Molecular mechanisms of cutis laxa and distal renal tubular acidosis-causing mutations in V-ATPase a subunits, ATP6V0A2 and ATP6V0A4*

Sally Esmail², Norbert Kartner², Yeqi Yao†, Joo Wan Kim², Reinhart A. F. Reithmeier§ and Morris F. Manolson‡§†

From the ‡Dental Research Institute, Faculty of Dentistry, University of Toronto, Toronto, Ontario M5G 1G6 Canada, and §Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Running title: Functional domains of ATP6V0A2 and ATP6V0A4

To whom correspondence should be addressed: Dr. Morris F. Manolson, Faculty of Dentistry, University of Toronto, 124 Edward St., Toronto, Ontario M5G 1G6, Canada, Tel.: 416-864-8234; Fax: 416-979-4936; Email: m.manolson@utoronto.ca

Keywords: ATPase, Proton pump, ER-associated degradation, N-glycosylation, Membrane protein, Protein degradation, Trafficking, 3D modeling

The a subunit is the largest of 15 different subunits that make up the vacuolar H⁺-ATPase (V-ATPase) complex, where it functions in proton translocation. In mammals, this subunit has four paralogous isoforms, a1–a4, which may encode signals for targeting assembled V-ATPases to specific intracellular locations. Despite the functional importance of the a subunit, its structure remains controversial. By studying molecular mechanisms of human disease-causing missense mutations within a subunit isoforms, we may identify domains critical for V-ATPase targeting, activity and/or regulation. cDNA-encoded FLAG-tagged human wild type ATP6V0A2 (a2) and ATP6V0A4 (a4) subunits and their mutants, a2P405L (causing cutis laxa), and a4R449H and a4G820R (causing renal tubular acidosis, dRTA), were transiently expressed in HEK 293 cells. N-glycosylation was assessed using endoglycosidases, revealing that a2P405L, a4R449H and a4G820R were fully N-glycosylated. Cycloheximide (CHX) chase assays revealed that a2P405L and a4R449H were unstable relative to wild type. a4R449H was degraded predominantly in the proteasomal pathway while a2P405L was degraded in both proteasomal and lysosomal pathways. Immunofluorescence studies disclosed retention in the endoplasmic reticulum and defective cell-surface expression of a4R449H and defective Golgi trafficking of a2P405L. Co-immunoprecipitation studies revealed an increase in association of a4R449H with the V₀ assembly factor VMA21, and a reduced association with the V₁ sector subunit, ATP6V1B1 (B1). For a4G820R, where stability, degradation and trafficking were relatively unaffected, 3D molecular modeling suggested that the mutation causes dRTA by blocking the proton pathway. This study provides critical information that may assist rational drug design to manage dRTA and cutis laxa.

Vacuolar H⁺-ATPases (V-ATPases) are conserved, multisubunit rotary proton pumps that play crucial roles in regulating the pH of cells and their intracellular compartments (1-5). They can be categorized as endomembrane or plasma membrane V-ATPases, based on their subcellular localization (6,7). Endomembrane V-ATPases are expressed in all eukaryotic cells in the membranes of acidic organelles like lysosomes, endosomes and the Golgi apparatus, where they translocate protons to acidify the luminal compartments of the organelles (8). Plasma membrane V-ATPases traffic to the surfaces of some specialized cells, such as osteoclasts, kidney intercalated cells and metastatic cancer cells, where they secrete protons into the extracellular fluid (6,9-11).

The V-ATPase complex consists of 15 different subunits arranged into two major sectors, the
cytoplasmic $V_1$ sector and the membrane-integrated $V_0$ sector. $V_1$ is responsible for ATP hydrolysis that provides the energy to rotate a central shaft that powers proton translocation (3). $V_0$ contains a coupled rotor that carries protons for transport through a proton channel pathway formed largely by the c. 100-kDa $a$ subunit (2,12). The $a$ subunit is the largest $V$-ATPase subunit, and in mammals there are four isoforms, $a1$–$a4$. The N-terminal half of the protein (NT$a$) is hydrophilic and associates with subunits of the $V1$ sector in the $V$-ATPase complex, and the C-terminal half (CT$a$) is an integral membrane domain consisting of 8 transmembrane $\alpha$-helices (TMs) and a cytoplasmic (C-terminal) tail domain (CTD). Whereas $a1$ and $a2$-containing $V$-ATPase complexes are targeted to endomembranes, $a3$ and $a4$ complexes are targeted to plasma membranes in some specialized cells (13,14).

Human missense mutations of the $a$ subunits are implicated in diverse diseases (1,15). For example, mutations affecting the function of $a2$ result in cutis laxa (wrinkled skin syndrome), where aberrant Golgi function results in glycosylation defects with consequent abnormal elastin processing that affects skin and internal organs (16-18). Mutations that affect $a3$ function result in inability of osteoclasts to resorb bone, causing autosomal malignant osteopetrosis that is characterized by dense, brittle bone (19,20). Loss of $a4$ function due to mutation results in distal renal tubular acidosis (dRTA) with occasional hearing loss (21,22). Here we focus on the effect of human mutations on $a2$ traffic to Golgi and $a4$ traffic to the plasma membrane based on their proposed in vivo locations and functions.

Despite such important implications for $a$ subunit functions in disease, the structures of human $a$ subunit isoforms are still controversial because of a lack of high-resolution structural data. Recently, however, a 6.4-Å model of the membrane-integrated domain of the yeast $a$ subunit (Vph1p) has been published (23,24). This model is based on a synthesis of data derived from cryo-EM 3D reconstruction, evolutionary covariance mapping of key residues, and low resolution X-ray crystallography. It confirms that the $a$ subunit membrane domain consists of 8 TMs, as has been previously shown (2,12), with TM7 and TM8 highly tilted and forming an interface with the $V_0$ rotor $c$-ring that enables proton translocation at the $a$ subunit/$c$-ring interface.

In spite of such recent advances, knowledge of $a$ subunit folding, targeting, and assembly into the $V$-ATPase holocomplex remains sparse. Considerably more investigation will be required to elucidate issues such as, for example, the mechanism of plasma membrane $a$ subunit targeting, the resolution of which will be required before efforts at designing strategies for targeted therapeutic interventions can realistically be considered. To that end, we conjectured that human disease-causing missense mutations within $a$ subunits could be used to identify critical domains essential for $V$-ATPase targeting, activity and/or regulation. As an approach to testing this, we have studied the molecular consequences of introducing the cutis laxa-causing mutation, p.Pro405Leu (P405L) in $a2$, and the dRTA-causing mutations, p.Arg449His (R449H) and p.Gly820Arg (G820R) in $a4$, into epitope-tagged human $a$ subunit constructs for expression and characterization in the HEK 293 mammalian expression system. We present here results of these studies with respect to subunit glycosylation, stability, degradation, incorporation into $V$-ATPase complexes, and subcellular localization.

