Sorting of the Yeast Vacuolar-type, Proton-translocating ATPase Enzyme Complex (V-ATPase)

IDENTIFICATION OF A NECESSARY AND SUFFICIENT GOLGI/ENDOSOMAL RETENTION SIGNAL IN Stv1p

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Background: The cytosolic N terminus of Stv1p (subunit a) of the V-ATPase is responsible for Golgi localization.

Results: We have identified residues that are necessary and sufficient for Golgi retrieval of the Stv1p V-ATPase.

Conclusion: We propose a structural model that highlights the accessibility of this peptide signal.

Significance: This represents the first sorting signal for any V-ATPase complex.

Subunit a of the yeast vacuolar-type, proton-translocating ATPase enzyme complex (V-ATPase) is responsible for both proton translocation and subcellular localization of this highly conserved molecular machine. Inclusion of the Vph1p isoform causes the V-ATPase complex to traffic to the vacuolar membrane, whereas incorporation of Stv1p causes continued cycling between the trans-Golgi and endosome. We previously demonstrated that this targeting information is contained within the cytosolic, N-terminal portion of V-ATPase subunit a (Stv1p). To identify residues responsible for sorting of the Golgi isoform of the V-ATPase, a random mutagenesis was performed on the N terminus of Stv1p. Subsequent characterization of mutant alleles led to the identification of a short peptide sequence, W83KY, that is necessary for proper Stv1p localization. Based on three-dimensional homology modeling to the Meiothermus ruber subunit I, we propose a structural model of the intact Stv1p-containing V-ATPase demonstrating the accessibility of the W83KY sequence to retrograde sorting machinery. Finally, we characterized the sorting signal within the context of a reconstructed Stv1p ancestor (Anc.Stv1). This evolutionary intermediate includes an endogenous W83KY sorting motif and is sufficient to compete with sorting of the native yeast Stv1p V-ATPase isoform. These data define a novel sorting signal that is both necessary and sufficient for trafficking of the V-ATPase within the Golgi/endosomal network.

The highly conserved vacuolar-type, proton-translocating ATPase enzyme complex (V-ATPase) is a multisubunit molecular machine responsible for organelle acidification across the tree of life (1). The V-ATPase converts chemical energy stored within ATP into rotational motion to translocate protons across membranes (2, 3). This process is analogous to the rotary function of the structurally similar F1F0-ATP synthase enzyme (4, 5). Acidification of vesicle and organelle compartments plays a crucial role in a plethora of biological processes, including ion homeostasis, membrane fusion, pH regulation, and protein transport through the endomembrane system, including endo- and exocytosis (1, 6, 7). Disruption of V-ATPase function is lethal in most organisms except a few species of fungi. Along these lines, many reports have demonstrated that aberrant V-ATPase function contributes to human diseases and pathologies, including viral entry, tumor invasiveness, pathogen virulence, multidrug resistance in cancer, diabetes, Alzheimer disease, and Parkinson disease (8–14).

Most of our understanding of the V-ATPase complex can be attributed to studies within the model organism Saccharomyces cerevisiae. Within budding yeast, the V-ATPase structure is composed of 14 different subunits and two major subdomains (1). The V1 portion (subunits A, B, C, D, E, F, G, and H) is responsible for hydrolysis of ATP and is assembled within the cytosol, whereas the V0 portion (subunits a, c, c’, c”, d, and e) serves to shuttle protons across the lipid bilayer and is assembled within the endoplasmic reticulum (ER) (1). To date, five other proteins have been identified that participate in the early assembly of the V0 subcomplex within the ER yet are not integrated within the final V-ATPase enzyme (15–19). Once assembled, the completed V0 is transported out of the ER (and associates with the assembled V1 subcomplex) to ultimately reside on the membranes of the Golgi, endosomes, and vacuole within yeast, where it serves to acidify these organelles (20).

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The abbreviations used are: V-ATPase, vacuolar-type, proton-translocating ATPase enzyme complex; ALP, yeast alkaline phosphatase encoded by the PHO8 gene; Anc.a, the reconstructed, ancestral subunit a of vacuolar H+-ATPase within the fungal clade; Anc.Stv1, the first evolutionary intermediate subunit a isoform containing the W83KY sorting signal along the evolutionary trajectory leading to modern Stv1p; mCherry, a mutated form of monomeric red fluorescent protein; YEPD, yeast extract peptone dextrose; TRITC, tetramethylrhodamine isothiocyanate; ER, endoplasmic reticulum.
Stv1p V-ATPase Sorting Signal for Golgi Localization

Loss of V-ATPase function in yeast results in conditional viability and a set of unique growth phenotypes, including sensitivity to elevated pH and elevated divalent cations, including calcium and zinc (20, 21). Proton/metal antipoters that reside on the Golgi and vacuole utilize the pH gradient generated by the V-ATPase to regulate cytosolic levels of various cations that can be toxic at high concentrations (22).

In yeast, all of the V-ATPase subunits are encoded by single genes except for subunit a, and there are two isoforms of this subunit, Stv1p and Vph1p (1). Due to their sequence and predicted secondary structure homology, it is likely that these two isoforms evolved from a common ancestral subunit within a subgroup of fungal species (23). Subunit a is the largest V0 subunit and contains both a cytosolic domain and membrane-embedded domain. This subunit serves to (i) link the V1 and V0 subdomains, (ii) act as a stationary stalk (stator) against which the hexameric proteolipid ring can rotate, and (iii) aid in translocation of protons across the membrane (24–26). Either Stv1p or Vph1p can be incorporated within the V-ATPase complex, and the identity of this subunit influences a number of biochemical and cellular properties of the V-ATPase complex (27, 28). These two populations of V-ATPase enzyme differ in V0 assembly, coupling efficiency, protein abundance, and reversible dissociation (29–32). One of the major differences between these complexes is the subcellular localization of the V-ATPase; inclusion of Vph1p causes the enzyme to localize to the vacuole membrane, whereas the Stv1p dictates localization within the Golgi/endosomal network (33). The Stv1p complex has been found to continually cycle between the Golgi and endosome, similar to other protein cargo that are retained and/or retrieved from a post-Golgi compartment (33). Sorting of Stv1p within the Golgi/endosome also requires the presence of the conserved heteropentameric “retromer” complex (23) responsible for retrograde traffic from the late endosome to the trans-Golgi (reviewed in Refs. 34 and 35).

The importance of deciphering the molecular differences between Stv1p and Vph1p V-ATPase complexes is evident in the evolutionarily diverse set of subunit a isoforms that have emerged in many eukaryotes. Similar to budding yeast, other organisms also utilize alternate forms of the stator subunit to impart localization information to the V-ATPase complex within specialized cell types to the Golgi, endosomes, lysosome, and even the plasma membrane (36). Arabidopsis thaliana has three differentially localized subunit a isoforms, Mus musculus and Caenorhabditis elegans have four isoforms, and Paramecium contains 17 different copies (37–41). Of note, several human genetic diseases have been linked to mutations in subunit a isoforms; renal tubule acidosis is linked to mutations in the a4 isoform (42), whereas osteopetrosis is linked to mutations in the a3 isoform (43). However, little is known about the mechanisms governing trafficking of various V-ATPase complexes to unique cellular compartments.

