. In the absence of doxycycline, strains THE1-Clp10 and tetR-VPS1 produced similar amounts of extracellular BSA degradation. In the presence of doxycycline, the tetR-VPS1 strain produced much less BSA degradation than strain THE1-Clp10. **Proteolysis assay in liquid BSA (B).** Overnight cultures were shifted to medium containing BSA as a sole nitrogen source and incubated at 30 °C for 24 hours, with and without doxycycline. The degree of proteolysis of BSA was analyzed by reducing SDS-PAGE and Coomassie blue staining. The tetR-VPS1 strain produced much less proteolysis in the presence of doxycycline compared to tetR-VPS1 without doxycycline or the control strain. Lanes from the same gel containing standard protein markers (M) and intact BSA are shown for reference. **Western analysis of Sap2p secretion (C).** Cell-free supernatants prepared as described in (B) were analyzed by Western blotting using anti-Sap2p antibodies (from M. Monod). Compared to the parental strain THE1-Clp10 (with and without doxycycline) and tetR-VPS1 (without doxycycline), much less Sap2 protein was secreted by the tetR-VPS1 strain when doxycycline was present. The triple deletion mutant strain *sap1-3Δ* (from B. Hube) was used as a negative control. rSap2 indicates purified recombinant Sap2p (from M. Monod) used as a positive control. **Analysis of lipase secretion (D).** Overnight cultures were spotted onto YNB-Tween 80 agar plates and incubated at 37 °C. The relative amount of lipolytic degradation after 3 and 6 days is indicated by the halo surrounding the fungal colony. The tetR-VPS1 strain produced much less extracellular lipolytic activity in the presence of doxycycline than corresponding controls. In contrast, no major differences in phospholipase activity were seen when assayed on egg-yolk agar plates (data not shown).

Fig. 6. Filamentation of strains grown in hyphal-inducing media

Filamentation on BSA and M199 agar plates (A). Overnight cultures of strains THE1-CIp10 and tetR-VPS1 were spotted onto BSA plates and incubated at 30 °C for 8 days. Robust filamentous structures emerging from the edge of each colony were observed in all strains, except in the tetR-VPS1 strain in the presence of doxycycline. Similar results were observed when these strains were spotted onto Medium 199 agar containing Earle's salts, buffered with 150 mM HEPES to pH 7.5, and incubated at 37 °C for 5 days. **Filamentation on Spider and 10% FCS agar plates (B).** Overnight cultures were spotted onto either YPD containing 10% fetal calf serum (FCS) or Spider agar medium and incubated at 37 °C for 5 days. On Spider agar, robust filamentation extending above the surface of the plate can be seen in all strains except for the repressed tetR-VPS1 strain, which did not produce a raised colony. Similarly, on YPD agar with 10% FCS, filamentation was robust in all strains except in the tetR-VPS1 strain grown in the presence of doxycycline. **Filamentation in liquid RPMI-1640 (C).** Filamentation was assessed in RPMI-1640 medium buffered to pH 7.3 with 165 mM MOPS, with or without doxycycline. Medium was inoculated with 5×10^6 cells/ml and incubated at 37 °C for 24 hr with constant shaking at 200 rpm. Strain THE1-CIp10 produced hyphae when grown in the presence or absence of doxycycline. In contrast, strain tetR-VPS1 produced masses of hyphae when grown under derepressing conditions, but not under repressing conditions. **Filamentation in YPD containing 10% FCS (D).** Pre-warmed medium was inoculated with 5×10^6 cells/ml and incubated at 37 °C with constant shaking at 200 rpm. Induction of filamentation was followed by light microscopy at fixed time points from the time of inoculum, with a four hour time point shown. The tetR-VPS1 strain remained predominantly

in the yeast form when grown in the presence of doxycycline, whereas, in contrast the other strains produced longer and greater numbers of filamentous structures.

Fig. 7.

Light microscopy of biofilms formed by strains THE1-CIp10 and tetR-VPS1 (A). The gross morphology of biofilms formed by strains THE1-CIp10 and tetR-VPS1, grown with and without doxycycline, were visualized with light microscopy. Relative values from the XTT-reduction assay are indicated. A statistically significant reduction (* $p < 0.001$) in biofilm mass was observed in the tetR-VPS1 strain grown under repressed conditions (with doxycycline).

Scanning electron microscopy of biofilms formed by the tetR-VPS1 strain (B). Biofilms formed by the tetR-VPS1 strain were visualized using scanning electron microscopy. Under derepressing conditions (without doxycycline), the tetR-VPS1 strain produced a thick biofilm that consisted predominantly of mature hyphae. Under repressing conditions (with doxycycline) the tetR-VPS1 strain produced a biofilm that was markedly less dense, and composed predominantly of yeast and pseudohyphal cells. The figures shown depict representative structures at 2,000x magnification with a 10 μ m bar indicated.

Table 1

Candida albicans strains used in this study.