Results

Amino acid residues $a2$ P405, $a4$ R449 and $a4$ G820 are highly conserved—Alignments of $a$ subunit polypeptide sequence segments affected by the human mutations causing cutis laxa and dRTA that are under study in the present work are shown in Fig. 1A and 1B. The mutated residues (highlighted in red) are identical in all four human and mouse $a$ subunit isoforms, and also in the yeast $a$ subunit isoform, Vph1p (highlighted in yellow). Fig. 1A shows a segment of the integral membrane domain of the $a$ subunit, where human mutations in $a2$ P405 (in TM1; TMs highlighted in blue) and $a4$ R449 (in TM3) result in cutis laxa and dRTA, respectively. Fig. 1B shows alignments for a C-terminal segment of the $a$ subunit comprising the CTD, where the human mutation in $a4$ G820 results in dRTA. Thus, the three mutations under consideration here, $a2$P405L, $a4$R449H and $a4$G820R, all affect highly conserved amino acid residues.

Glycosylation and stability of cutis laxa mutant, $a2$P405L—We have previously shown that all human
a subunit isoforms are N-glycosylated and that N-glycosylation is required for their stability (25,26). We have also shown in previous work that in the case of the osteopetrosis mutation, a2P405L, the a subunit is misfolded, unglycosylated, retained in the ER, and ultimately subjected to proteolytic degradation (20). It was of interest, therefore, to determine whether the cutis laxa and dRTA mutations have similar impacts on a2 and a4 subunits, respectively, using methods for assessing N-glycosylation and stability that were previously described (25). Briefly, glycosylation and stability were tested in HEK 293 cells by transient transfection and expression of FLAG-tagged wild type and mutation-bearing a2 and a4 subunit constructs. Whole-cell lysates prepared 24 h post-transfection were treated with PNGase F to assess whether mutant proteins, a2P405L, a4R449H and a4G820R were N-glycosylated, and with Endo H to determine whether any bound glycans were of the high mannose or hybrid type (27,28). Stability was assessed using the CHX chase method previously described (25,26). Briefly, cells were treated, 24 h post-transfection, with CHX (10 μg/ml) for up to 12 h, and whole-cell lysates prepared, immunoblotted and quantified (GAPDH was used as a loading control; see Experimental Procedures). Fig. 1C shows immunoblots of wild type FLAG-tagged a2 protein (WT a2-2FLAG), and the similarly epitope-tagged cutis laxa mutant subunit, a2P405L (a2P405L-2FLAG) expressed transiently in HEK 293 cells, with and without Endo H treatment of the wholecell lysates. WT a2-2FLAG was observed as a 110-kDa band, and upon Endo H treatment its relative mobility was reduced to 105 kDa, representing the deglycosylated a2-2FLAG. The mutant a2P405L-2FLAG was also observed as a 110-kDa band, and upon Endo H treatment its relative mobility was reduced to 105 kDa, representing the deglycosylated a2P405L-2FLAG.

In the same manner, protein stability of a2P405L was assessed by transient expression of the mutant protein or its wild type counterpart. After allowing 24 h of expression, the cells were incubated with or without 10 μg/ml CHX, and were harvested after the indicated times for whole-cell lysate preparation (see Experimental Procedures). Glycans were removed from all proteins, wild type and mutant, prior to immunoblotting, by treatment with PNGase F. Fig. 1D shows quantitative band analysis of the immunoblots used to assess stability of a2P405L-2FLAG transiently expressed in HEK 293 cells. All band intensities were normalized to GAPDH as a loading control, and to zero time controls. These data showed that a2P405L-2FLAG was degraded at a significantly faster rate than WT a2 (p < 0.05), the mutant protein having a half-life of 13.4 ± 1.0 h compared to 23.8 ± 4.3 h for WT a2-2FLAG (see Supplemental Data, Tables S1 and S2, for data and statistics for all stability assays in the present work).

Glycosylation and stability of dRTA mutants, a4R449H and a4G820R—Transient expression of FLAG-tagged human WT a4 and dRTA mutants was performed as for the a2 constructs. On immunoblotting, as shown in Fig. 1E, WT a4-2FLAG was observed as a 105-kDa band and, upon PNGase F and Endo H treatment, its relative mobility was reduced to 98 kDa, representing the deglycosylated a4-2FLAG. Similarly, a4R449H-2FLAG and a4G820R-2FLAG were observed as 105-kDa bands, and upon PNGase F or Endo H treatments their relative mobilities were reduced to 98 kDa, representing deglycosylated a4R449H-2FLAG and a4G820R-2FLAG. Thus, both a4R449H and a4G820R appeared to be N-glycosylated with Endo H-sensitive glycans, consistent with what was observed for WT a4.

To determine stability, WT a4 and the mutant proteins a4R449H and a4G820R were compared using CHX chase experiments, as described above; whole-cell lysates were prepared at indicated time intervals and analyzed. Glycans were removed from all proteins, wild type and mutants, by PNGase F treatment of cell lysates prior to immunoblotting; Fig. 1F shows quantitative band analysis of the immunoblots. All band intensities were normalized, as described above. Analysis of the data graphed in Fig. 1F showed that stability of a4R449H-2FLAG, with a half-life of 13.7 ± 1.7 h, was reduced by only 20% (p < 0.05) relative to WT a4-2FLAG (17.0 ± 1.2 h); however, the half-life of a4R449H-2FLAG (4.8 ± 0.39 h) was greatly reduced, by over 70% (p < 0.01) relative to WT a4-2FLAG.