In eukaryotes, trafficking through the secretory pathway (ER-Golgi-endosome-vacuole) requires packaging into specific coated vesicle compartments through recognition of short peptide sequences (termed sorting signals) (44). A variety of short signals (usually 3–5 residues) have been described for active transport of different protein cargo to specific compartments along the secretory pathway in eukaryotes (45–52). For example, in yeast, retrieval of Ste13p from the late endosome to the Golgi requires the cytosol-exposed sequence FXFXD (45, 53). Interestingly, the Stv1p peptide also contains an FXFXD motif, yet deletion of these residues does not alter V-ATPase sorting (33). To date, no recognition signal has been described for the Stv1p subunit of the V-ATPase complex or any other subunit a isoform within eukaryotes.

Previous work using protein chimeras between Stv1p and Vph1p found that the location of the targeting information within Stv1p must reside within the cytosolic, N-terminal portion (amino acids 1–455) (33). However, additional in-frame deletions of Stv1p resulted in non-functional V-ATPase complexes with gross V0 assembly defects (33). The difficulty in creating functional protein chimeras and/or deletion mutants of Stv1p/Vph1p suggested that correct folding of the subunit a N terminus is required for proper V0 assembly and ER exit. Therefore, we have taken an alternative genetic approach to identify residues required for trafficking of the Stv1p-containing V-ATPase. We randomly mutagenized the Stv1p N terminus and utilized a growth phenotype associated with mislocalization of the Stv1p-containing V-ATPase to select for candidate mutants. In this way, we have generated mutant copies of Stv1p that result in (i) a properly assembled V0 subcomplex, (ii) normal V-ATPase enzyme function, and (iii) mislocalization of the Stv1p-containing V-ATPase to the vacuole membrane. Further characterization of putative mutant copies has led to the identification of a small stretch of residues unique to Stv1p that are required for continued cycling between the Golgi and endosome. This tripeptide motif (WXXK) represents a unique sorting signal for the Stv1p V-ATPase that is similar in function to the FXFXD motif depicted for retrieval of Ste13p. This sorting signal is also predicted to be within an accessible, cytosolic surface of the Stv1p N-terminal structure as compared with the buried canonical FXFXD motif based on three-dimensional homology modeling. Finally, an evolutionary ancestor (Anc.Stv1) that also contains the modern sorting signal is competent to compete with yeast Stv1p for retrieval from a post-Golgi compartment. This study presents the first sorting signal described for any subunit a isoform of the V-ATPase that is both necessary and sufficient for transport within the Golgi/endosomal network.

EXPERIMENTAL PROCEDURES

Yeast Strain and Plasmid Construction—Molecular biology procedures were followed in this study (54). Yeast strains used in this study can be found in Table 1. To create GCY9, wild-type yeast (SF383–1Da) (55) was transformed with a PCR-amplified fragment containing the NatMX4 cassette (56) and bases containing homology to the 3′- and 5′-UTR of the VPH1 locus. For GCY17, GCY9 was transformed with a PCR fragment containing the KanMX4 cassette to replace the N terminus of STV1 (codons 1–455). This strain (vph1Δ stv1-NTΔ) was determined to phenocopy a full Vma− strain (vph1Δ stv1Δ) on elevated calcium and elevated zinc (Fig. 1A). For GFY311, GFY310 was deleted for VPH1 using a similar strategy as GCY9. For strains GFY398–GY403, GFY407, GFY411, GFY412, GFY416–GFY427, and GFY439, the following method was used. A CEN-
based vector (pRS316) containing the full-length STV1 (3xHA tag at codon 227), 544 bp of 5’-UTR, and 135 bp of 3’-UTR was generated. A modified QuikChange protocol (57) was used to introduce two unique restriction sites: an Nhel site located 74 bp upstream of the start codon and an SnaBI site (silent) 1403 bp downstream of the start codon. The STV1 locus was subcloned from pRS316 to an integration vector pRS306 (that lacks the ability to replicate in yeast) to create pGC34. The STV1 gene within pRS306 was truncated 630 bp of coding sequence upstream of the stop codon and also contained a unique MluI restriction site (silent) beginning at nucleotide 1859 after the start codon. Next, the STV1 N terminus (containing the Nhel/SnaBI sites) was cloned into a pCR4Blunt-TOPO (Invitrogen) vector. QuikChange™ PCR was used to introduce one, two, or three amino acid substitutions using the TOPO vector as a template (pGC44). For GY430, the polyalanine stretch was inserted using inverse PCR. Following confirmation of the mutational change(s) by DNA sequencing, the STV1 N terminus was subcloned to a pRS306 vector (pGC34) using the Nhel/SnaBI sites. Finally, integration vectors were linearized with MluI, transformed into GCY17 yeast (vph1Δstv1Δ-r-Δm), and selected for growth on medium lacking uracil. Clonal integrants were tested on medium buffered with elevated calcium to ensure proper restoration of mutagenized, full-length STV1 within the genome. GCY11 (unmutagenized STV1), GCY12, and GCY13 were all generated using this strategy (see “Random Mutagenesis of Stv1 N Terminus and Mutant Screening” for GCY12 and GCY13).

To create GY442 and GY443, the following methods were used. A PCR fragment containing PrSTV1::STV1::3xHA::GFP::ADH::HygR from pGF674 (wild-type STV1) or pGF675 (STV1 W83KYLHL→AKAAAA) was transformed into vph1Δ::NatR stv1Δ::KanR yeast (GY271) to integrate at the STV1 locus. Second, a PCR fragment containing only the KanR cassette was transformed to switch the vph1Δ::NatR locus to vph1Δ::KanR. Third, a PCR fragment containing PrVPH1::VPH1::mCherry::ADH::NatR (from pGF22) was integrated at the VPH1 locus. To generate strain GY579, the Anc.Stv1 intermediate was chosen from the phylogeny of subunit isoforms used to generate the sequence of Anc.a (23) because it was the first ancestor within our evolutionary tree to include the W83KY sorting signal. The open reading frame of Anc.Stv1 was synthesized (Genscript, Piscataway, NJ), including a double HA epitope tag codon 208. A triple in vivo ligation was used to link (i) PrVPH1 (from pGF382), (ii) full-length Anc.Stv1 (PCR-amplified from pGF611), and (iii) ADH::NatR. Next, the entire Anc.Stv1 cassette was amplified and integrated into vph1Δ::HygR STV1::GFP::KanR (GY327) to create GY579.

Plasmids used in this study are listed in Table 1. Vector pGC8 was generated by subcloning STV1::3xHA (epitope tag at position 227) from pGC3 to YEp352 using sites XbaI/Sall. pGF775 was created by in vivo ligation of STV1::2xHA::GFP::ADH::HygR (amplified from pGF383) to PrVPH1 (from pGF382).

