Deletion of Vacuolar Proton-translocating ATPase V\textsubscript{o}\textsubscript{a} Isoforms Clarifies the Role of Vacuolar pH as a Determinant of Virulence-associated Traits in Candida albicans*

Summer M. Raines\textsuperscript{1}, Hallie S. Rane\textsuperscript{5}, Stella M. Bernardo\textsuperscript{4*}, Jessica L. Binder\textsuperscript{7}, Samuel A. Lee\textsuperscript{4*}, and Karlett J. Parra\textsuperscript{4,5,1}

From the \textsuperscript{4}Department of Biochemistry and Molecular Biology and the \textsuperscript{5}Department of Internal Medicine of the School of Medicine, University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131 and the \textsuperscript{1}Section of Infectious Diseases, New Mexico Veterans Healthcare System, Albuquerque, New Mexico 87108

Received for publication, October 9, 2012, and in revised form, December 20, 2012. Published, JBC Papers in Press, January 11, 2013, DOI 10.1074/jbc.M112.426197

Vaccular proton-translocating ATPase (V-ATPase) is a central regulator of cellular pH homeostasis, and inactivation of all V-ATPase function has been shown to prevent infectivity in Candida albicans. V-ATPase subunit a of the V\textsubscript{o} domain (V\textsubscript{o}\textsubscript{a}) is present as two fungal isoforms: Stv1p (Golgi) and Vph1p (vacuole). To delineate the individual contribution of Stv1p and Vph1p to C. albicans physiology, we created stv1Δ/Δ and vph1Δ/Δ mutants and compared them to the corresponding reintegrant strains (stv1Δ/ΔR and vph1Δ/ΔR). V-ATPase activity, vacuolar physiology, and in vitro virulence-related phenotypes were unaffected in the vph1Δ/Δ mutant. The vph1Δ/Δ mutant exhibited defective V1V0 assembly and a 90% reduction in concanamycin A-sensitive ATPase activity and proton transport in purified vacuolar membranes, suggesting that the Vph1p isoform is essential for vacuolar V-ATPase activity in C. albicans. The vph1Δ/Δ cells also had abnormal endocytosis and vacuolar morphology and an alkalinated vacuolar lumen (pH\textsubscript{vph1}, 6.8 versus pH\textsubscript{stv1}, 5.8) in both yeast cells and hyphae. Secreted protease and lipase activities were significantly reduced, and M199-induced filamentation was impaired in the vph1Δ/Δ mutant. However, the vph1Δ/Δ cells remained competent for filamentation induced by Spider media and YPD, 10% FCS, and biofilm formation and macrophage killing were unaffected in vitro. These studies suggest that different virulence mechanisms differentially rely on acidified vacuoles and that the loss of both vacuolar (Vph1p) and non-vacuolar (Stv1p) V-ATPase activity is necessary to affect in vitro virulence-related phenotypes. As a determinant of C. albicans pathogenesis, vacuolar pH alone may prove less critical than originally assumed.

Vaccular proton-translocating ATPase (V-ATPase)\textsuperscript{2} is critically involved in pH homeostasis in eukaryotic organisms and has been most thoroughly studied in Saccharomyces cerevisiae. V-ATPase is present both in the vacuole and throughout the endomembrane system, where it functions to energize membranes, acidify organelles, and regulate cytosolic pH (1, 2). V-ATPase is a multisubunit enzyme that is organized into two domains; the V\textsubscript{i} complex is bound peripherally to the cytosolic leaflet of the membrane, whereas the V\textsubscript{o} complex is membrane-bound. V\textsubscript{i} hydrolyzes ATP, causing rotation of a central rotor connected to a proteolipid ring structure within the V\textsubscript{o} complex. During rotation, this proteolipid ring binds protons from the cytosol and transfers them through a membrane channel and into organelles, thereby acidifying the organelle lumen (1).

The “a” subunit of V\textsubscript{o} (V\textsubscript{o}\textsubscript{a}) aids in assembly of the V\textsubscript{i} and V\textsubscript{o} complexes into a functional V-ATPase enzyme (3, 4). V\textsubscript{o}\textsubscript{a} is also involved in catalysis, as two semi-channels located within this subunit form the path for protons to enter and exit the complex (5). V\textsubscript{o}\textsubscript{a} is the only fungal V-ATPase subunit encoded by two functional homologs: STV1 and VPH1. These isoforms allow for organelle targeting of V-ATPase; in S. cerevisiae, complexes containing Stv1p localize to the Golgi and pre-vacuolar compartments, whereas Vph1p targets V-ATPase to the vacuole (6, 7). The S. cerevisiae isoforms also differ in their regulation and kinetics, as Vph1p-containing complexes uniquely dissociate in

---

\textsuperscript{*} This work was supported, in whole or in part, by National Institutes of Health Grants SR01GM086495 (to K. J. P.), K12GM088021 (to S. M. R.), and T32AI007538-13 (to S. M. B.). The work was also supported by a Department of Veterans Affairs MERIT Award (to S. A. L.) and a Biomedical Research Institute of New Mexico grant (to S. A. L.).

\textsuperscript{1} To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of New Mexico, MSC08 4670, Albuquerque, NM 87131. Tel.: 505-272-1633; Fax: 505-272-6587; E-mail: kjparra@salud.unm.edu.

\textsuperscript{2} The abbreviations used are: V-ATPase, vacular proton-translocating ATPase; V\textsubscript{o}\textsubscript{a}, a subunit of the V-ATPase V\textsubscript{o} complex; vma, vacular membrane ATPase; CMAC, 7-amino-4-chloromethylcoumarin; FDA-64, N\textsubscript{3}-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide; YPD, yeast extract/petepone/dextrose; BCECF-AM, 2\textsuperscript{-}7\textsuperscript{-}bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester; M199, Medium 199.

---

JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 288 • NUMBER 9 • MARCH 1, 2013

6190
response to glucose deprivation and display higher ratios of proton transport/ATP hydrolysis than complexes containing Stv1p (8).

Vph1p- and Stv1p-containing V-ATPase pumps may have specialized roles in fungal infections, because vacuolar and endocytic processes have been implicated in Candida albicans pathogenesis (9). C. albicans is the most frequently diagnosed fungal pathogen (10). The fungus normally exists commensally with humans; it colonizes the skin and mucosal membranes and can cause superficial infections even in healthy individuals. However, C. albicans can also enter the bloodstream via tissue damage or release from biofilms that form on medical implants and, once there, circulates throughout the body to cause sepsis and organ failure (11). These severe cases of systemic candidemia often occur during immunodeficiency, and patient mortality rates can reach 35% even with anti-fungal treatment (12).

C. albicans infectivity involves mechanisms interrelated with vacuolar and/or V-ATPase functions (2, 13) that depend on the expression of virulence factors, a set of genes and proteins that influence the numerous pathways used by C. albicans (14). For example, C. albicans secretes aspartyl proteinases and lipases that are involved in nutrient acquisition, host cell degradation, and immune evasion (15). In addition to protein secretion, other virulence-related pathways intertwined with V-ATPase-dependent events include iron acquisition from the environment, protection against reactive oxygen species produced by immune cells, production of host cell adhesion molecules, and formation of biofilms (14).