Pathways for degradation of unstable mutant proteins, a2P405L and a4R449H—The stability of the a4G820R mutant subunit was not greatly different from wild type, but the a2P405L and a4R449H mutants were clearly unstable. It was of interest to further characterize whether degradation of the latter two mutants was via the proteasomal pathway or the lysosomal pathway. After expression of a2P405L-2FLAG and a4R449H-2FLAG in HEK 293 cells,
CHX chase experiments were done with and without either an inhibitor of proteasomes (10 μM MG132), or lysosomes (25 mM NH₄Cl), as previously described (25). Fig. 2 shows quantitative band analyses for the immunoblots loaded with WT a2-2FLAG and a2P405L-2FLAG, or WT a4-2FLAG and a4R449H-2FLAG, before and after MG132 treatment. Analysis of data in Fig. 2A showed that stability of the a2P405L-2FLAG construct (half-life 13.4 ± 1.4 h) was 64% that of WT a2-2FLAG (half-life 21.0 ± 1.6 h); however, after proteasomal inhibition, the degradation rates of a2P405L-2FLAG (half-life 17.7 ± 0.69 h) and WT a2-2FLAG (half-life 17.8 ± 1.2 h) were indistinguishable (p = 0.89). Data for Fig. 2B showed that without proteasomal inhibition, the half-life of the mutant a4R449H-2FLAG (5.6 ± 0.12 h) was 26% that of WT a4-2FLAG (21.3 ± 3.7 h; p < 0.05). After proteasomal inhibition, there was a highly significant decrease (p < 0.01) in the degradation rate of a4R449H-2FLAG (half-life 5.6 ± 0.12 h before treatment, 21.5 ± 1.5 h after), with restoration of stability to levels exceeding that of WT a4-2FLAG with the same treatment (half-life 17.2 ± 1.0 h). Data for Fig. 2C showed that lysosomal inhibition partially restored stability of a2P405L-2FLAG (half-life 7.8 ± 0.51 h before and 11.4 ± 1.0 h after treatment, p < 0.01), by about half (56%) of the difference between untreated mutant levels and treated wild type levels. Finally, data from Fig. 2D showed that lysosomal inhibition had no significant effect (p = 0.10) on the degradation rate of a4R449H-2FLAG (half-life 5.1 ± 0.15 h before treatment, 5.6 ± 0.34 h after treatment). Taken together, this suggested that degradation of a2P405L occurs both in the proteasomal and lysosomal pathway, while the degradation of a4R449H predominantly occurs in the proteasome.

a2 P405 is required for Golgi trafficking, and a4 R449 for ER exit—The apparently significant degradation of both a2P405L and a4R449H suggests that the mutant subunits fail to assemble into the V-ATPase complex; therefore, we conducted immunofluorescence localization experiments to establish whether there is colocalization of these mutant proteins with ER and/or Golgi compartment markers. Fig. 3A and B show colocalization studies of WT a2-2FLAG and a2P405L-2FLAG with calnexin (ER marker) and syntaxin 6 (Golgi marker). Fig. 3A shows representative fluorescence photomicrography images of HEK 293 cells transfected with empty vector (left-most panel), WT a2-2FLAG (middle panel) and a2P405L-2FLAG (right-most panel), probed with anti-FLAG antibody (green) and antibodies to the ER marker, calnexin (red). These images showed that a2P405L-2FLAG (green) colocalized with calnexin at a rate similar to that seen for WT a2-2FLAG (p = 0.073). A similar experiment is shown in Fig. 3B, but using the Golgi marker protein, syntaxin 6 (red). The a2P405L-2FLAG mutant protein appeared to colocalize with the Golgi marker at a rate lower than was apparent for WT a2-2FLAG (p < 0.05).

Fig. 3C shows representative fluorescence photomicrography images of control, empty vector-transfected cells (left-most panel), WT a4-2FLAG (2nd from left), a4R449H-2FLAG (2nd from right) and a4G820R-2FLAG (right-most panel) probed with anti-FLAG (green) and anti-calnexin (red) antibodies. The data suggested that a4R449H-2FLAG colocalized with the ER marker, calnexin, at a rate exceeding that of WT a4-2FLAG (p < 0.05), whereas a4G820R-2FLAG was similar to WT a4-2FLAG in this respect (p = 0.081). Fig. 3D shows representative micrographs of the same cell series as in Fig. 3C, but probed with anti-FLAG (green) and anti-syntaxin 6 (red) antibodies. The mutant protein, a4R449H-2FLAG, colocalized with syntaxin 6 at a lower rate than the WT a4-2FLAG (p < 0.05), whereas a4G820R-2FLAG was again similar to the WT a4-2FLAG in this respect (p = 0.090).

Fig. 3E shows colocalization analysis of images represented in Fig. 3A and B, revealing that a2P405L-2FLAG colocalized with calnexin in the ER, the same as WT a2-2FLAG. The localization of a2P405L-2FLAG to Golgi (syntaxin 6), however, was reduced with reference to the wild type (p < 0.001; r = 0.5–0.8). Similarly, Fig. 3F shows colocalization analysis of images represented in Fig. 3C and D, revealing significant retention of a4R449H-2FLAG in the ER, and significantly lower association with the Golgi marker, compared with WT a4 (p < 0.001; r = 0.5–0.8). The a4G820R mutant, on the other hand, was indistinguishable from wild type in these respects (p = 0.081 for calnexin, p = 0.090 for syntaxin 6).

Defective cell surface expression of a4R449H—As shown above, a4R449H was unstable relative to WT a4, was retained in the ER, and was ultimately degraded in the proteasome. To further its characterization, it was of interest to determine whether any of the mutant protein was able to
traffic to its normal location at the cell surface. To assess cell surface expression (Fig. 4), a4 is tagged with both HA in extracellular loop II (ELII) and FLAG at the end of the C-terminus. We have shown ELII is the site of N-glycosylation within subunit a1-a4 (25,26) indicating that ELII is luminal/extracellular. In contrast, we and others have shown that the C-terminal domain is cytoplasmic(12,25,26). Comparing the accessibility of either epitope in permeabilized vs non-permeabilized cells can determine if a4 is expressed on the cell surface. In permeabilized cells, one would expect that both cytoplasmic and extracellular epitopes would be assessable to fluorescently-labeled antibodies; in non-permeabilized cells, only HA on the extracellular EL2, would be available. Fig. 4A–D show representative fluorescence micrographs of HEK 293 cells transfected with either WT a4-HA-2FLAG, a4R449H-3HA-2FLAG or a4G820R-3HA-2FLAG, double-stained with anti-HA (red) on non-permeabilized cells followed by cell permeabilization and staining with anti-FLAG (green). Total protein expression is represented by anti-FLAG (green) staining, and cell-surface expression by anti-HA (red) staining. Fig. 4A shows empty vector-transfected (control) cells stained, Fig. 4B shows intracellular as well as cell surface expression for cells transfected with WT-a4-3HA-2FLAG, and Fig. 4C shows only intracellular expression in cells transfected with WT-a4R449H-3HA-2FLAG, with no cell surface expression detected. Fig. 4D shows intracellular, as well as cell-surface, expression for cells transfected with WT-a4G820R-3HA-2FLAG, a mutant that has a half-life similar to that of WT a4.