**Random Mutagenesis of Stv1 N Terminus and Mutant Screening**—A CEN-based vector (pRS316) containing the full-length STV1::3xHA, flanking UTR, and unique Nhel/SnaBI sites was digested, purified, and co-transformed into vph1Δ stv1Δ yeast along with a randomly mutagenized PCR fragment (Genemorph II random mutagenesis kit, Agilent Technologies, La Jolla, CA) of the STV1 N terminus (codons 1–455) containing 40-bp overlapping tails. Colonies were selected on synthetic medium lacking uracil to select for recircularization of the vector and subsequently tested on rich medium containing ZnCl2 using a combination of replica plating and robotic plating (Singer Instrument, Roadwater, UK). Isolates that scored as zinc-resistant were retested using a robotic plating strategy. Putative strains were arrayed into 96-well plates and printed onto permissive medium in a 1536 array format (allowing for 16-fold degeneracy per strain). Colonies were then plated from YEPD medium to medium buffered with a range of zinc conditions. The robotic plating technique ensured a controlled, reproducible method of transferring yeast to zinc plates in great quantities. Finally, images were scored following 1–2 days on zinc for subtle differences in growth. Plasmids were isolated from putative zinc-resistant and calcium-resistant clones and prepared for direct integration into the genome at the STV1 locus by subcloning into a pRS306 vector containing a C-terminal truncation and unique MluI site (pGC34). GCY12 and GCY13 were created using this strategy.

**Culture Conditions**—Yeast was grown in liquid culture containing 1% yeast extract, 2% peptone, and 2% dextrose (YEPD), buffered to pH 5.0 using 50 mM succinate/phosphate. All YEPD media also contained 0.01% adenine. Synthetic media containing dextrose (and appropriate amino acid drop-out mixtures) were used for strains containing plasmids. Growth tests were performed by growing yeast to saturation overnight and diluting into fresh rich medium to an A600 of ~4–5 h (or until an A600 of 0.8–1.0 was obtained). Growth tests were performed on medium containing 100 mM CaCl2, 1.0 mM ZnCl2, or 1.5 mM ZnCl2 and incubated at 30 °C for 1–2 days.

**p13 Fractionation and Immunoblotting**—Overnight yeast cultures were grown to saturation and back diluted to an A600 of 0.25 and grown for 4–5 h. Approximately 50 A600 of yeast were harvested, washed, and resuspended into 1 ml of phosphate-buffered saline (PBS) (126 mM NaCl, 2.5 mM KCl, and 10 mM NaHPO4/KHPO4, pH 7.1) and 2× EDTA-free protease inhibitor mixture (Roche Applied Science). Glass beads were added and vortexed for 8–9 min at 4 °C. Next, cells were spun at 2,250 rpm for 5 min, and the supernatant was transferred to a fresh tube and spun at 13,000 × g for 15 min at 4 °C. The p13 fraction was washed a second time with ice-cold PBS and centrifuged at 13,000 × g, and the pellet was resuspended into ~250 μl of Thorner buffer (8 μM urea, 5% SDS, and 50 mM Tris, pH 6.8). A modified Lowry protocol was performed to equalize total protein loaded for all samples (58). Samples were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-HA antibodies (Sigma-Aldrich), anti-Dpm1p antibodies (5C5, Invitrogen), and secondary horseradish peroxidase-conjugated anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Blots were visualized by ECL.

**Fluorescent Microscopy**—Quinacrine staining of yeast cells was done as described previously (23). Briefly, cells were grown to midlogarithmic phase in YEPD (unbuffered), cooled on ice, and stained with 200 μM quinacrine, 100 μM HEPES (pH 7.6), and 50 μg/ml concanavalin A-tetramethylrhodamine (Invitro-
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TABLE 1
Yeast strains and plasmids used in this study

| Strain   | Description                                      | Reference   |
|----------|--------------------------------------------------|-------------|
| SF388-1De | MATa ura3-52 leu2-3,112 his4-519 ade6 pep4-3 gal2 |             |
| KEBY4    | SF388–1De stv1ΔKan6                         | Ref. 55     |
| GCY9     | SF388–1De vph1ΔNat6                        | Ref. 33     |
| GYF325   | SF388–1De STV1:2xHA-GFP:ADH:HoG6              | Ref. 23     |
| GYF311   | SF388–1De vph1ΔHoG6 STV1::GFP:ADH:Nat6        | This study  |
| GCY17    | SF388–1De vph1ΔNat6 stv1-N-termΔKan6          | This study  |
| GCY11    | SF388–1De vph1ΔNat6 stv1-N-termΔKan6          | This study  |
| GCY12    | GCY11 F50L/Q152L/L203/L259S/F409D            | This study  |
| GYF398   | GCY11 K77A                                   | This study  |
| GYF399   | GCY11 H78A                                   | This study  |
| GYF400   | GCY11 E81A                                   | This study  |
| GYF401   | GCY11 T82A                                   | This study  |
| GYF402   | GCY11 W83A                                   | This study  |
| GYF403   | GCY11 W83F                                   | This study  |
| GYF404   | GCY11 K84A                                   | This study  |
| GYF405   | GCY11 Y85A                                   | This study  |
| GYF412   | GCY11 Y85F                                   | This study  |
| GYF416   | GCY11 I86A                                   | This study  |
| GYF417   | GCY11 L87A                                   | This study  |
| GYF418   | GCY11 H88A                                   | This study  |
| GYF419   | GCY11 I89A                                   | This study  |
| GYF420   | GCY11 D90A                                   | This study  |
| GYF421   | GCY11 D91A                                   | This study  |
| GYF422   | GCY11 E92A                                   | This study  |
| GYF423   | GCY11 G93A                                   | This study  |
| GYF424   | GCY11 N94A                                   | This study  |
| GYF425   | GCY11 W83F/Y85F                              | This study  |
| GYF426   | GCY11 W83A/Y85A                               | This study  |
| GYF427   | GCY11 W83KY → AAA                             | This study  |
| GYF430   | GCY11 W83KYLH → AKAAAA                      | This study  |
| GYF432   | SF388–1De STV1::3xHA::GFP:ADH:HoG6 VPH1::mCherry::ADH::Nat6 | This study  |
| GYF442   | SF388–1De STV1::3xHA::GFP:ADH:HoG6 VPH1::mCherry::ADH::Nat6 | This study  |
| GYF443   | SF388–1De STV1::3xHA::GFP:ADH:HoG6 VPH1::mCherry::ADH::Nat6 | This study  |
| GYF317   | SF388–1De STV1::GFP:ADH:Kan6 Prvpr::Anc.a2xHA::ADH::Nat6 | Ref. 23     |
| GYF579   | SF388–1De STV1::GFP:ADH:Kan6 Prvpr::Anc.a2xHA::ADH::Nat6 | This study  |
| GYF316   | SF388–1De STV1::GFP:ADH:Kan6 Prvpr::STV1:2xHA::ADH::Nat6 | Ref. 23     |
| GYF315   | SF388–1De STV1::GFP:ADH:Kan6 VPH1::2xHA::ADH::Nat6 | Ref. 23     |

| Plasmid  | Description                                      | Reference   |
|----------|--------------------------------------------------|-------------|
| pGCR     | YEp352 STV1::3xHA                                | This study  |
| pG775    | pRS415 Prvpr::STV1::2xHA::GFP:ADH:HoG6           | This study  |
| pGP242   | pRS316 Prvpr::mCherry::ALP:ADH::Nat6            | Ref. 23     |

The quality of the generated models was assessed in I-TASSER based on two major criteria, the C- and the TM-scores. The C-score is calculated based on the significance of the threading alignments and the convergence of the I-TASSER simulations. C-scores typically range from −5 to 2, with higher scores reflecting a model of better quality. The TM-score is a measure of structural similarity between the predicted model and the native or experimentally determined structure, with a value close to 0.5 indicating a model of correct topology. Assessments for the various V-ATPase subunit N-terminal models obtained in this study are presented in supplemental Table 1.