V-ATPase is a master regulator of pH, and notably, many C. albicans virulence pathways are regulated by pH. Activation and secretion of proteinases and lipases and the activity of the enzymes themselves occur over a range of pH optima (15). C. albicans exhibits pH-dependent morphological dimorphism; the fungus can exist both as a yeast form that is needed for dissemination throughout the environment and as a hyphal form that contributes to host tissue damage and invasion (16). The ability of C. albicans to form hyphae is tightly controlled by extracellular pH; an acidic environment favors growth of the non-pathogenic yeast form of C. albicans, although increasing pH triggers the morphological switch to hyphal growth and increased virulence (16). C. albicans has adapted pH-sensing mechanisms to accommodate its need to respond rapidly to pH changes (17).

Vacuolar pH appears to be particularly important in C. albicans virulence. Maintenance of a proton gradient across the vacuolar membrane allows for transport, storage, and detoxification of metabolites and ions (18). Numerous C. albicans mutants that display vacuolar alkalinization also display defective filamentation and a loss of in vivo virulence (9, 19–23). The most commonly used class of anti-fungal drugs, the azoles, appears to function in part through disruption of vacuolar acidification (23).

Given the importance of pH in C. albicans virulence and the fact that the fungal V-ATPase subunit c’ (VMA11) does not have mammalian orthologs, V-ATPase is an attractive anti-fungal drug target (13). Previous genetic studies in pathogenic fungi have associated a loss of V-ATPase activity and vacuolar acidification with virulence defects in Histoplasma capsulatum (24), Cryptococcus neoformans (25), and C. albicans (9, 26). Recent work has also shown that treatment of C. albicans with the anti-fungal drug fluconazole reduces V-ATPase activity and alkalinizes vacuoles; alternatively, restoration of vacuolar acidification causes fluconazole resistance and normal fungal growth (23). Importantly, Erickson et al. (25) have established a specific role for Vph1p in C. neoformans virulence. However, the isoform-specific roles of Stv1p versus Vph1p have not previously been examined in any pathogenic fungus, including C. albicans.

In the present study we created stv1Δ/Δ and vph1Δ/Δ C. albicans mutants to study the contribution of each V₀,a isoform to vacuolar function and in vitro virulence-related phenotypes. We demonstrate that loss of the individual V₀,a isoforms does not affect growth of C. albicans, although vacuolar V-ATPase activity and acidification are dramatically reduced specifically in the vph1Δ/Δ mutant. The vph1Δ/Δ mutant also exhibits defective secreted aspartyl protease and lipase activity. Despite the major disturbance in vacuolar pH, the vph1Δ/Δ cells are competent for filamentation, biofilm formation, and macrophage killing in vitro, suggesting that vacuolar acidification is not essential for these processes. Importantly, our work suggests that V-ATPase activity and pH homeostasis in both vacuoles and non-vacuolar compartments play key roles in these fungal virulence-related pathways, at least in vitro.

EXPERIMENTAL PROCEDURES

Targeted Disruption of C. albicans STV1 and VPH1—The C. albicans STV1 (orf 19.1190) and VPH1 (orf 19.6863) open reading frames were identified using the Candida Genome Database based on sequence homology to S. cerevisiae Stv1p (YMR054W) and Vph1p (YOR270C), respectively. C. albicans stv1Δ/Δ and vph1Δ/Δ mutants were created by disrupting both genomic alleles using a PCR-based homologous recombination strategy (27, 28). URA3-containing first-allele targeting cassettes were generated using DR PCR primers (see Table 1) and plasmid pDDB57 as a template (28). To create heterozygous null mutants, the C. albicans BWP17 background strain was transformed directly with the PCR reaction mixtures using the lithium acetate method, and uridine prototrophs were selected using synthetic media lacking uracil and uridine (0.67% yeast nitrogen base without amino acids, 0.079% complete synthetic mixture lacking uracil and uridine, 2% glucose).

ARG4-containing second-allele targeting cassettes were generated using DR PCR primers (see Table 1) and plasmid pRS-Arg4ΔSpel as a template (27). To create homozygous null mutants, selected heterozygous null mutants were transformed directly with the PCR reaction mixture, and arginine prototrophs were selected using synthetic media lacking arginine. Histidine prototrophy was restored by transforming the homozygous null strains with pGEM-HIS1 linearized with NruI and selecting using synthetic media lacking histidine (27).

The open reading frames of STV1 (plus 273 bp upstream and 227 bp downstream) and VPH1 (plus 401 bp upstream and 297 bp downstream) were amplified using primers found in Table 1 and subcloned into pGEM-HIS1. The cloned STV1 and VPH1 genes were sequenced and compared with the Candida Genome Database Assembly 21 annotated sequence. These
The V-ATPase V1A subunit was visualized with a 1:000 dilution by SDS-PAGE and transferred to nitrocellulose overnight at 150 mA. The V1A subunit was cross-reacts with the presence of anti-human V1A rabbit polyclonal antibody (30); the anti-

reintegrant constructs were digested with NruI, transformed into the homozygous null mutants, and selected using synthetic media lacking histidine to generate isogenic, complemented reintegrant strains.

Genomic DNA was extracted from multiple independent transformants using the Masterpure Yeast DNA Kit (Epicenter). Integration of the gene targeting cassettes and reintegrant constructs was verified by PCR product size using gene-specific detection primers (DET) that span the targeted region. Integration of the gene targeting cassettes and reintegrant constructs was verified by PCR product size using gene-specific primers (INT) targeting an internal region of the open reading frame, whereas the presence of HIS1 was confirmed with HIS primers (Table 1 and see Fig. 1).

C. albicans strains used in this study are outlined in Table 2. Phenotypes were verified in two independent clonal lines of each strain; data from only one clonal line are presented in this study. Strains were routinely maintained at 30 °C in YPD (1% yeast extract, 2% peptone, 2% glucose) supplemented with 80 μg/ml uridine.

Growth Phenotyping—Overnight cultures were washed twice with phosphate-buffered saline. Cells were counted with a hemocytometer. Cultures were diluted to a concentration of 10⁶ cells/ml. 5-Fold serial dilutions were stamped onto pH 4 and pH 5 complete synthetic media plates (buffered with 50 mM HEPES, 50 mM Na₂PO₄ (pH 7.6), 2% glucose). Stained cells were incubated for 48–72 h at 30 °C.

V-ATPase Assembly and Activity Assays—Vacuolar membranes were purified by Ficoll density gradient centrifugation (29). For Western blots, vacuolar protein (80 μg) was separated by SDS-PAGE and transferred to nitrocellulose overnight at 150 mA. The V₁A subunit was visualized with a 1:000 dilution of anti-human V₁A rabbit polyclonal antibody (30); the antibody cross-reacts with the C. albicans protein.