In order to confirm the above findings for a4 subunits, which are expected to traffic ultimately to the plasma membrane, cell-surface proteins of intact cells were biotinylated, and the biotinylated proteins were then affinity purified for further assessment (see Experimental Procedures). Fig. 4E shows an immunoblot of the whole-cell lysates and cell-surface fraction from cells that were transfected with WT a4-2FLAG, a4R449H-2FLAG, or a4G820R-2FLAG. WT a4-2FLAG and a4G820R-2FLAG were expressed on the surface, as expected, but there was no cell surface expression of a4R449H-2FLAG. This result confirms the immunofluorescence findings in Fig. 4A–D, suggesting that a4R449H is largely retained in the ER. a4R449H shows increased association with VMA21—As demonstrated above, a4P405L-2FLAG and a4R449H-2FLAG had substantially shorter half-lives and defective Golgi and ER localization, as compared with their wild type counterparts. It was of interest to characterize the effect of these mutations on their incorporation into the V-ATPase complex. The assembly of human V-ATPase is not well characterized, but studies in yeast have revealed that biosynthesis of V₀ in the ER is dependent on three assembly factors, Vma12p, Vma21p, and Vma22p (7). Due to the high homology between mammalian a subunit and the yeast ortholog, Vph1p, a similar biosynthetic mechanism was expected. VMA21, the human ortholog of yeast Vma21p, is the only characterized human V-ATPase assembly factor (29). VMA21 is required for incorporation of the a subunit into the V₀ subcomplex, but the dissociation of a subunit from V₀ is required for further V₁–V₀ assembly. Thus, prolonged association of VMA21 with V₀ inhibits the formation of the V-ATPase holocomplex (30,31). Fig. 5A–C show representative immunoblots loaded with immunoprecipitate fractions that were pulled down with anti-FLAG antibody from lysates of HEK 293 cells transfected with WT a2-2FLAG, a2P405L-2FLAG, WT a4-2FLAG, a4R449H-2FLAG, or a4G820R-2FLAG and immunoblotted with either anti-VMA21 or anti-B1 antibodies. Protein band quantification analysis (Fig. 5D and E) showed a difference between the mutant protein, a4R449H-2FLAG, and its wild type counterpart. The mutant had a significantly higher association (p < 0.05) with VMA21 (representing a–V₀ assembly), and a lower association with B1 (representing V₁–V₀ assembly) compared with wild type a4 (Fig. 5E). Interestingly, there was no significant difference (p = 0.12) between the association of a4G820R-2FLAG or a2P405L-2FLAG with either B1 or VMA21, compared with their wild type counterparts.

a4 G820 resides within the putative proton pathway—As shown above, the a4G820R-2FLAG, compared with WT, showed only a small or insignificant difference in terms of protein stability, localization in the secretory pathway, or cell surface expression. Therefore, it remained of interest to determine the mechanism by which the a4 G820R mutation causes dRTA. In an attempt to address this, we constructed a homology model for the CTA domain of the human a4 subunit, based on a recent
model for the CTA domain of yeast Vph1p. The latter was built based on low-resolution X-ray crystallography, high-resolution cryo-EM, mutagenesis studies, and analysis of evolutionary covariance (23). This model showed the locations of highly conserved, key functional residues within the proton translocation pathway, or proton channel. In a similar manner, we reconstructed the same residues within our human a4 model, and found that the a4 G820 residue was located within the putative interface of the proton translocation pathway (Fig. 6A and B). We created a second homology model for the a4G820R mutant protein (Fig. 6C) and showed that the positively charged side chain of the mutant a4 R820 residue possibly interferes with the proton pathway by forming a salt bridge (3.2 Å) with the adjacent negatively-charged residue, E729. The latter amino acid has been previously recognized as an important residue for proton translocation (32).

**Discussion**

**a2 P405, a4 R449 and a4 G820 are conserved and crucial for function**—Mutation of the V-ATPase a2 subunit amino acid residue P405 results in cutis laxa, and a4 mutations in the residues R449 and G820 result in dRTA. In an effort to understand how these missense point mutations can lead to disease, we first conducted multiple amino acid alignments, which revealed that the residues of interest were highly conserved (Fig. 1A and B). The a2 P405, a4 R449 and a4 G820 residues reside within TM1, TM2 and the CTD, respectively, which are highly conserved domains in species ranging from human to yeast. By characterizing the effects that these mutations have on a subunit glycosylation, structural stability, trafficking and assembly, we hoped to elucidate their disease mechanisms and also add to the as yet limited understanding of structural/functional domains within human V-ATPases, ultimately to provide a basis for rational drug design.

**Human a2P405L and a4R449H are N-glycosylated, but are unstable and a4R449H degraded predominantly in the proteasomal pathway**—We have previously shown that human a1–a4 subunits are N-glycosylated and that this is important for subunit stability (25,26). In the present study, results of Fig. 1C and E showed that mutant proteins, a2P405L, a4R449H and a4G820R, were all N-glycosylated, and all with Endo H-sensitive high-mannose or hybrid glycan moieties. Moreover, a2P405L and a4R449H, but not a4G820R, showed a much higher rate of turnover (i.e. decreased stability) relative to their respective wild type subunit (Fig. 2). These results also showed that turnover rates of a2P405L and a4R449H could be restored to wild type levels by treatment with the proteasomal inhibitor, MG132. Treatment with the lysosomal inhibitor, NH4Cl, had no significant effect on the turnover rate of a4R449H and modestly reduced the turnover rate of a2P405L. This suggested that a4R449H was degraded predominantly in the proteasomal pathway, which is activated in response to the presence of misfolded proteins in the ER (33), with some degradation of the former occurring also in the lysosomal pathway.

Within this study, we tagged both WT and mutant subunits with C-terminal epitopes. We, and other, have shown that a variety of different epitope types and sizes inserted at the extreme C-terminal domain of the mammalian V-ATPase a subunit does not appear to affect activity or stability (25,26,34,36). In yeast, we were able to show that introducing green fluorescent protein (GFP), a 238 amino acid, 26.9 kDa polypeptide, to the C-terminal of Vph1p, the yeast V-ATPase a subunit, did not affect subunit stability, assembly, function and trafficking with respect to endogenous Vph1p (12).