Fitting the Predicted Stv1p N-terminal Model into Electron Microscopy Reconstruction Density Maps—The predicted Stv1p N-terminal model and the x-ray structures of yeast subunits H (Protein Data Bank code 1H08) (62) and yeast subunit C (Protein Data Bank code 1U7L) (63) were modeled into the density of the 25 Å negative stain electron microscopy (EM) reconstruction of the intact yeast V-ATPase (EMDB ID 1640) (64) using the University of California San Francisco molecular modeling program Chimera (65). All of the fits were unique, corresponding to the 99th percentile for volume inclusion and real-space correlation.

gen) at 30 °C for 10 min. Cells were washed three times in ice-cold 100 mM HEPES with 2% glucose before being visualized (maintained on ice).

For visualizing cells expressing either GFP-tagged or mCherry-tagged constructs, yeast strains were grown to midlog phase in YEPD, pH 5.0, centrifuged at 6,000 rpm, washed once with water, and visualized on the microscope. Images were obtained using a ×100 objective lens on an Axioplan fluorescence microscope (Carl Zeiss, Thornwood, NY). Modifications were performed using Axiosio Vision software (Carl Zeiss). Between 150 and 350 cells were counted from images taken for Figs. 4 and 6.

Structural Homology Modeling of the Stv1p N Terminus—Coordinates of the N terminus of the Meiothermus ruber subunit I structure (Protein Data Bank code 3RRK) (59) were used as a starting template for obtaining structural models of the N-terminal cytoplasmic domains of Stv1, Vph1, Anc.a through the online server I-TASSER (60). Because no electron density for the M. ruber subunit I was observed beyond residue 301, the three V-ATPase subunit I sequences (Stv1p, Vph1p, and Anc.a N termini) were aligned by ClustalW2 (61) and ended at the corresponding residue (supplemental Fig. 1). These sequences were used as inputs for the homology modeling. Sequence template alignments were generated using the program MUSTER, which is built into I-TASSER. The quality of the generated models was assessed in I-TASSER based on two major criteria, the C- and the TM-scores. The C-score is calculated based on the significance of the threading alignments and the convergence of the I-TASSER simulations. C-scores typically range from −5 to 2, with higher scores reflecting a model of better quality. The TM-score is a measure of structural similarity between the predicted model and the native or experimentally determined structure, with a value close to 0.5 indicating a model of correct topology. Assessments for the various V-ATPase subunit N-terminal models obtained in this study are presented in supplemental Table 1.
RESULTS

Mislocalization of Stv1p-containing V-ATPase Enzyme to the Vacuole Membrane Causes Vacuolar Acidification and Zn\(^{2+}\) Resistance—To examine sorting of the Stv1p containing V-ATPase, we have tested a number of growth conditions that are dependent upon the presence and trafficking of the two V-ATPase isoforms in budding yeast. V-ATPase function can be assayed in yeast through resistance to elevated levels of divalent cations, such as calcium or zinc or elevated pH (20). Specific antiporters reside on organelle membranes (such as the Golgi and/or vacuole) that utilize the proton gradient generated by the V-ATPase to sequester ions away from the cytoplasm (66, 67). Loss of V-ATPase function renders yeast fully sensitive to excess metals (calcium and zinc) and elevated pH (20). Previous work has shown there are a number of phenotypic differences in mutants lacking the \(STV1\) or \(VPH1\) isoforms (23, 27, 68).

Whereas strains lacking the Golgi/endosome V-ATPase (\(stv1\)) are resistant to elevated levels of zinc, \(vph1\) yeasts are sensitive to elevated zinc (Fig. 1A). The presence of either isoform is sufficient to convey resistance to elevated calcium (Fig. 1A).

The more abundant Vph1p V-ATPase is present on the vacuole membrane, whereas the Stv1p-containing complex localizes to the Golgi and, to a lesser extent, the endosome (33). Previous work demonstrated that overexpression of Stv1p causes mislocalization of the Stv1p V-ATPase to the vacuole membrane in the absence of Vph1p (27, 31). Stv1p-containing V-ATPase enzymes were fully functional when present on the vacuole membrane and allowed for zinc resistance in a \(vph1\) strain (Fig. 1A, lane 5). Stv1p showed a dramatic shift to the vacuolar membrane upon overexpression and rescued the vacuole acidification defect of \(vph1\) cells, as shown by the accumulation of the pH-sensitive fluorescent dye quinacrine (Fig. 1B). Stv1p tagged at the N terminus with GFP displayed a punctate localization that has been previously shown to localize to the late Golgi in wild-type yeast (Fig. 1C) (27, 33). Together, these results demonstrate that (i) the mechanism of Stv1p V-ATPase retention within the Golgi/ endosome is saturable, and (ii) missorting of the Stv1p V-ATPase to the vacuole restores acidification as well as zinc resistance. We have developed a novel strategy to generate Stv1p mutants that mislocalize a fully functional V-ATPase enzyme to the vacuole.

Forward Genetic Screen for Stv1p Mutants Defective for Retention within the Golgi/Endosome—The sorting information that dictates the subcellular localization of the yeast V-ATPase is found within the cytosolic, N-terminal domain of subunit \(a\) (33). Protein chimeras between Stv1p and Vph1p demonstrated that the first 455 amino acids of Stv1p are sufficient to cause the V-ATPase to be localized to the Golgi/endosome (33). However, previous attempts to identify the specific region within the N terminus of Stv1p responsible for V-ATPase localization have not been successful. Only a single mutant (Stv1p 165–208) has been described that removes a portion of the N terminus yet does not affect the localization of the Golgi isoform (33). One contributing factor to the difficulty in assaysing Stv1p mutants is that ER quality control mechanisms prevent proper association of Stv1p/Vph1p chimeras with the remaining \(V_0\) subunits. Defects in the assembly proc-
Sovol retention has not significantly disrupted $V_0$ assembly that a loss of function allele of Stv1p that disrupts Golgi/endo-
utagenized the N terminus of Stv1p and performed a forward identify the Stv1p-sorting signal, we have randomly rapid degradation of subunit a (21, 69). Therefore, in order to 

dent transformants were tested on ZnCl2, and 842 were scored 

table utilized a vector expressing 

canonical FNA, not applicable.