For ATP hydrolysis, vacuolar vesicles (15 μg) were added to an enzymatic assay in which the rate of ATP hydrolysis is coupled to the oxidation of NADH, measured as a loss of A₃₄₀ over time (31). The V-ATPase inhibitor concanamycin-A (100 nm) was used to assess V-ATPase-specific activity.

Proton transport of purified vacuolar vesicles (30 μg) was measured via quenching of 1 μM 9-aminoo-6-chloro-2-methoxyacridine upon the addition of 0.5 mM ATP, 1 mM MgSO₄ (MgATP) as described previously (32, 33). Fluorescence at 410-nm excitation/490-nm emission was monitored for 1 min before MgATP addition and for an additional 40 s after. Proton transport was calculated as the change in fluorescence for the first 15 s after MgATP addition. Concanamycin-A inhibitor (100 nm) was used to assess V-ATPase-specific activity.

Vacuolar Morphology—Live cells were co-stained with the lipophilic dye N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylylamino)phenyl)hexatrienyl)pyridinium dibromide (FM4-64, from Invitrogen) and with 7-amino-4-chloromethylcoumarin (CMAC, from Invitrogen). Cells were grown to early log phase and resuspended at 2–4 ×10⁶/ml in YPD. FM4-64 (40 μM) was added, and cells were incubated for 15 min at 30 °C, 250 rpm. For the 15-min time course, cells were then resuspended in 100 μM CMAC in 10 mM HEPES, 5% glucose (pH 7.4) and incubated for 15 min at room temperature without shaking. Cells were resuspended in 10 mM HEPES, 5% glucose (pH 7.4) and vacuolar morphology was observed via fluorescence microscopy using a Zeiss Observer Z1. For the 1 h time-course, cells were resuspended in fresh YPD after FM4-64 staining and incubated for an additional 45 min at 30 °C, 250 rpm before CMAC staining was performed as described above.

Vacuolar pH Measurements—Quinacrine accumulation was used to qualitatively assess vacuolar pH in live C. albicans cells; quinacrine is only fluorescent under acidic conditions. Cells were grown in YPD to early log phase, and 1 ml of cell suspension was cooled for 1 min on ice and pelleted. Cells were resuspended in 200 μM quinacrine in YPD buffered with 50 mM Na₂PO₄ (pH 7.6) and incubated at room temperature for 5 min. Cells were then cooled on ice for 5 min and washed twice (100 mM HEPES, 50 mM Na₂PO₄ (pH 7.6), 2% glucose). Stained cells were resuspended in 200 μl of wash buffer and visualized via fluorescence microscopy using a Zeiss Observer Z1.

### TABLE 1

| Primer     | Sequence (5’-3’)                                      | Purpose                                      |
|------------|-------------------------------------------------------|----------------------------------------------|
| STV1–5DR   | CAGTCTTTCACAAATTCGCCATATCTACGTGATA                   | Generate STV1 targeting cassette             |
| STV1–3DR   | TATAACACGCTACCAGCTGTAACGATAGGCGCAGGTT                | Generate STV1 targeting cassette             |
| VPH1–5DR   | CAACTTACCTACCTGTGTTATTTCCGCGATGCTGAT                | Generate VPH1 targeting cassette             |
| VPH1–3DR   | TTATATGCTTTATATTATTTTGAGTTATTTTAGGTTGCT             | Generate VPH1 targeting cassette             |
| 5Ndel-STV1 | AGCTCTCAATGTAATCAGTTCGCGACAC                           | Generate wild-type STV1 reintegrant          |
| 3Mdel-STV1 | TATAACACGCTACCAGCTGTAACGATAGGCGCAGGTT                | Generate wild-type STV1 reintegrant          |
| 3Mdel-VPH1 | AGCTCTAACGCTACCAGCTGTAACGATAGGCGCAGGTT               | Generate wild-type VPH1 reintegrant          |
| STV1–5DET  | GCGTGAGATCCACGACGATGC                                 | Detect size of allele in STV1 genomic locus  |
| STV1–3DET  | CAGTCTTTCACAAAATTCGCCATATCTACGTGATA                  | Detect size of allele in STV1 genomic locus  |
| VPH1–5DET  | GATACGCTTACCTGTGTTATTTCCGCGATGCTGAT                 | Detect size of allele in VPH1 genomic locus  |
| VPH1–3DET  | CACTCCAAATGCGATGAGT                                 | Detect size of allele in VPH1 genomic locus  |
| STV1–INTF  | GAGCGGATGACGACGATGCA                                  | Detect presence/absence of STV1              |
| STV1–INTR  | GCCATCTTCACTTGCTGAC                                   | Detect presence/absence of STV1              |
| VPH1–INTF  | TACCTGAAATGCGACCAAC                                   | Detect presence/absence of VPH1              |
| VPH1–INTR  | GTTGGTGCCCTCCGTGAGCAA                                 | Detect presence/absence of VPH1              |
| GEM3HISR   | CTCCTCCACGCAACGTTGAC                                  | Detect presence of HIS1                      |
| HIS3AMP    | GTCAGGTTGGCGCAGGAC                                   | Detect presence of HIS1                      |

Primers used in this study

**V-ATPase Vₐ Subunit Isoforms in C. albicans**
Cells were also stained with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM, from Invitrogen), a pH-sensitive fluorophore that accumulates in the fungal vacuole. The fluorescence excitation profile of BCECF changes with pH, and the ratio of fluorescence intensities measured at two different excitation wavelengths can, therefore, be used to quantitatively measure vacuolar pH (33–35). Cells were grown to 0.4–0.8 A600/mL, harvested (60 A600), resuspended in 120 μL YPD, and stained with 50 μM BCECF-AM for 30 min at 30 °C with shaking. Stained cells were harvested, washed, and resuspended in 120 μL of complete synthetic media. To generate a pH calibration curve for each strain, 20 μL of stained cells were incubated for 30–45 min at 30 °C with 2 mL of each pH calibration buffer (50 mM MES, 50 mM HEPES, 50 mM KCl, 50 mM NaCl, 200 mM ammonium acetate, 10 mM sodium azide, 10 mM 2-deoxyglucose; pH range from 5.5–8.0) and 50 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) ionophore; this allows the calibration buffer to equilibrate with the vacuolar lumen. The fluorescence intensity at 535 nm was measured at both 490-nm and 450-nm excitation, and the 490/450-nm ratio versus pH was used to generate a calibration curve. Stained cells (20 μL) were then added to 2 mL of sample buffer (1 mM HEPES/MES (pH 5.0), 2% glucose). Fluorescence intensity was measured for 10 min as described above, and the average fluorescence over the last 7 min was used to calculate the 490/450-nm ratio. Background fluorescence (10 A600 unstained cells in 2 mL sample buffer) was subtracted, and vacuolar pH was calculated from the calibration curve.