**a2 P405 is required for Golgi trafficking, and a4 R449 for ER exit**—The relatively high degradation rates of both a2P405L and a4R449H in the proteasomal pathway suggested that these subunits fail to assemble into the V-ATPase complex and therefore fail to traffic to their normal destinations. In spite of the higher turnover rate of a2P405L relative to the wild type (Fig. 2A and C), however, quantification of colocalization of a2P405L with calnexin showed no significant difference in association of a2P405L with calnexin, compared to WT a2 (p = 0.073). Additionally, however, a2P405L showed significantly less association (p < 0.05) with the Golgi marker, syntaxin 6 (Fig. 3E), which suggested that the a2P405 mutation results in misprocessing that leads to defective Golgi trafficking, but not ER retention. In contrast,
quantification of colocalization analysis for a4R449H and a4G820R with the ER-resident marker, calnexin (Fig. 3C and F), revealed a significantly higher colocalization of a4R449H with calnexin (p < 0.05), suggesting ER retention of a4R449H, but not of a4G820R which was not different from wild type a4 in that respect (p = 0.081). However, the exact mechanism of a4R449H ER retention remained to be investigated. Taken together, these observations suggest that the a2 P405 and a4 R449 residues within TM1 and TM2, respectively, are essential for human a2 and a4 stability, and for their trafficking in the secretory pathway.

a4 R449 is crucial for cell surface expression and a-Vo association—We have previously shown experimentally that the exogenously expressed WT a4 is able to traffic to the plasma membrane of HEK 293 cells (25). In the current study we have used the same strategy to determine the effect of the mutations in a4R449H and a4G820R on cell surface expression. Fig. 4B and D showed that both WT a4 and a4G820R were able to traffic to the cell surface, while a4R449H showed defective cell surface expression (Fig. 4C). The same findings were subsequently confirmed by cell surface biotinylation (Fig. 4E).

It was of interest also to determine the effect of the mutations under investigation on formation of the V-ATPase holocomplex. To that end, we specifically characterized the association of a2P405L, a4R449H and a4G820R with the only characterized human V-ATPase assembly factor, VMA21. Protein band quantification of co-immunoprecipitates (Fig. 5E) revealed that a4R449H had a significantly higher association (p < 0.05) with VMA21. In yeast, the assembly chaperone Vma21p assembles with V0-associated a subunits, and dissociates only after V0 exits the ER (37); dissociation of Vma21p from V0 is required for V1–V0 assembly, and prolonged Vma21p–V0 association reduces V1–V0 assembly. We propose that the significantly higher association observed between a4R449H and VMA21 indicates a prolonged association of a4R449H–V0 with VMA21 that leads to failure of V1–V0 assembly, ER retention of a4R449H, and ultimately its proteasomal degradation, resulting in defective cell surface expression.

a4 G820 is a functional residue residing in the putative proton pathway—The a4 G820 residue is highly conserved among species (Fig. 1B). Due to the lack of a mammalian model, the mechanism of the a4G820R dRTA-causing mutation has been studied previously only in yeast. One of these studies reported that the a4G820R mutation in the yeast homolog, Vph1p, did not affect pump assembly or targeting but decreased V-ATPase hydrolytic and proton pumping activities by 83–85% (10). Another study in the yeast a subunit showed that the a4G820R homologous mutation (Vph1pG812R) was associated with severe loss of proton translocation (by 78%) and a moderate decrease in ATPase activity (by 36%). This study also showed that the a4 G820 residue lies within the domain that interacts with the glycolytic enzyme, phosphofructokinase-1 (PFK-1) and that the a4G820R-equivalent mutation inhibited this interaction (38). In the present work we used exogenous expression in HEK 293 cells to investigate the role of this mutation in protein stability, glycosylation and trafficking in the secretory pathway and to the plasma membrane, and our results showed that the stability of a4G820R was only mildly affected (Fig. 1F) and trafficking to the Golgi and plasma membrane were not discernably altered (Fig. 3D and Fig. 4D).

In an attempt to obtain further insights into how a4 G820R might impact V-ATPase function, we used the recently published atomic model synthesized from studies of T. thermophilus and S. cerevisiae V-ATPase and bovine F-ATPase (23) as a template to construct a 3D human a4 C-terminal domain model (Fig. 6A). The model revealed that the a4 G820 residue interfaces with the proposed proton transport pathway. Furthermore, swapping arginine for glycine (a4 G820R) resulted in a putative salt bridge (3.2 Å) with the adjacent negatively charged residue a4 E729 (compare Fig. 6B with Fig. 6C), which is also highly conserved and is thought to be important for proton translocation (32). Therefore, we proposed that the a4G820R mutation likely causes dRTA by forming a salt bridge that sterically interferes with the structure of the proton channel and consequently with proton translocation. It might do this directly, by blocking the proton channel physically (i.e. in its immediate vicinity), or allosterically by altering the conformation of the CTD more extensively. Some conformational change must be occurring to be consistent with the previous observation that the mutation also inhibits the interaction of the a subunit with PFK-1 (38), the binding of which must occur at a cytoplasmically accessible site; however,
this does not preclude the possibility that the putative salt bridge resulting from the G820R mutation both blocks the proton pathway directly and causes extended conformational changes in the CTD.

**Conclusion**—Characterization of highly conserved residues implicated in diseases has been successfully used by others as a strategy for determining protein domain function and to inform targeted drug discovery (39-41). For example, deletion of the highly conserved residue F508 (ΔF508) in the human cystic fibrosis transmembrane-conductance regulator (CFTR) leads to cystic fibrosis. The ΔF508 mutation results in protein misfolding, misprocessing and aberrant trafficking (42). Characterization of the molecular mechanism of ΔF508 CFTR disease causation has led to the development of molecular chaperone approaches to correct CFTR folding and promote its trafficking to its normal functional destination, yielding a promising approach for treatment of ΔF508 cystic fibrosis (41).