TABLE 2
Randomly generated zinc-resistant Stv1p mutant alleles
Mutants are integrated at the STV1 locus in strains lacking VPH1.

| Mutant number | Amino acid changes |
|---------------|-------------------|
| 9             | K299R/K310N/H331R/K362N/Q379K |
| 25            | E127V/L314Q/H344Y/H351Q |
| 31            | A49V/L875/R246K/N268Y/V312F |
| 57            | I43P/K212R |
| 79            | N72I/L1175/E329M/N415D |
| 89            | D205V/D401Y/N415D |
| 95            | A49V/K362N/Q379R/I393V |
| 99            | L86G/N212D |
| 101           | H88L/I93S/Q209H/V303A/D337Y/I417N |
| 104/249       | L47/314D/E340D/Q341K/H344Y/I403V |
| 132           | E76V/A79P/N164D/E340D/Q341K/H344Y/I403V |
| 146           | L86F |
| 155           | F90/155/Q215/L259R/E409D |
| 172           | R59G/L218I/N415D |
| 176           | T124S/T219A/I417N |
| 187           | A49T/E127V/L314Q/H344Y/H351Q |
| 200           | N174S/Q152L/L276M/F414S |
| 213           | N576T/P110H/I193S/Q209H/V303A/D337Y/I417N |
| 254           | L47/314D/E340D/Q341K/H344Y/I403V |
| 270           | H88L/Q150L/W257L/I304L/T324A |
| 301           | D46E/F50L/S198R/D217V/Y238F/K299E/T345A/T405M/L418P |

PCR-based mutagenesis was performed on amino acids 1–455 of Stv1p to generate a library of mutant alleles. Mutagenized fragments were inserted into a centromere-based vector to express full-length Stv1p under its endogenous promoter. The screen was performed in a yeast strain lacking both STV1 and VPH1; therefore, clones that failed to sort Stv1p to the Golgi/endosomal retention would not significantly disrupted $V_0$ assembly and/or V-ATPase enzyme function.

Further characterization was performed on this set of mutants to ensure that Stv1p-containing V-ATPase was mis-
sessed prevent the $V_0$ subcomplex from exiting the ER and cause rapid degradation of subunit a (21, 69). Therefore, in order to identify the Stv1p-sorting signal, we have randomly mutagenized the N terminus of Stv1p and performed a forward genetic screen for mislocalization of the Golgi isoform using zinc resistance as a phenotypic readout. This approach ensured that a loss of function allele of Stv1p that disrupts Golgi/endosomal retention has not significantly disrupted $V_0$ assembly and/or V-ATPase enzyme function.

FIGURE 2. Randomly mutagenized STV1 alleles cause vacuolar acidification and resistance to Zn2+. A, cultures of wild-type (SF8381–Δvph1), vph1Δ STV1::3xHA (GCY11), and integrated, randomly mutagenized STV1 alleles 155 (GCY12) and 301 (GCY13) were spotted onto YE and medium containing 1.5 mM Zn2+. B, strains from A were stained with quinacrine and concanavalin A-TRITC and visualized by fluorescent microscopy. C, Western blot analysis of strains from A. vph1Δ STV1 (GCY9) served as a negative control for the HA epitope tag. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to HA and Dpm1p (loading control). The nearest molecular marker (kDa) is shown on the left.

TABLE 3
Targeted mutational alanine scanning across Stv1p N terminus
Mutants are integrated at the STV1 locus in strains lacking VPH1.

| Genotype | Mutations | 100 mM Ca2+ | 1.0 mM Zn2+ |
|----------|-----------|-------------|-------------|
| STV1 WT  | NA        | +           | +           |
| vph1Δ STV1 | NA        | –           | –           |
| D–H1AF → AAAA | 4        | +           | +           |
| N9–Q16R → AAAA | 3        | +           | +           |
| W8–K120 → AKAQA | 4        | +           | ++          |
| Y18–L150 → AAAA | 3        | +           | ++          |
| K27–S120 → AAAA | 3        | +           | +           |
| D166–E170 → AAAA | 3        | +           | +           |
| F171–F175 → AAAA | 2        | +           | +           |
| D217–T219 → AAAA | 3        | +           | +           |
| L163–K165 → AAAA | 3        | +           | +           |
| V202–S204 → AAAA | 3        | +           | +           |
| K310–V312 → AAAA | 3        | +           | +           |
| D327–T329 → AAAA | 4        | +           | +           |
| D340–V342 → AAAA | 3        | +           | +           |
| L345–E347 → AAAA | 1        | +           | +           |

* The number of different sites found within our randomly mutagenized allele pool (Table 2).
* NA, not applicable.
cannot be attributed to overexpression and saturation of retrieval from the Golgi.

**Targeted Alanine Scanning across the Stv1p N Terminus Reveals the Necessity of a Tripeptide Signal, W83K84Y85**—In order to investigate the potential contribution of individual residues within the Stv1p mutant pool, we defined 13 smaller regions throughout the N terminus (Table 3). Small stretches (3–7 amino acids) were chosen to include substitutions found by random mutagenesis (Table 3). Rather than recapitulating the original substitution, polyalanine stretches replaced native residues by targeted mutagenesis, and the mutant Stv1p was integrated into the genome at the STV1 locus in vph1/H9004 yeast. Additional mutants were also tested across the N terminus to provide further coverage (supplemental Table 2). The growth results on zinc revealed a single site, Y85ILH, to be required for Golgi localization of Stv1p when mutated to alanines (Table 3). Also, an upstream residue, Trp83, was found within our random mutant pool. The quintuple mutant W83KYILH displayed a strong growth phenotype on zinc. These sequences are located within a small insertion within the Stv1p isoform that is not present within the Vph1p isoform (Fig. 3A) (70). Thus, we performed an analysis of amino acids found in close proximity to this Stv1p-specific insertion that is flanked by two conserved pairs of residues, K77H78 and E92G93 (Fig. 3A). Single amino acids of Stv1p were substituted for alanine and tested for zinc resistance; the strongest effects were seen for Trp83, Lys84,

**FIGURE 3.** Targeted alanine scanning reveals the necessity of the unique sequence, W83K84Y85, for proper Stv1p sorting. A, alignment of a small section of the N termini of yeast Stv1p, Vph1p, and Anc.a (23). Identical residues are shown against a black background. The amino acid positions relative to each protein are labeled. B, cultures of wild type (SF838–1Dv), vph1Δ STV1::3xHA (GCY11), and strains containing a single amino acid substitution (Lys77–Asn94) to alanine (GFY398–GFY402, GFY407, GFY411, and GFY416–GFY424) were spotted onto rich medium and medium containing 1.0 mM Zn2+. The dotted white line separates strains that were spotted onto different plates with identical controls; images are merged for clarity. C, cultures of wild-type, vph1Δ STV1::3xHA, and Stv1p alleles expressing the mutations W83A (GFY402), K84A (GFY407), Y85A (GFY411), A83KA (GFY426), A83AA (GFY427), and A83KAAAA (GFY430) were spotted onto YEPD and medium containing 1.0 mM Zn2+. D, a summary of growth data from mutants containing conservative amino acids changes to Trp83, Lys84, and Tyr85. Strains were scored as causing strong zinc resistance (smallest letter size), partial zinc resistance (medium sized letter), or zero growth on zinc (largest sized letter).