For quinacrine staining in C. albicans hyphae, liquid YPD + 10% fetal calf serum (FCS) was inoculated at a starting density of 5 × 10^6 cells/mL (5 A600/mL). For BCEF staining, YPD + 10% FCS was inoculated to achieve a target density of 0.4–0.8 A600/mL at the start of the assay (24 h post-hyphal induction). For both quinacrine and BCECF, these starter filamentation cultures were incubated at 37 °C with shaking for up to 24 h, and vacuolar pH was measured at selected time points using the above methods.

**Analysis of C. albicans Virulence-related Phenotypes**—For plate assays, 3 μL of overnight cultures grown for ~18 h were used for spotting. Extracellular aspartyl protease secretion/activity was assayed on BSA plates after incubation at 30 °C for 3 days (36). Lipase secretion/activity was assayed on yeast nitrogen base plates containing 2.5% Tween 80 after incubation at 37 °C for 6 days (37). The amount of extracellular enzyme secretion/activity was quantified by measuring the diameter of the degradation halo surrounding the fungal colony; halo diameters were normalized to the diameter of the fungal colony. Filamentation was assayed at 37 °C for 7 days on the following solid media: Medium 199 (M199) containing Earle’s salts (Invitrogen) supplemented with L-glutamine and buffered to pH 7.5 with 150 mM HEPES, Spider medium (38), and YPD + 10% FCS. For embedded agar assays, cells were grown at 25 °C overnight, washed, and mixed with molten YPD agar (~250 cells per plate). Solidified plates were incubated at 30 °C for 3 days.

**RESULTS**

To study the importance of the individual Vα subunit isoforms of V-ATPase in C. albicans physiology, we generated both stv1/stv1 (stv1Δ/stv1 hereafter) and vph1/vph1 (vph1Δ/vph1 hereafter) homozygous null mutants via PCR-mediated gene disruption. We also constructed isogenic, complemented reintegrand strains (stv1Δ/R and vph1Δ/R); these strains should restore wild-type characteristics and are used to confirm the specificity of the genetic mutations. All strains are described and validated in Table 2 and Fig. 1.

**Loss of V-ATPase Vα Isolforms Vph1p and Stv1p Separately Does Not Reduce Growth of C. albicans**—The S. cerevisiae vph1Δ mutant displays a mild vmaΔ (vacuolar membrane ATPase) phenotype characterized by normal growth at acidic pH and reduced growth at neutral pH (41), whereas the stv1Δ strain does not exhibit vmaΔ mutant growth. Loss of both Vα isofoms is needed to elicit a strong vmaΔ phenotype (41), possibly due to isomer redundancy in S. cerevisiae. We anticipated that the counterpart C. albicans Vα mutants would display similar growth characteristics. To test this hypothesis, we plated each strain on pH-buffered media and grew them at 30 °C. In contrast to S. cerevisiae, both C. albicans Vα mutants displayed normal growth at every pH tested (pH 4.0–8.5) (Fig. 2). Thus, either Vph1p or Stv1p is sufficient to sustain normal C. albicans growth across a broad pH range.

**TABLE 2**

| Strain | Parent | Genotype | Source |
|--------|--------|----------|--------|
| DAY185 (prototrophic wild-type) | | his1:G::HIS1, his1::HIS1, ura3::LAMb, hisG::ARG4, ura3::LAMb, arg4::hisG | (66) |
| BWP17 (auxotrophic background) | | his1:G::HIS1, ura3::ARG4, arg4::hisG, STV1::STV1, VPH1::VPH1 | (27) |
| stv1Δ+/− (heterozygous null) | BWP17 | | This study |
| stv1Δ−/− (homozygous null − HIS1) | | his1::HIS1, ura3::ARG4, arg4::hisG, STV1::STV1, VPH1::VPH1 | This study |
| vph1Δ+/− (heterozygous null) | BWP17 | | This study |
| vph1Δ−/− (homozygous null − HIS1) | | his1::HIS1, ura3::ARG4, arg4::hisG, STV1::STV1, VPH1::VPH1 | This study |

**V-ATPase Vα Subunit Isoforms in C. albicans**
V-ATPase Vα Subunit Isoforms in C. albicans

Loss of VPH1 Drastically Reduces Vacuolar ATPase Activity and Disrupts Vacuolar Physiology in C. albicans—Vph1p localizes to the vacuole in S. cerevisiae (6). However, S. cerevisiae Strains with Vph1p partially contributes to vacuolar V-ATPase activity if Vph1p is absent despite its primary localization in Golgi and pre-vacuolar compartments (42). To determine the contribution of each Vα isoform to vacuolar biogenesis in C. albicans, we assessed V-ATPase complex assembly and measured ATP hydrolysis and proton transport in purified C. albicans vacuolar membrane vesicles.

In S. cerevisiae, loss of any Vα subunit prevents assembly of the integral Vα domain. As a result, the peripheral V1 domain is not present at the membrane (43). To assess assembly of vacuolar V1Vα complexes in our Vα C. albicans mutants, we performed Western blots with anti-V1A antibodies; detection of the catalytic V1A subunit in isolated vacuolar membranes will occur if the V-ATPase complex (V1Vα) is assembled. Subunit V1A was detected in all C. albicans strains except vph1Δ/Δ, which showed a complete lack of V1A subunit at the membrane (Fig. 3A), indicating that as in S. cerevisiae, Vph1p likely functions at the vacuolar membrane, and lack of a Vα subunit prevents V1Vα assembly.

The V1 complex is responsible for hydrolyzing ATP, whereas the Vα complex is involved in proton pumping (1). Active transport of protons by V-ATPase pumps requires properly assembled V1Vα complexes. In agreement with the lack of V1Vα complex assembly, we noted an ~90% reduction in concanamycin A-sensitive ATP hydrolysis specifically in the vph1Δ/Δ mutant vacuolar membranes (Fig. 3B). Proton transport was also dramatically impaired (~90% reduction) in the vph1Δ/Δ mutant (Fig. 3C). As expected, its isogenic reintegrant strain, vph1Δ/ΔR, recovered full activity (Fig. 3, B and C). Vacular membranes from the stv1Δ/Δ strain retained wild-type levels of concanamycin A-sensitive ATP hydrolysis and proton transport, suggesting that as in S. cerevisiae, complexes containing Stv1p regulate non-vacuolar V-ATPase activity. Together, these data imply that Vph1p is likely the predominant Vα isoform in the C. albicans vacuole.

Vph1p has been associated with vacuolar membrane fusion and endocytic processes in S. cerevisiae (42, 44), and deficiencies in vacuolar trafficking often lead to alterations in vacuolar morphology in C. albicans (45). We examined vacuolar morphology in the Vα mutants by staining live cells with FM4-64 and CMAC. Upon endocytosis, the lipophilic dye FM4-64 stains vacuolar membranes (46), whereas CMAC passively accumulates in the vacuolar lumen (47). Fig. 4 illustrates that vph1Δ/Δ cells displayed abnormal endocytic trafficking, as much of the FM4-64 staining remains at the cell membrane, even after 1 h of incubation. Vacuolar morphology also appears abnormal in the vph1Δ/Δ cells, as FM4-64 dye that does internalize to the vacuole accumulates in multiple vesicular structures instead of in a defined vacuolar membrane. These results resemble those seen in the S. cerevisiae vph1Δ mutant (42). The corresponding reintegrant strain, vph1Δ/ΔR, had apparent normal endocytosis and vacuolar membrane structure, similar to DAY185 (wild-type), stv1Δ/Δ, and stv1Δ/ΔR cells. These observations are consistent with the notion that membranes having Vph1p- and Stv1p-containing V-ATPase complexes function differently.