V-ATPase a isoforms are potential targets for therapeutics directed towards a number of diseases (1). Thus, a further understanding of the structural domains affecting a subunit folding, trafficking, membrane targeting, function and regulation will enhance our ability to target specialized V-ATPases. We previously showed that N-glycosylation is required for a subunit stability, assembly and trafficking to the plasma membrane (25,26). In the present work we showed that a2P405L and a4R449H resulted in cutis laxa and dRTA through interfering with protein stability, and subsequent ER retention and degradation. a4R449H was degraded predominantly in the proteasomal pathway while a2P405L was degraded in both proteasomal and lysosomal pathways. In summary, we have proposed a model for how we believe that the N-glycosylated a4 subunit is assembled, trafficked in the secretory pathway, and delivered to the plasma membrane (see Fig. 7). Our data also suggest routes to drug discovery such as screening for chemical chaperons to rescue a subunit folding to allow ER exit for treatment of cutis laxa and dRTA.

**Experimental Procedures**

**Enzymes and reagents**—Restriction enzymes, endo-β-N-acetylglucosaminidase H (Endo H; catalog no. P0702S), and peptide N-glycosidase F (PNGase F; P0704S) were from New England Biolabs (Whitby, Canada). Octaethylene glycol mono-n-dodecyl ether (C12E8) was from NIKKO Chemicals (Barnet Products, Englewood Cliffs, NJ). Bradford protein assay reagent (500-0006) was from Bio-Rad (Mississauga, Canada), 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; 10236276001) was from Roche Diagnostics (Mississauga, Canada), and cycloheximide (CHX; CYC003) was from BioShop (Burlington, Canada). Phenylmethylsulfonyl fluoride (PMSF; P7626), Protease Inhibitor Cocktail (P8340), and the proteasome inhibitor N-(benzoyloxycarbonyl)leucinylleucinylleucinal (MG132; C2211) were from Sigma-Aldrich (Oakville, Canada). Dulbecco’s modified Eagle’s medium (DMEM; 11965092), Dulbecco’s phosphate buffered saline (DPBS; 1404182), heat-inactivated fetal bovine serum (FBS; 16140071), penicillin/streptomycin mix (15140122), phosphate buffered saline (PBS; 10010023), 1X Trypsin/EDTA (25200056), and the Novex ECL horseradish peroxidase (HRP) chemiluminescent substrate reagent kit (WP20005) were obtained from Gibco (Fisher Scientific, Whitby, Canada). GenJet In Vitro DNA Transfection Reagent (SL100488) was purchased from SignaGen Laboratories (Rockville, MD).

**Antibodies**—Mouse monoclonal IgG2b anticalnexin (3H4A7; sc-130059), HRP-conjugated goat polyclonal IgG anti-rabbit IgG (sc-2004), mouse monoclonal IgG1 anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH, 0411; sc-47724), and HRP-conjugated goat polyclonal IgG anti-mouse IgG (sc-2005) were purchased from Santa Cruz (Dallas, TX). Rabbit IgG anti-VMA21 antibody (HPA010972) was from Sigma-Aldrich. Rabbit polyclonal IgG anti-FLAG (ab1162) and mouse monoclonal IgG1 anti-syntaxin 6 (ab56656) were from Abcam (Cambridge; Burlingon, Canada). Alexa Fluor 568-conjugated goat polyclonal IgG anti-rabbit IgG (A-11011), Alexa Fluor 488-conjugated goat polyclonal IgG anti-rabbit IgG (A-11034), Alexa Fluor 568-conjugated goat polyclonal anti-mouse IgG (A-11004), and Alexa Fluor 488-conjugated goat polyclonal IgG anti-mouse IgG (A11001) were from Molecular Probes, Fisher Scientific.
cDNA constructs, plasmids and cells—The pCMV6-XL4 plasmid carrying human a2-coding cDNA was purchased from Origene (SC115366). To prepare wild type (WT) a2-2FLAG (with tandem C-terminal FLAG epitope tags), the insert was transferred from pCMV6-XL4 to pBluescript SK+, then tagged with 2FLAG (2 × DYKDDDDK) at its carboxy-terminus between XbaI/HindIII restriction sites. The 2FLAG-tagged construct was then transferred back to pCMV6-XL4 between APal/HindIII sites. To prepare the a2P405L,2FLAG mutant construct, the pCMV6-XL4 carrying WT a2-2FLAG was modified by inserting a synthetic fragment bearing the P405L mutation between BmgBI/pf1F1 sites (human a2 cDNA bp 1916–2487); the mutant synthetic cDNA was obtained from GeneArt in the PMA-T vector. WT a4-3HA-2FLAG was prepared as described previously (25). To prepare a4R449H-3HA-2FLAG and a4G820R-3HA-2FLAG, GeneArt synthetic cDNA bearing the R449H mutation was inserted between EcoRI/SapI sites, and the a4G820R fragment was inserted into the Apal site in pcDNA3.1(+). Accession numbers for source sequences used in constructs are: human a2 (ATP6V0A2), NM_012463; human a4 (ATP6V0A4), NP_570856. The DNA sequences of all constructs were confirmed by commercial sequencing (ACGT; Toronto, Canada). Human embryonic kidney cells (HEK 293; CRL-1573TM) were from the American Type Culture Collection (ATCC; Manassas, VA).

Cell culture and transfection—Liquid nitrogen-stored HEK 293 cells were rapidly thawed in a water bath at 37 °C followed by incubation in 75 cm² tissue culture flasks containing 17 ml DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin mix, in a humidified 5% CO₂ incubator for 4 days at 37 °C. The cells, at 70–80% confluence, were trypsinized with 1 ml of 1X Trypsin/EDTA and seeded into 6-well plates at a density of 4–7 × 10⁵ cells/well and incubated for 24 h. Cells were subsequently transiently transfected with 1 μg/well of plasmid construct in a transfection complex containing GenJet reagent and plasmid DNA in a 3:1 ratio. The transfection complex was diluted to 200 μl final volume with serum-free DMEM and incubated for 10 min prior to transfection. Post-transfection cells were incubated for 24 h and then harvested for protein expression analysis. There was no significant difference (p<0.05) in cell viability between HEK 293 cells transfected with either WT, mutant, or empty vectors (data not shown).

Protein expression analysis and assessment of glycosylation—Whole-cell lysates were prepared as previously described (25). Briefly, cells were harvested in 0.2 ml/well lysis buffer (PBS containing 1% C12E8, 1 mM PMSF and 1:100 (v/v) Protease Inhibitor Cocktail) and incubated on ice for 30 min. Lysates were then centrifuged at 15,000g for 30 min at 4 °C, and supernatants were collected for further analysis. Protein concentrations of the supernatants were quantified using the Bradford protein assay.