Strain	Parent	Relevant genotype	Source/Reference
SC5314		wild-type	(Kurtz et al., 1986)
BWP17	SC5314	<i>ura3Δ/ura3Δ arg4Δ/arg4Δ hisΔ/his1Δ VPS1/VPS1</i>	(Wilson et al., 1999)
SLZK1	BWP17	<i>ura3Δ/ura3Δ arg4Δ/arg4Δ hisΔ/his1Δ VPS1/vps1Δ::dpl200-URA3-dpl200</i>	This study
THE1	CA18	<i>ade2Δ::hisG/ade2Δ::hisG ura3Δ::imm434/ura3Δ::imm434 ENO1/eno1Δ::ENO1-tetR-ScHAP4AD-3xHA-ADE2 VPS1/VPS1</i>	(Nakayama et al., 2000)
THE1-Cip10	THE1	<i>ade2Δ::hisG/ade2Δ::hisG ura3Δ::imm434/ura3Δ::imm434 ENO1/eno1Δ::ENO1-tetR-ScHAP4AD-3xHA-ADE2 RP10/RP10::URA3 VPS1/VPS1</i>	This study
SMB1	THE1	<i>ura3Δ::imm434/ura3Δ::imm434 VPS1/vps1Δ::dpl200-URA3-dpl200 ade2Δ::hisG/ade2Δ::hisG ENO1/eno1Δ::ENO1-tetR-ScHAP4AD-3xHA-ADE2</i>	This study
SMB1F	SMB1	<i>ura3Δ::imm434/ura3Δ::imm434 VPS1/vps1Δ::dpl200 ade2Δ::hisG/ade2Δ::hisG ENO1/eno1Δ::ENO1-tetR-ScHAP4AD-3xHA-ADE2</i>	This study
tetR-VPS1	SMB1F	<i>ura3Δ::imm434/ura3Δ::imm434 vps1Δ::dpl200/99t-VPS1-URA3 ade2Δ::hisG/ade2Δ::hisG ENO1/eno1Δ::ENO1-tetR-ScHAP4AD-3xHA-ADE2</i>	This study

Table 2

Primer sequences used in this study.

Primer	Primer sequence (5' – 3')
VPS1-CP5	CGAACATTTACTGTTGGTTTAGTC
VPS1-CP3	AACTGTCACTGTCAGTTTAGTGAG
ScVPS1-5	GCTCGAGGGTAAACCACTTGTGC
ScVPS1-3	GTATTGGACCAATTGAGGTATTAGG
VPS1-5DR	GTCCACCAAAATCAATAATTTACCAAAAAACGATAACAAACGAGTACTTTATTGACAACCGTTTTCACGTCACGACGTT
VPS1-3DR	AAGATAAATAATATACCACCGACTTCTGAAATAAAAAAGAAATTACTCTACTCTATAATACCTGTGGAATTGTGAGCGGATA
VPS1-5DRB	GAATGCATAGAGACAGTGCCACCAAAATCAATAATTTACCAAAAAACGATAACAAACGAGTACTTTATTGACAACCAATTGGTTTCCAGTCACGACGTT
VPS1-3DRB	ATAAACACACACTTATATTTATAAATTTGGTTTCATTAACTAAGCAGAAATTAATAAGAAGATAAATATATACCACCGACTTCTGTGTGGAAATTGTGAGCGGATA
VPS1-5DRC	GGTAGAAAAAATAATCTTTTATATATATTTCCCATACAAAGCAATTAAATCAAAACGATAGAAAGTTATATCAAAAATGGATGGTTTTCACGTCACGACGTT
VPS1-3DRC	AGAAATTACTCTACTCTATAATACCTCTAAACACTAGAAAACAATTTCACTAGCAATTTCTCAACACCTCAACCAATTTCTAAGTGGAAATTGTGAGCGGATA
VPS1-5DetA	GATCAAAACCGATGGTCGTGTG
VPS1-3DetA	ATTGCTCACAAAGTTCACAGGCC
VPS1-3DetC	GTGGATCAATTTCTTGCTTGC
VPS1-5SOU	CAAAACACCAGAACAAATTTGCCATTTCACC
VPS1-3SOU	AGCAAGACAAGAAATGATCCACTTACACAG
TetVPS1-5DR	TGTAAGTGGATCAATTTCTTGCTTGCTTAAATTTTGTGAATGCATAGAGACAGTGCCACC AAAATCGTAAATACGACTCACTATAGGG
TetVPS1-3DR	CCTCCACCTAAAGGAGCCAAATGCACTTGTAATTTGTGTAATGGTGGCAATCAATGTCCTCATCCATTTTCTAGTTTCTGAGATAAAAGCTG
TetVPS1-5Det	GTTCAAATGGAGGCTGGGTTTGTG
TetVPS1-3Det	CTAGTTTCTGAGATAAAGCTG
VPS1-5NB	GGCTGGACGTGTCATCCCAT
VPS1-3NB	GGTGATGTGGCTTGTGCTGG