**FIGURE 4.** Disruption of the W83KY sorting signal causes mislocalization of Stv1p V-ATPase to the vacuole. A, strains expressing integrated copies of VPH1-mCherry and either wild-type STV1-GFP (GFY442) or STV1-GFP A83KAAAA (GFY443) were visualized by fluorescent and differential interference contrast microscopy (DIC). The merged image displays the overlap of the GFP (green) and mCherry (magenta) signals as white. B, quantification of the percentage of cells displaying vacuolar membrane localization from strains in A was performed in triplicate. 200 cells were counted for each strain, and the error is expressed as the S.D. (error bars). Lack of vacuole membrane signal indicates the presence of cellular puncta. C, cultures expressing STV1 mutant A83KA (GFY426) or A83KAAAA (GFY430) were stained with quinacrine and concanavalin A-TRITC and visualized. D, Western blot analysis was performed on the strains from B (using vph1Δ STV1 as a negative control). Following SDS-PAGE and transfer to nitrocellulose, blots were probed with anti-HA and anti-Dpm1p (loading control) antibodies. The molecular marker is shown on the left (kDa).
and Tyr\textsuperscript{85} (Fig. 3B). Additionally, a subtle, but reproducible level of growth was also seen for single mutants of Ile\textsuperscript{86}, Leu\textsuperscript{87}, and His\textsuperscript{88} on lightly lower zinc conditions (supplemental Table 2). These Stv1p mutants were all tested on medium containing 100 mM Ca\textsuperscript{2+} to ensure that a lack of zinc resistance was not due to loss of V-ATPase enzyme assembly and/or enzyme function; all single mutants tested showed identical growth on calcium as the control strain (supplemental Table 2). A closer examination of residues 83–88 within Stv1p demonstrated that the double mutant (A\textsuperscript{83}KA) or quintuple mutant (A\textsuperscript{83}KAAAA) showed slightly higher levels of growth on ZnCl\textsubscript{2} compared with any of the single mutant substitutions (Fig. 3C). Inclusion of the K84A substitution in the context of either A\textsuperscript{83}AA (Fig. 3D) or W\textsuperscript{83}AA (supplemental Table 2) caused a decrease in growth on both calcium and zinc, indicative of a possible V\textsubscript{0} assembly or enzyme function defect. Therefore, subsequent analyses have focused primarily on the A\textsuperscript{83}KA and A\textsuperscript{83}KAAAA mutant alleles. Finally, we introduced conservative mutations of Trp\textsuperscript{83}, Lys\textsuperscript{84}, and Tyr\textsuperscript{85} to structurally similar amino acids (Fig. 3D). Both Lys\textsuperscript{84} and Tyr\textsuperscript{85} showed some degree of flexibility in amino acid identity because substitution to Arg and Gin (for Lys\textsuperscript{84}) or to Phe and Trp (for Tyr\textsuperscript{85}) did not cause any zinc resistance. Trp\textsuperscript{83} was less tolerant of alternate amino acids, although it displayed only a partial level of zinc resistance when mutated to Phe or Tyr (Fig. 3D). Together, these results demonstrate that the tripeptide signal beginning at residue 83 is necessary for Golgi retention and that a modest degree of amino acid flexibility exists within this sorting signal: WXZ ((where X is a large positively charged or polar residue and Z is a bulky aromatic residue)).

We next tested whether the Stv1p W\textsuperscript{83}KYILH polyalanine mutant mislocalized to the vacuole membrane. Because the mutagenesis screen was designed to utilize zinc resistance as a phenotypic readout for mislocalized Stv1p, yeast had to be deleted for endogenous Vph1p. However, because deletion of either V-ATPase isoform does not disrupt the localization of the other,\textsuperscript{3} we sought to assay the localization of Stv1p mutants in the presence of Vph1p in otherwise wild-type yeast. In Fig. 4A, we visualize Vph1p-mCherry and Stv1p-GFP, both expressed from the genome. Wild-type Stv1p localized to discrete puncta as shown previously (23), including perivacuolar puncta, whereas Vph1p exclusively localized to the vacuole membrane (Fig. 4). The Stv1p mutant A\textsuperscript{83}KAAAA can be seen co-localized to the vacuole membrane with Vph1p (Fig. 4A), and over 90% of cells display vacuole membrane localization (Fig. 4B).

Cells expressing Stv1p mutants (lacking a GFP tag) also showed comparable levels of vacuolar acidification to randomly generated Stv1p mutants (Fig. 4C). Finally, Western blot analysis showed no significant variability in the levels of Stv1 protein in these mutant strains (Fig. 4D). These results demonstrate that the W\textsuperscript{83}K\textsuperscript{84}Y\textsuperscript{85} sequence within the Stv1p N terminus is necessary for proper retention within the Golgi/endosome.

Three-dimensional Homology Modeling of the Stv1 N Terminus Reveals the Structural Accessibility of the W\textsuperscript{83}KY Sorting Signal but Not the Canonical FXFXD Golgi Retrieval Motif—Although no complete high resolution structure exists for any of the yeast V-ATPase subunit isoforms, including Vph1p or Stv1p, recent work has provided the first crystal structure for the prokaryotic M.\textit{ruber} subunit I (homologous to the eukaryotic V-ATPase subunit a) (59). Despite a low sequence identity between Stv1p/Vph1p and the M.\textit{ruber} subunit I, there is a very high degree of secondary structure conservation across lineages (supplemental Fig. 1) (59). Hence, we utilized the prokaryotic structure as a template to generate a structural model of the yeast Golgi isoform Stv1p N-terminal residues 1–408 (Fig. 5A).

The W\textsuperscript{83}KY sorting signal sequence is located in the loop between α helix IV and V (prokaryotic α helices II and III) (Fig. 5A). A space-filling model (Fig. 5B) clearly shows this three-residue segment to be exposed and solvent-accessible. To visualize the placement of the W\textsuperscript{83}KY signal within the entire V-ATPase complex, we also fit the predicted Stv1p N-terminal model into the EM reconstruction density map of the intact yeast V-ATPase (Fig. 5C) (64) along with the crystal structures of yeast subunits C and H (62, 63). The two aromatic residues Trp\textsuperscript{83} and Tyr\textsuperscript{85} are predicted to be on the protein exterior as well as facing away from the central core of the V\textsubscript{0} domain (Fig. 5C).