In agreement with the lack of proton transport noted in Fig. 3C, the vacuolar pH of the vph1Δ/Δ mutant was significantly more alkaline than the vph1Δ/ΔR strain, as assessed qualitatively by quinacrine staining (Fig. 4B) and quantitatively by measuring florescence of the ratiometric pH-sensitive dye BCECF (Fig. 4C). Alkalization of the vph1Δ/Δ vacuolar lumen from pH 5.95 ± 0.01 to pH 6.76 ± 0.05 validated the quinacrine observations. The stv1Δ/Δ mutant was normal for all markers of vacuolar physiology tested, corresponding to a likely non-vacuolar cellular distribution and its minimal effect on vacuolar V-ATPase function (Fig. 3). From these studies, we conclude that like S. cerevisiae,
C. albicans Vph1p (but not Stv1p) is required for proton transport across the vacuolar membrane and vacuolar biogenesis. Loss of VPH1 Leads to Alterations in Some C. albicans Virulence-related Traits without Major Changes in in Vitro Filamentation, Biofilm Formation, or Macrophage Killing—C. albicans uses a variety of virulence pathways, many of which

**FIGURE 3.** Vacuolar ATPase activity is drastically reduced in *C. albicans* vph1ΔΔ mutants. A, VvoV1 V-ATPase complexes are not properly assembled at the vacuolar membrane in *vph1ΔΔ* mutants. Purified vacuolar vesicles were analyzed by Western blot using an anti-V1A antibody. A representative image (from three blots of three independent vacuolar preps) is shown. B, ATP hydrolysis is dramatically reduced in *vph1ΔΔ* mutants. Concanamycin-A-sensitive ATP hydrolysis of purified vacuolar vesicles was measured spectrophotometrically using an enzyme assay coupled to NADH oxidation at 340 nm. The average DAY185-specific activity was 0.95 μmol of Pi/min/mg. C, proton transport is dramatically reduced in *vph1ΔΔ* mutants. ATP-dependent proton transport of purified vacuolar vesicles was measured via fluorescence quenching of 1 μM 9-amino-6-chloro-2-methoxyacridine upon the addition of 0.5 mM ATP, 1 mM MgSO4. Proton transport was calculated as the change in fluorescence for the first 15 s after ATP addition. The average DAY185 slope was 1340.75 fluorescence units/15 s. Percentage activities are expressed relative to DAY185 and are shown as the average ± S.D. for n = 3–6 separate vacuolar purifications; percent reductions in activity are indicated. ***, p < 0.001; ****, p < 0.0001 versus the reintegrant strain (R) as measured by a two-tailed unpaired Student’s t test.

**FIGURE 4.** Vacuolar biogenesis is disrupted in *C. albicans* vph1ΔΔ mutants. A, endocytosis to the vacuole and vacuolar morphology was impaired in *vph1ΔΔ* mutants. Live *C. albicans* cells were stained with FM4-64 dye (red) and CMAC dye (blue) to mark the vacuolar membrane and the vacuolar lumen, respectively. Representative differential interference contrast and fluorescence images are shown. FM4-64 internalization to the vacuole was assessed after 15 min and after 1 h. B and C, the vacuolar lumen is alkalinized in *vph1ΔΔ* mutants. Quinacrine accumulation (B) was used to qualitatively assess vacuolar acidification in live *C. albicans* cells; quinacrine fluorescence is quenched as pH increases. Representative differential interference contrast and fluorescent images are shown. BCECF accumulation (C) was used to quantitatively assess vacuolar pH in live *C. albicans* cells; BCECF fluorescence increases with pH. Cells were stained with 50 μM BCECF-AM for 30 min, and the average fluorescence over 7 min was compared with a standard curve to generate absolute pH values. Vacuolar pH is expressed as the average ± S.D. for n = 3–6 replicates. ****, p < 0.0001 versus the reintegrant strain (R) as measured by a two-tailed unpaired Student’s t test.

*C. albicans* Vph1p (but not Stv1p) is required for proton transport across the vacuolar membrane and vacuolar biogenesis.

Loss of VPH1 Leads to Alterations in Some *C. albicans* Virulence-related Traits without Major Changes in in Vitro Filamentation, Biofilm Formation, or Macrophage Killing—*C. albicans* uses a variety of virulence pathways, many of which
require an acidic vacuole for activity. Thus, maintenance of vacuolar pH is considered a critical component of \textit{C. albicans} virulence (9, 13, 19 –23), and we hypothesized that the alkalinized vacuoles noted in the \textit{vph1ΔΔ} mutants (Fig. 4), should result in reduced virulence-related phenotypes \textit{in vitro}.

To initiate host cell invasion, \textit{C. albicans} secretes aspartyl proteases and lipases responsible for degrading the host cell extracellular matrix and cell membrane, respectively. These enzymes are synthesized as pro-proteins that require activation by vacuolar proteases and secretion with an acidic pH optimum (15). Protease secretion can be assayed \textit{in vitro} on plates containing BSA, whereas lipase secretion is assayed on plates containing Tween 80. In each assay, the amounts of extracellular enzyme activity are quantified by the size of the halo surrounding the fungal colony, which corresponds to the extracellular enzyme activity present. Halo diameters were normalized to the diameter of the colony itself; therefore, a ratio of 1 indicates a lack of detectable halo. Halo size is shown as the average ± S.E. for \( n = 8 \) – 16 replicates. *, \( p < 0.05 \); **, \( p < 0.01 \) versus the reintegrant strain (R) as measured by a two-tailed unpaired Student’s t-test.

extracellular protease and lipase activities were unaffected in the \textit{stv1ΔΔ} mutant, indicating that only Vph1-containing membranes play a part in these processes.