Protein glycosylation was assessed by treatment of samples with either PNGase F or Endo H. Briefly, 30 μg of whole-cell lysate was denatured in 3 μl of 10X glycoprotein denaturation buffer (5% sodium dodecyl sulfate, 0.4 M dithiothreitol; New England Biolabs), the reaction mixture was adjusted to 20 μl and incubated at 65 °C for 10 min, then 2 μl 10X Glyco Buffer was added (for PNGase F, 0.5 M sodium phosphate, pH 7.4 at 25 °C; for Endo H, 0.5 M sodium citrate, pH 7.5 at 25 °C). Subsequently, 2 μl 10% (w/v) NP-40 (New England Biolabs) and 2,000 u PNGase F, or Endo H, were added. The final volume was adjusted to 40 μl with distilled H₂O, incubated for 1 h at 37 °C, and then analyzed by immunoblotting.

Immunoblotting—Immunoblotting was conducted as previously described (25). Briefly, 30 μg of whole-cell lysate was loaded per well and subjected to 7% SDS-PAGE. Proteins were then transferred to nitrocellulose membrane and incubated overnight at 4 °C with 1:2,000–1:3,000 diluted primary antibodies (anti-FLAG, anti-B1, or anti-VMA21). 1:5,000 diluted anti-GAPDH was used in some experiments to provide loading controls. The blots were then incubated for 1 h at room temperature with 1:5,000 HRP-labeled secondary antibody and bands were developed with chemiluminescent substrate reagent.

Protein stability and protein band quantification—Protein stability was evaluated using the cycloheximide (CHX) chase assay. Briefly, HEK 293 cells were transfected with WT and mutant cDNA constructs and 24 h post-transfection the cells were treated with 10 μg/ml CHX with or without proteasomal inhibitor (10 μM MG132), or lysosomal inhibitor (25 mM NH₄Cl), for up to 12 h. The cells were subsequently harvested and whole-cell lysates were prepared for analysis.
immunoblotting with anti-FLAG, and anti-GAPDH as a loading control.

Protein band quantification of CHX immunoblots was performed using Bio-Rad Quantity One 4.6.9 software. Briefly, band intensities were quantified after subtracting background signals from band signals using the rolling-disc method. Relative protein levels were estimated after normalizing band intensities relative to GAPDH loading controls and zero-time controls. Glycoproteins tend to run in SDS-PAGE as diffuse bands so, for more accurate comparison of unglycosylated bands with the more similar deglycosylated protein bands, whole-cell lysates were treated with PNGase F prior to immunoblotting to remove glycan moieties, yielding uniformly sharp protein bands.

Statistical analysis of the CHX chase data was done using GraphPad Prism 5 software. Non-linear curve fitting was done assuming a simple exponential one-phase decay model. Prior subtraction of background was accomplished by modifying the default model equation in GraphPad Prism as follows: Exponential/one-phase decay model, \( Y = (Y_0) \times \exp(-K \times X) \). Automated curve fitting and non-linear regression analysis provided half-life times (h). For time zero, mean and SD were obtained from data normalized to GAPDH only, then applied proportionately to the zero points normalized for GAPDH and zero time (i.e. 1.0). Data in figures were plotted point-to-point rather than as fitted exponential curves to preserve clarity of the original data. Mean and SD values were derived from three independent experiments, and P-values, representing significance of differences for comparisons, were derived from unpaired, 2-tailed Student's t-tests. Data analyses, including raw data, correlation coefficients (R^2) of fit of the one-phase exponential decay model, and half-life values and their standard deviations, are tabulated in Supplementary Material, Table S1. The derived P-values for comparisons are tabulated in Supplementary Material, Table S2.

**Co-immunoprecipitation**—HEK 293 cells were transfected with WT and mutants cDNA constructs and whole-cell extracts were prepared in IP buffer (150 mM NaCl, 25 mM Tris HCl, pH 7.2 at 25 °C, containing 1% C12E8, 1:100 (v/v) Protease Inhibitor Cocktail and 1 mM PMSF), as previously described (25). Co-immunoprecipitation of WT and mutants was conducted by treating 50 μg whole-cell lysate with 5 μg of anti-FLAG antibody and incubating overnight at 4 °C with agitation. Antigen-anti-FLAG antibody immunocomplexes were pulled down by incubation with 100 μl (50% packed volume) of protein A agarose beads for 2 h at room temperature with agitation. The antigen-coated beads were then incubated 5 min with SDS-PAGE sample buffer at 95 °C to elute the antigens. Antigen-containing supernatants were collected after centrifugation at 2,500 g for 3 min and then were immunoblotted with anti-FLAG, anti-B1 and anti-VMA21 antibodies.

**Immunofluorescence and colocalization analysis**—HEK 293 cells were grown on glass coverslips and transiently transfected with WT and mutant cDNA constructs. The cells were washed with DPBS and fixed with 3.7% (w/v) paraformaldehyde for 15 min at room temperature. Subsequently, cells were permeabilized with DPBS containing 0.2% Triton X-100 at room temperature for 15 min. Cells were then blocked with DPBS containing 5% bovine serum albumin for 1 h at room temperature, followed by immunostaining with anti-FLAG (1:1,000), anti-calnexin (1:500), or anti-syntaxin 6 (1:500) antibodies in DPBS containing 5% bovine serum albumin for 45 min at room temperature. Cells then were washed 3 times with DPBS and immunostained with fluorescent second antibodies (1:500) for 45 min at room temperature. Nuclei were stained with 0.1 mg/ml DAPI in DPBS for 10 min and cells were mounted with ProLong Gold Antifade Reagent (Fisher Scientific). Photomicrography images were acquired using a Quorum Spinning Disk Confocal System equipped with a Hamamatsu C9100-13 EM-CCD, Yokogawa CSU X1 scan head, and Improvision Piezo focus drive (Imaging Facility, Hospital for Sick Children, Toronto, Canada).

Colocalization quantification of 20 images (10-15 cells/image) each from three independent experiments was conducted using Volocity v6.3 3D image analysis software (PerkinElmer, Woodbridge, Canada). Colocalizations of two fluorescent signals (red and green) were quantified and expressed as Pearson’s correlation coefficients (r). Significance of differences between WT and mutants were estimated using two-tailed Student’s t tests.