Interestingly, the Stv1p N terminus also contains a canonical FXFXD sorting motif (45) beginning at residue 201. Previous work has shown that deletion of this motif within a highly variable loop of Stv1p did not affect the sorting of Stv1p to the Golgi/endosome (33). Our targeted mutagenesis of the Stv1p N terminus did not detect any sorting defect when the F\textsuperscript{201}SFDD sequence was substituted with alanines (Table 3). Within the context of our model of Stv1p, the two aromatic residues (Phe\textsuperscript{201} and Phe\textsuperscript{203}) are positioned facing inward, buried within one of the globular head domains (Fig. 5B), and also face the interior of the V\textsubscript{0} core (Fig. 5C). We tested whether the F\textsuperscript{201}XF motif within Stv1p can substitute for the W\textsuperscript{83}KY sorting signal within the more exposed, outward facing loop. If these substitutions allow for a fully functional sorting signal, we would expect (i) proper retention within the Golgi, (ii) a failure to mislocalize Stv1p to the vacuole, and (iii) a lack of zinc resistance. In Fig. 5D, we show that mutation of both Trp\textsuperscript{83} and Tyr\textsuperscript{85} to phenylalanine does not cause mislocalization of the Stv1p V-ATPase to the vacuole and causes little to no zinc resistance in \textit{vph1Δ} yeast. No decrease in calcium resistance was detected compared with the control strains, demonstrating that the W83F/Y85F mutant is functional. These results demonstrate that (i) the W\textsuperscript{83}KY sorting motif within Stv1p may represent an FXFXD-like motif, and (ii) the structural orientation and context of the sorting motif are critical to function.

The Reconstructed Ancestral Intermediate (Anc.Stv1) Containing W\textsuperscript{83}KY Is Sufficient to Compete with Native Yeast Stv1p for Retrograde Trafficking—Studies of other canonical sorting signals within cargo proteins have demonstrated that these short peptide sequences are sufficient to dictate the trafficking of other cargo (45, 49, 53). Given the complex orientation of the N terminus of Stv1p within the entire complex (Fig. 5C) (64) and the difficulties inherent in generating protein chimeras between different subunit isoforms (33), we utilized a unique approach to test sufficiency of the W\textsuperscript{83}KY signal. We recently characterized the Anc.a protein, the most recent common ancestor of modern Stv1p and Vph1p, and demonstrated that it

\textsuperscript{3}G. C. Finnigan, G. E. Cronan, H. J. Park, and T. H. Stevens, unpublished results.
was able to functionally replace the two modern yeast isoforms and localized to both Golgi and vacuole membranes (23). To predict the sequence of Anc.a, a phylogeny of the evolution of both Vph1p and Stv1p isoforms in other species was generated (23). Along the trajectory from Anc.a leading to modern yeast Stv1p, a number of evolutionary intermediates are also predicted to have existed (Fig. 6A). We examined the various ancestral subunits along the branches leading toward Stv1p and determined the intermediate that first incorporated the short variable loop (connecting helices IV and V) containing the W83KY signal; we have termed this ancestor “Anc.Stv1.” We have chosen to examine the trafficking of this ancestor for several reasons. First, our results have provided strong evidence for the importance of the structural context for the subunit A signal (Fig. 5). Second, our extensive analysis of both random and targeted mutant Stv1p alleles (Tables 2 and 3) supports a complex model for presentation of the W83KY signal that is atypical among well studied protein cargo. Our strategy directly addresses both of these concerns; the predicted ancestral isoform contains both the W83KY signal and the assortment of residues that have co-evolved within the N-terminal region (supplemental Fig. 2).

We tested the ability of the Anc.Stv1 isoform to engage retrograde machinery similar to modern Stv1p. We have developed an assay that utilizes the observation that sorting of endogenous levels of Stv1p-GFP is saturable (Fig. 1). By expressing various isoforms (Stv1p, Vph1p, and ancestral isoforms) at the VPH1 locus, we can test whether native retrieval of the Stv1p V-ATPase has been perturbed, through competition with transport machinery. Overexpression of untagged Stv1p caused a complete shift in cellular localization of endogenous Stv1p-GFP to the vacuole, whereas expression of wild-type Vph1p or Anc.a did not affect the sorting of Stv1p-GFP (Fig. 6B). Alkaline phosphatase (ALP), a cargo protein that traffics to the vacuole independently of the late endosome (71–73), was tagged with mCherry and marked the vacuole in these cells. Both Anc.a and Anc.Stv1 proteins were expressed at the same level.3 Compared with control strains, Anc.Stv1 was sufficient to dramatically alter the localization of endogenous Stv1p-GFP to the vacuole, with 70% of cells showing vacuolar membrane localization in addition to cellular puncta (Fig. 6C). These results demonstrate that the W83KY signal is sufficient to shift the sorting of the ancestral isoform when present within the context of a reconstructed ancestral intermediate.

DISCUSSION

The overwhelming evolutionary trend across Eukarya has been to diversify the function and localization of the V-ATPase within specialized cells. One strategy has been to evolve additional isoforms of V-ATPase subunit A that allow for differential regulation, enzyme activity, and subcellular localization of distinct V-ATPase complexes (36, 74). Given the central role of the V-ATPase in many cellular functions and its emerging role in human disease (reviewed in Ref. 7), it is critical to understand the sorting of this molecular machine within the secretory pathway. The V-ATPase has also been an attractive target for drug design and therapeutic possibilities for a variety of condi-

FIGURE 5. Three-dimensional homology modeling of the Stv1p N terminus demonstrates the accessibility of the W83KY signal compared with the canonical FXFDX motif. A, a structural model of Stv1p (residues 1–408) was obtained using coordinates from the bacterial M. ruber subunit I cytosolic domain as a threading template (59). The model is color-coded, and labels indicate α-helices and β-sheet strands with Roman and Arabic numbers, respectively. Side chains of residues W83KY and F201SFDD are highlighted in red. B, a space-filling model of the Stv1p N terminus with residues W83KY and F201SFDD labeled in red to illustrate the accessibility of these two motifs. C, a fit of the Stv1p homology model (from A) and crystal structures of yeast subunit H (magenta) (62) and C (cyan) (63) into the 25 Å negative stain EM reconstruction density of the yeast V-ATPase (64). Blue triangles indicate the vertical columns of density of two of the three peripheral stalks. Sequences corresponding to W83KY and F201SFDD are shown in red with the Trp83 and Phe201 side chains indicated by arrows. The inset is a 90° clockwise rotation of the EM fit along the vertical axis and illustrates the Trp83 residue as more peripheral within the V-ATPase complex and therefore more accessible than residue Phe201. For the sake of clarity, subunits H and C are not shown. D, cultures of wild type (SF838-1D), vph1Δ STV1:3×3xHA (GCY11), and strains containing amino acid substitutions W83F (GFY403), Y85F (GFY412), and W83F/Y85F (GFY425) were spotted onto rich medium containing either 100 mM Ca2+/H11001 or 1.0 mM Zn2+/H9251.
Stv1p V-ATPase Sorting Signal for Golgi Localization

FIGURE 6. The W83KY peptide in a reconstructed ancestral isoform (Anc.Stv1) is sufficient to compete with yeast Stv1p-GFP. A, the phylogeny of fungal V-ATPase subunit a (23). The positions of the reconstructed, preduplicated ancestral isoform (Anc.a) and the first ancestral intermediate containing the W83KY sorting signal (Anc.Stv1) are labeled. For simplicity, only the evolutionary trajectory leading to S. cerevisiae Stv1p is shown; breaks in the tree correspond to the evolution of additional fungal subunit a proteins. Other ancient intermediates along this lineage are represented by gray circles. The branch lengths shown are not proportional to evolutionary sequence change. B, yeast strains all expressing endogenous STV1-GFP and isoforms integrated at the VPH1 locus (STV1 (GFY316; STV1 under VPH1 promoter control), VPH1 (GFY315), Anc.a (GFY317), and Anc.Stv1 (GFY579)) were visualized by fluorescent and differential interference contrast microscopy. A vector expressing N-terminally tagged mCherry-ALP (pGF242) served as a marker for the vacuole membrane. The overlay of the GFP signal (green) and mCherry signal (magenta) indicates cells where there is strong colocalization of GFP and mCherry signals. C, quantification of cells displaying GFP signal on the vacuole membrane. Experiments were performed in triplicate, and the error is expressed as the S.D. (error bars). Cells that did not show Stv1p-GFP on the vacuole membrane localization showed cellular puncta.