Vacuolar acidification appears to be particularly important for the formation of hyphae (9, 19 –23), when \textit{C. albicans} undergoes the dimorphic transition from a yeast form at acidic environmental pH to a filamentous form at neutral-to-alkaline pH (16). Despite strikingly abnormal vacuolar morphology and luminal pH (Fig. 4), \textit{vph1ΔΔ} filamentation was only partially altered compared with the \textit{vph1ΔΔR} strain (Fig. 6A); the cells responded differently to different stressors. The \textit{vph1ΔΔ} filaments formed in embedded agar were shorter, and loss of hyphal formation was noted when the cells were plated on M199 media, an amino acid-rich media that weakly induces filamentation. All \( V_\text{a} \) mutant strains, including the \textit{vph1ΔΔ} mutant, displayed wild-type levels of hyphal formation on \textit{Spider} media and YPD + 10% FCS, both known to strongly induce filamentation (Fig. 6A). All \( V_\text{a} \) mutant strains also showed normal filamentation in liquid induction media, including RPMI 1640,\textsuperscript{3} \textit{Spider},\textsuperscript{2} and YPD + 10% FCS (Fig. 7A). Together, these data suggest that individual filamentation pathways differentially rely on vacuolar pH homeostasis and challenge the

\textsuperscript{3} S. M. Raines, H. Rane, S. M. Bernardo, J. L. Binder, S. A. Lee, and K. J. Parra, unpublished results.
dig that vacuole acidification is crucial for *C. albicans* filament formation, at least in vitro.

To further elucidate the contributions of Vph1p and vacuolar pH to virulence-related processes in *C. albicans*, we examined the ability of *vph1Δ/Δ* cells to form biofilms and kill mammalian macrophages in vitro. Biofilms are microbial communities surrounded by a thick extracellular matrix; *C. albicans* biofilms are highly resistant to anti-fungal therapy (49). Macrophages play an important role in the defense against *C. albicans* infection (50). There was no significant change in biofilm density (Fig. 6B) or macrophage survival (Fig. 6C) in the *vph1Δ/Δ* mutants, providing additional evidence that maintenance of an acidic vacuolar lumen is not essential for *C. albicans* virulence-related phenotypes in vitro.

**Induction of Hyphal Formation Does Not Rescue Vacuolar pH in *vph1Δ/Δ* C. albicans**—The work presented here suggests that vacuolar alkalization (via loss of Vph1p in the *vph1Δ/Δ* mutant) is not sufficient to disrupt the ability of *C. albicans* to form filaments in vitro. However, this assumption is based on measurements of vacuolar pH made only in the yeast form of the *vph1Δ/Δ* mutant (Fig. 4). In *S. cerevisiae*, functional Stv1p can transit to the vacuole in *vph1Δ* cells (42), especially when *STV1* is overexpressed (8, 41). We asked whether induction of hyphal formation triggers a mechanism to restore vacuolar acidification and assure continued filamentation. One possible mechanism is Stv1p compensation for loss of Vph1p, similar to the phenomenon seen in *S. cerevisiae*. We could expect up-regulation of *C. albicans STV1* expression or ectopic localization.
provide strong evidence that vacuolar acidification is not essential for filamentation in \textit{C. albicans} as measured \textit{in vitro}. This work also implies that the Stv1p-containing V-ATPase complexes in non-vacuolar organelles of the \textit{vph1Δ/Δ} cells play important, previously under-appreciated roles in \textit{C. albicans} filamentation and several virulence-associated traits.

**DISCUSSION**

We have demonstrated that the Vph1p isoform of the V\textsubscript{o,a} subunit is specifically required for vacuolar V-ATPase activity and acidification of the \textit{C. albicans} vacuole. We also show that vacuolar pH dysfunction is not sufficient to prevent cell growth, filamentation, or a number of virulence-related phenotypes \textit{in vitro}, including biofilm formation and macrophage killing.Thus, although vacuolar acidification contributes to \textit{C. albicans} virulence (9, 19–23), it is not essential, at least \textit{in vitro}. In addition, this study suggests that despite its lack of function in vacuolar processes, the Stv1p isoform of V\textsubscript{o,a} contributes significantly to many virulence-related pathways when its Vph1p homolog is not functional.

With the exception of \textit{VPH1} and \textit{STV1}, deletion of a V-ATPase structural gene causes the \textit{vma} growth phenotype in \textit{S. cerevisiae}. However, the normal growth of the \textit{vph1Δ/Δ} \textit{C. albicans} strain (Fig. 2) was surprising, given that \textit{S. cerevisiae} \textit{vph1Δ} mutants display a mild \textit{vma} phenotype (41). \textit{C. albicans} \textit{vma7Δ/Δ (V1F)} mutants, which presumably lack all cellular V-ATPase function, display significant pH-dependent growth defects in \textit{C. albicans} (9), thereby resembling \textit{S. cerevisiae} \textit{vmaΔ} mutants (2, 13). We anticipate that loss of both V\textsubscript{o,a} isoforms, which also eliminates V-ATPase function completely, will be necessary to develop pH-dependent growth impairment (\textit{vma} phenotype) in \textit{C. albicans}. However, constructing the \textit{vph1/vph1::stv1/stv1} double deletion strain will be challenging due to a lack of readily available dominant selection markers for \textit{C. albicans} and the established difficulty of creating \textit{C. albicans} cells lacking all cellular V-ATPase activity.\textsuperscript{3} Therefore, \textit{vph1/vph1::stv1/stv1} double deletion studies remain to be performed.

Normal growth of \textit{C. albicans} \textit{vph1Δ/Δ} does not result from Stv1p compensation at the vacuole, as V-ATPase assembly and activity in vacuolar membranes is virtually absent in \textit{vph1Δ/Δ} mutant cells (Fig. 3). Rather, Stv1p may be involved in maintenance of an acidic pH in late Golgi compartments, a requirement for growth in \textit{S. cerevisiae} (51). Finnigan \textit{et al.} (7) recently identified a localization sequence within \textit{S. cerevisiae} Stv1p, W(83)KY, that targets V-ATPase complexes to the Golgi/endosomal network. Although no such sequence exists near amino acid position 569. Thus, further study is needed to definitively identify the cellular localization of Stv1p in \textit{C. albicans} and to determine its physiological function and activity in wild-type and \textit{vph1Δ/Δ} mutant cells.

\textit{C. albicans} \textit{vma7Δ/Δ} mutants accumulate FM4-64 in intra-vacuolar structures, implying a block in vacuolar degradation (9). The \textit{vph1Δ} mutants in both \textit{C. albicans} (Fig. 4A) and \textit{S. cerevisiae} (42) display impaired endocytosis and abnormal vacuolar morphology characterized by the lack of definitive vacuolar membrane staining and accumulation of FM4-64 in

**FIGURE 7.** Induction of hyphal formation does not rescue vacuolar pH in \textit{vph1Δ/Δ C. albicans}. \textbf{A–C}. \textit{C. albicans} filamentation occurs despite elevated vacuolar pH. Hyphae formation was induced over 24 h in liquid YPD +10% FCS. Quinacrine accumulation into hyphae was used to qualitatively assess vacuolar pH during filamentation as described in the legend to Fig. 4B. Representative differential interference contrast and fluorescent images are shown at 4 h (A) and 24 h (B) post-hyphae induction; results were similar at 1 and 2 h.\textsuperscript{5} BCECF accumulation into hyphae was used to quantitatively assess vacuolar pH during filamentation as described in the legend to Fig. 4C. Vacuolar pH was calculated at 24 h post-hyphae induction (C) and is expressed as the average ± S.D. for \( n = 3–6 \) separate readings. ****, \( p < 0.0001 \) versus the reintegrant strain (R) as measured by a two-tailed unpaired Student’s t test.

of Stv1p to the vacuole, thereby rescuing the vacuolar pH phenotype.

To address this issue and clarify whether vacuolar acidification is essential for \textit{C. albicans} filamentation \textit{in vitro}, we measured vacuolar pH in the hyphal form of our \textit{C. albicans} V\textsubscript{o,a} mutants to directly correlate filamentation status with vacuolar pH. All V\textsubscript{o,a} mutant strains formed filaments in response to liquid YPD +10% FCS (Fig. 7, A and B), a media that induces filamentation in \textit{C. albicans}. Importantly, the vacuolar pH of the \textit{vph1Δ/Δ} mutants remained significantly elevated over 24 h (pH 6.8 compared with pH 5.8 for all other strains) despite displaying filamentous morphology (Fig. 7, A–C). These data
vesicular structures. The finding that endocytosis and vacuolar membrane structure are unaffected in C. albicans stv1/Δ cells (Fig. 4A) distinguishes C. albicans from S. cerevisiae. S. cerevisiae stv1/Δ displays normal endocytosis but fragmented vacuoles (42), which may be due to a role for Stv1p in the budding of endosomal-bound vesicles from the Golgi (42). Despite the commonalities between the two fungal species in regard to Vph1p functions, these results highlight an important difference in Stv1p function and underscore that caution must be taken when directly extrapolating results from S. cerevisiae to C. albicans.

Augsten et al. (52) previously demonstrated that loss of FAB1, a phosphatidylinositol 3-phosphate 5-kinase, leads to defective vacuolar acidification in C. albicans. Notably, this vacuolar pH defect did not affect virulence as measured by epithelial cell adhesion, kidney fungal burden, and survival in a murine model of systemic candidemia. The fab1Δ/Δ results provide precedence that vacuolar acidification is not necessarily a prerequisite for C. albicans virulence. Our conclusion that an acidic vacuolar lumen is not essential for C. albicans biofilm formation and macrophage killing in vitro supports this concept. However, it should not diminish the importance of vacuolar functions in pathogenesis, including vacuolar expansion (40, 53), trafficking (45, 54), and heavy metal sequestration (9, 26).

The results presented here imply that loss of vacuolar acidification is necessary (hence lack of any measureable phenotype in our stv1/Δ mutants) but not sufficient to entirely prevent virulence-related phenotypes in vitro. The vph1Δ/Δ mutants display significantly reduced extracellular protease and lipase secretion and abnormal filamentation in M199 medium and embedded agar. In contrast, filamentation in response to both Spider media and YPD + 10% FCS was unaffected, and in vitro biofilm formation and macrophage killing were normal (Fig. 6). Our findings suggest that different virulence-associated traits differentially rely on vacuolar physiology. Enzyme secretion and activity appear to require an acidified vacuole. In contrast, hyphal formation in response to strong induction signals does not require vacuolar acidification, suggesting that vacuolar function is less critical when multiple stressor signals are triggered. In vitro biofilm formation and macrophage killing also do not require vacuolar acidification. These various pathways may then collaborate to yield a complete virulence-related phenotype.

S. cerevisiae V-ATPase is induced during starvation and filamentous growth (55). In addition to their role in virulence (16), hyphae improve nutrient foraging during times of starvation due to their larger surface area and ability to penetrate the extracellular environment (55). Vacuolar function is important for mobilization of nutrient stores and amino acid sequestration (56), and V-ATPase is involved in amino acid sensing and storage at the vacuole (57, 58). This could help explain the selective defect in filamentation seen in vph1Δ/Δ mutants plated on M199 (Fig. 6A), a media that mimics nutrient deprivation through use of amino acids as its main nutrient source.

At first glance, our data appear to contradict previous work by Erickson et al. (25) demonstrating that loss of Vph1p in C. neoformans leads to both vacuolar alkalization and defective virulence, as measured both in vitro and in vivo. However, the V_α subunit appears to be encoded by a single gene in C. neoformans; a STV1 homolog is not present in the C. neoformans genome (25). Therefore, in contrast to C. albicans vph1/Δ mutants, the virulence-related defects seen upon loss of C. neoformans VPH1 are due to depletion of total cellular V-ATPase, again emphasizing the roles that V-ATPases in both vacuolar and non-vacuolar organelles play in fungal virulence.

The idea that the presence of V-ATPase complexes in non-vacuolar organelles is sufficient to maintain virulence-related mechanisms in the vph1Δ/Δ is solidified by findings in the C. albicans vma7Δ/Δ strains lacking all cellular V-ATPase. Like the vph1Δ/Δ mutant, this strain displays defective vacuolar acidification; however, unlike the vph1Δ/Δ mutant, filamentous growth is severely impaired in the vma7Δ/Δ strain, and this mutant is non-pathogenic in vivo (9). These strains differ in the continued presence of Stv1p in the vph1Δ/Δ mutant, and this likely explains the persistence of in vitro virulence-associated traits in the vph1Δ/Δ strain. Previous studies delineating the importance of pre-vacuolar secretory pathways in C. albicans virulence (19, 59) also illustrate the critical role that non-vacuolar organelles play in this process. Lack of any detectable phenotype in the stv1Δ/Δ mutant can be explained if, as in S. cerevisiae, the C. albicans Golgi apparatus has a strong buffering capacity (60) and membrane transport and trafficking pathways are maintained.

By extrapolation, our findings that vacuolar acidification is not essential for in vitro filamentation, biofilm formation, or macrophage killing suggest that vacuolar acidification may also be dispensable for in vivo virulence. A previous C. albicans genetic screen has identified avirulent mutants that display normal levels of filamentation in vitro (61), demonstrating that in vitro filamentation status does not always directly correlate with virulence in vivo. Therefore, future murine systemic candidemia studies must be performed to fully clarify the role that V1p and Stv1p play in physiological virulence.

Future studies will also focus on the mechanism(s) underlying why the C. albicans vph1Δ/Δ mutant strain is competent for in vitro filamentation in the face of vacuolar pH disruption. Germ tube formation during hyphal development requires alkalization of the cytoplasm (62). In S. cerevisiae, V-ATPase mutants display an abnormally acidified cytosol because they either lack the Pma1p proton efflux pump at the cell surface (35, 63, 64) or Pma1p is not fully active (60). Stv1p-containing V-ATPase complexes present in the vph1Δ/Δ mutant may be sufficient for proper localization of Pma1p and normal cytosolic alkalization during hyphal development. Alternatively, an established role for the Golgi and exocytosis machinery in polarized growth of hyphae (16) may help explain how Stv1p contributes to continued filamentation in the vph1Δ/Δ mutant.