No mutations were obtained in our screen for residues 1–40 as well as 418–455 of Stv1p. Although the extreme N terminus of subunit a is well conserved across species, it is unclear whether the extreme C-terminal portion of the cytosolic domain contributes to sorting. For one, our structural model does not extend beyond residue 408, yet the remaining N-terminal residues are in relatively close proximity; they are clustered within the globular domain opposite the F201SFDD motif and must eventually rejoin with the C-terminal domain embedded within the membrane (Fig. 5, A and B). Also, recent work has suggested that residues 362–407 of Vph1p represent a “tethering” domain for subunit d (Vma6p), and this corresponds to residues 407–453 for Stv1p (75). Furthermore, several residues within this region were identified within our mutant allele pool, including Asn415, Val416, Ile417, and Leu418. Additional characterization of these single mutants revealed a modest zinc phenotype for both N415A and I417A (supplemental Table 2). However, we also tested these mutations in combination with the strong A83KAAAAA mutant and found there was no additive effect on Stv1p mislocalization (supplemental Table 2). These results may help explain the wide variety of mutants obtained in our original Stv1p mutagenesis, many of which do not directly alter the W83KY sequence. One possibility is that additional residues (such as Asn415/Ile417) are required for proper positioning of the W83KY signal. Alternatively, other residues could provide additional contacts with retrograde trafficking machinery and contribute to a more complex sorting surface rather than short linear signal.

It is not uncommon for cargo proteins to contain more than one sorting signal. Multiple signals have been described for cargo at all stages of the secretory pathway from ER exit to entry into the vacuole (53, 76, 77). For instance, experimentation using the heterologous protein A-ALP (cytosolic domain of Ste13p fused to the transmembrane and lumenal domains of alkaline phosphatase) demonstrated that a combination of two signals resulted in Golgi localization (53). In this system, the FXFXD motif was responsible for retrieval from a post-Golgi compartment, whereas an additional signal caused slowed exit.
from the Golgi (53). This second signal was characterized as phosphorylation of Ser\(^{13}\) within the cytosolic domain of Ste13p that regulated transport within the endosomal network (52). Similarly, the resident vacuolar protein Sna3p has been found to have multiple sorting determinants for entry into multivesicular bodies (77). It still remains a possibility that Stv1p has maintained a second signal that causes slowed anterograde transport out of the Golgi similar to Anc.a (23); however, subsequent analysis will require the development of a kinetic assay for trafficking of the V-ATPase through the secretory pathway.

Interestingly, Stv1p also contains the canonical Golgi retrieval motif FXFXD within its N terminus. We have found that the W\(^{83}\)KY signal may represent an FXFXD-like motif because replacement of both aromatic residues to phenylalanine did not significantly compromise Stv1p sorting, although there is a preference for the first amino acid to be tryptophan rather than other residues (Fig. 3D). The FXFXD sequence has been characterized as both necessary and sufficient to dictate retrieval of Ste13p (or other cargo proteins and protein chimeras) from the late endosome to the trans-Golgi (45, 53). However, the structural positioning of the F\(^{201}\)SDD sequence within Stv1p demonstrates that it is buried within a globular domain of the N terminus, with both aromatic residues unlikely to be accessible to the cytosol (Fig. 5). Unlike other model cargo proteins, such as Pho8p (ALP), Ste13p, Kex2p, Vps10p, and Sna3p, that have a relatively simple embedded membrane topology and short cytosolic-facing linear portions (for example, 121 residues for Ste13p, 34 residues for ALP, and 164 residues for Vps10p), the Stv1p V-ATPase contains 14 separate subunits and is almost 1 MDa in size. Subunit a is predicted to interact with many of the other V-ATPase subunits within both the V\(_1\) and V\(_0\) domains (25, 78–80). Therefore, the structural context of any sorting signal would have to (i) not perturb other intersubunit interactions that are conserved between both Stv1p and Vhp1p complexes and (ii) still remain accessible to transport machinery that associates with sorting signal(s). For these reasons, the Stv1p sorting signal may require an ideal three-dimensional structural orientation to allow for proper V\(_0\) assembly, function, and subsequent sorting signal recognition. Our study suggests that rather than primary sequence comparison, the identification of other signal sequences in distant subunits (23) were not fully lost within the Anc.Stv1 intermediate. Future analyses using ancestral intermediates will aid in defining all the residues/surfaces required for Stv1p sorting and interaction with retrograde machinery.

We have previously shown the Golgi localization of Stv1p V-ATPase requires the presence of the retromer complex (23). It is unclear whether retromer binds directly to Stv1p to mediate retrieval from the endosome back to the trans-Golgi. The large retromer subunit Vps35p has been shown to directly associate with various cargo, including yeast Vps10p (84, 85), Ste13p (84, 85), human cation-independent mannose 6-phosphate receptor (86, 87), and sortilin (88) by genetic, co-immunoprecipitation, and yeast two-hybrid analyses. Given the diversity of retromer-binding signals found within cargo proteins (FXFXD for Ste13p, YSSL for Vps10p, and (W/F)L(M/V) for cation-independent mannose 6-phosphate receptor), it is not surprising that the W\(^{83}\)KY signal within Stv1p represents a novel sorting motif (45, 87, 89). Additionally, retromer-dependent cargo was shown to bind to distinct regions of Vps35p (reviewed in Ref 34). We tested whether two yeast Vps35p mutants, D123N and D528G, which are disrupted for A-ALP and Vps10p transport, respectively, also perturb Stv1p-GFP sorting. However, neither of these mutants caused any change in Stv1p localization compared with control strains.\(^{3}\) It is possible that Stv1p binds to a unique portion of Vps35p directly. Alternatively, Stv1p may require the presence of an accessory protein to modulate the interaction of the V-ATPase...
to retromer as in the case of the Fet3p-Frt1p complex that utilizes the cargo-specific adapter, Grd19p, to associate with retromer (90). A combination of biochemical and genetic approaches (including genome-wide screens) will differentiate between these possibilities for retromer-dependent V-ATPase transport.

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