In conclusion, we have shown that the V_α isoforms of V-ATPase contribute differentially to vacuolar biogenesis and bioenergetics of the human fungal pathogen C. albicans, with the Vph1p isoform controlling V-ATPase activity and acidification in the vacuole. We demonstrate that vacuolar acidification is not essential for C. albicans filamentation in vitro, as filamentous growth on several hyphae-inducing media was normal in the vph1Δ/Δ mutant. The vph1Δ/Δ cells also remain...
V-ATPase Vₐ Subunit Isoforms in C. albicans

competent to form biofilms and kill macrophages. This phenomenon likely results from the continued presence of V-ATPase in non-vacuolar organelles. We anticipate that the C. albicans vph1/vph1:stv1/stv1 double deletion strain will resemble the vma7Δ/Δ mutant, because in S. cerevisiae, vma7Δ mutants simultaneously lose both Vph1p- and Stv1p-containing V-ATPase complexes (65).

To the best of our knowledge this study is the first to propose a role specifically for Stv1p-containing V-ATPase complexes in C. albicans virulence-associated pathways, particularly if Vph1p is not functional. Importantly, our results suggest that as a determinant of C. albicans filamentation, vacuolar pH alone may be less critical than originally assumed, at least in vitro. It is likely that both Vph1p- and Stv1p-containing V-ATPase complexes must be inhibited to fully combat C. albicans infection.

Acknowledgments—We thank Dr. Aaron P. Mitchell (Carnegie Mellon University) for providing strain BWP17 and plasmids pDDB57, pRS-ARG4ΔSpeI, and pGEM-HIS1. We thank Dr. Vera Michel for developing and validating the anti-human Vₐ antibody. We thank Dr. Gloria Martinez for the helpful discussions.

REFERENCES

1. Toei, M., Saum, R., and Forgac, M. (2010) Regulation and isoform function of the V-ATPases. *Biochemistry* 49, 4715–4723
2. Kane, P. M. (2006) The where, when, and how of organellar acidification by the yeast vacuolar H⁺-ATPase. *Microbiol. Mol. Biol. Rev.* 70, 177–191
3. Manolos, M., F. Proteau, D., and Jones, E. W. (1992) Evidence for a conserved 95–120-kDa subunit associated with and essential for activity of V-ATPases. *J. Exp. Biol.* 172, 105–112
4. Kawasaki-Nishi, S., Nishi, T., and Forgac, M. (2001) Arg-735 of the 100-kDa subunit a of the yeast V-ATPase is essential for proton translocation. *J. Biol. Chem.* 267, 14294–14303
5. Kawasaki-Nishi, S., Nishi, T., and Forgac, M. (2001) Arg-735 of the 100-kDa subunit a of the yeast V-ATPase is essential for proton translocation. *Microbiol. Mol. Biol. Rev.* 70, 177–191
6. Kawasaki-Nishi, S., Bowers, K., Nishi, T., Forgac, M., and Stevens, T. H. (2001) The amino-terminal domain of the vacuolar proton-translocating ATPase subunit A controls targeting and in vivo dissociation, and the carboxyl-terminal domain affects coupling of proton transport and ATP hydrolysis. *J. Biol. Chem.* 276, 47411–47420
7. Finningan, G. C., Cronan, G. E., Park, H. J., Srivivasan, S., Quicho, F. A., and Stevens, T. H. (2012) Sorting of the yeast vacuolar-type, proton-translocating ATPase, encoded by the ERG24 gene, as a potential antifungal target site. *Antimicrob. Agents Chemother.* 46, 947–957
8. Eck, R., Nguyen, M., Günther, J., Künkel, W., and Zipfel, P. F. (2005) The phosphatidylinositol 3-kinase Vps34p of the human pathogenic yeast *Candida albicans* is a multifunctional protein that interacts with the putative vacuolar H⁺-ATPase subunit Vma7p. *Int. J. Med. Microbiol.* 295, 57–66
9. Bruckmann, A., Künkel, W., Härte, R., Wetzker, R., and Eck, R. (2000) A phosphatidylinositol 3-kinase of *Candida albicans* influences adhesion, filamentous growth, and virulence. *Microbiology* 146, 2755–2764
10. Baj, N., Arthington-Skagg, B., Lee, W., Pierson, C. A., Lees, N. D., Eckstein, J., Barbuch, R., and Bard, M. (2002) *Candida albicans* sterol C-14 reductase, encoded by the ERG24 gene, as a potential antifungal target site. *Antimicrob. Agents Chemother.* 46, 947–957
11. Zhang, Y. Q., Gamarra, S., García-Efron, G., Park, S., Perlin, D. S., and Rao, R. (2010) Requirement for ergosterol in V-ATPase function underlies antifungal activity ofazole drugs. *Eukaryot. Cell* 9, 266–277
12. Hilty, J., Smulian, A. G., and Newman, S. L. (2008) The Histoplasma capsulatum vacuolar ATPase is required for iron homeostasis, intracellular replication in macrophages, and virulence in a murine model of histoplasmosis. *Mol. Microbiol.* 70, 127–139
13. Erickson, T., Liu, L., Gueyjian, A., Zhu, X., Gibbons, J., and Williamson, P. R. (2001) Multiple virulence factors of *Cryptococcus neoformans* are dependent on VPH1. *Mol. Microbiol.* 42, 1121–1131
14. Weissman, Z., Shemer, R., Conibear, E., and Kornitzer, D. (2008) An endocytic mechanism for haemoglobin-iron acquisition in *Candida albicans*. *Mol. Microbiol.* 69, 201–217
15. Wilson, R. B., Davis, D., and Mitchell, A. P. (1999) Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. *J. Bacteriol.* 181, 1868–1874
16. Wilson, R. B., Davis, D., Enloe, B. M., and Mitchell, A. P. (2000) A recyclable *Candida albicans* URA3 cassette for PCR product-directed gene disruptions. *Yeast* 16, 65–70
17. Ovegi, M. A., Pappas, D. L., Finch, M. W., Jr., Bilbo, S. A., Resendiz, C. A., Jacobem, L. J., Warrier, A., Trombley, D. J., McIlvou, K. M., Margalef, K. L., Mertz, M. J., Storms, J. M., Damin, C. A., and Parra, J. K. (2006) Identification of a domain in the V0 subunit d that is critical for coupling of the yeast vacuolar proton-translocating ATPase. *J. Biol. Chem.* 281, 30001–30014
18. Michel, V., Licon–Munoz, Y., Trujillo, K., Bisoffi, M., and Parra, J. K. (2013) Inhibitors of vacuolar ATPase Protein pumps inhibit human prostate cancer cell invasion and prostate-specific antigen expression and secretion. *Int. J. Cancer* 132, E1–E10
19. Ovegi, M. A., Carenbauer, A. L., Wick, N. M., Brown, J. F., Terhune, K. L., Bilbo, S. A., Weaver, R. S., Shircliff, R., Newcomb, N., and Parra-Belky, K. J. (2005) Mutational analysis of the stator subunit E of the yeast V-ATPase. *J. Biol. Chem.* 280, 18393–18402
20. Forgac, M., Cantley, L., Wiedenmann, B., Altstiel, L., and Branton, D. (2003) Chlorothiaz-coated vesicles contain an ATP-dependent proton pump.