**Cell surface biotinylation**—Cell surface labeling was performed using EZ-Link N-HSSS-Biotin reagent (Pierce 21328; Fisher Scientific), as described previously (25). Briefly, HEK 293 cells...
were transiently transfected and, 24 h post-transfection, the cells were incubated with 1 mg/ml freshly prepared EZ-Link NHS-SS-Biotin for 1 h at 4 °C with gentle agitation. The cells were then incubated with ice-cold quenching buffer (192 mM glycine, 25 mM Tris, pH 8.3 at 25 °C) to remove excess biotin. Cells were harvested in 0.4 ml ice-cold RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM EDTA and 10 mM Tris-HCl, pH 7.5 at 25 °C) containing Protease Inhibitor Cocktail (1:100 v/v) and 1 mM PMSF, and were incubated for 30 min on ice, then centrifuged at 15,000g for 30 min at 4 °C. Supernatants were collected, and biotinylated cell-surface proteins were affinity purified by incubating supernatants with 100 µl (50% packed volume) of streptavidin agarose beads (Pierce 20347; Fisher Scientific) for 2 h at 4 °C. The eluted, biotinylated cell-surface proteins and total lysate proteins were analyzed by 7% SDS-PAGE and immunoblotted, as previously described (25).

Structural modeling of human a4 subunit—Homology modeling of the integral membrane domain of the human a4 subunit was generated by SWISS-MODEL, using the yeast a subunit ortholog, Vph1p, as a template (PDB:5H1M) (23,43). Subsequently, the model was corrected and the 3D representation was generated using the 3D graphical YASARA interface (44). A 3D representation of a4GS20R was generated after substituting Gly 820 with Arg, using the YASARA FoldX plug-in.

Acknowledgements—S.E. was supported in part by a scholarship from the Toronto Musculoskeletal Centre, and J.W.K. was supported by a scholarship from the Canadian Institutes of Health Research/Institute of Musculoskeletal Health and Arthritis.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions—S.E., N.K., R.A.F.R. and M.F.M. conceptualized, planned and analyzed experimental work. S.E. performed all experiments except those represented in Fig. 1C, which were performed by J.W.K.; S.E. and Y.Y. designed and prepared constructs used in the study, and Y.Y. provided additional technical expertise. S.E. and N.K. wrote the manuscript and prepared figures.

Footnotes
* This work was supported by the Canadian Institutes of Health Research (MOP-12333 and MOP-11119 to M.F.M.).
† To whom correspondence should be addressed: Faculty of Dentistry, University of Toronto, 124 Edward Street, Toronto, Ontario M5G 1G6, Canada. Tel.: 416-864-8234; Fax: 416-979-4936; Email: m.manolson@utoronto.ca
‡ The abbreviations used are: CFTR, cystic fibrosis transmembrane-conductance regulator; CHX, cycloheximide; CTa, C-terminal (integral membrane) half of V-ATPase a subunit; CTD, C-terminal (cytoplasmic) tail domain (of V-ATPase a subunit); dRTA, distal renal tubular acidos; Endo H, endo-β-N-acetylglucosaminidase H; ER, endoplasmic reticulum; NTA, N-terminal (cytoplasmic) half of V-ATPase a subunit; PFK-1, phosphofructokinase-1; PNGase F, peptide:N-glycosidase F; TM, transmembrane α-helix; V-ATPase, vacuolar-type H+-ATPase.

References
1. Kartner, N., and Manolson, M. F. (2016) The vacuolar proton ATPase (V-ATPase): regulation and therapeutic targeting, in Regulation of Ca2+-ATPases, V-ATPases and F-ATPases (Chakraborti, S., and Dhalla, N. S. eds.), Springer International Publishing, Switzerland. pp 407–437
2. Toei, M., Saum, R., and Forgac, M. (2010) Regulation and isoform function of the V-ATPases. Biochemistry 49, 4715–4723
3. Futai, M., Nakanishi-Matsui, M., Okamoto, H., Sekiya, M., and Nakamoto, R. K. (2012) Rotational catalysis in proton pumping ATPases: from E. coli F-ATPase to mammalian V-ATPase. Biochim. Biophys. Acta 1817, 1711–1721
4. Casey, J. R., Grinstein, S., and Orlowski, J. (2009) Sensors and regulators of intracellular pH. Nat. Rev. Mol. Cell Biol. 11, 50–61
5. Beyenbach, K. W., and Wieczorek, H. (2006) The V-type H\textsuperscript{+}-ATPase: molecular structure and function, physiological roles and regulation. J. Exp. Biol. 209, 577–589
6. Breton, S., and Brown, D. (2013) Regulation of luminal acidification by the V-ATPase. Physiology 28, 318–329
7. Forgac, M. (2007) Vacuolar ATPases: Rotary proton pumps in physiology and pathophysiology. Nat. Rev. Mol. Cell Biol. 8, 917–929
8. Sun-Wada, G.-H., and Wada, Y. (2013) Vacuolar-type proton pump ATPases: acidification and pathological relationships. Histol. Histopathol. 28, 805–815
9. Hinton, A., Bond, S., and Forgac, M. (2009) V-ATPase functions in normal and disease processes. Pfluegers Arch./Eur. J. Physiol. 457, 589–598
10. Ochotny, N., Van Vliet, A., Chan, N., Yao, Y., Morel, M., Kartner, N., von Schroeder, H. P., Heersche, J. N. M., and Manolson, M. F. (2006) Effects of human a3 and a4 mutations that result in osteopetrosis and distal renal tubular acidosis on yeast V-ATPase expression and activity. J. Biol. Chem. 281, 26102–26111
11. Kornak, U., Schultz, A., Friedrich, W., Uhlhaas, S., Kremens, B., Voit, T., Hasan, C., Bode, U., Jentsch, T. J., and Kubisch, C. (2000) Mutations in the a3 subunit of the vacuolar H\textsuperscript{+}-ATPase cause infantile malignant osteopetrosis. Hum. Mol. Genet. 9, 2059–2063
12. Kartner, N., Yao, Y., Bhargava, A., and Manolson, M. F. (2013) Topology, glycosylation and conformational changes in the membrane domain of the vacuolar H\textsuperscript{+}-ATPase a subunit. J. Cell. Biochem. 114, 1474–1487
13. Wagner, C. A., Finberg, K. E., Breton, S., Marshansky, V., Brown, D., and Geibel, J. P. (2004) Renal vacuolar H\textsuperscript{+}-ATPase. Physiol. Rev. 84, 1263–1314
14. Toyomura, T., Murata, Y., Yamamoto, A., Oka, T., Sun-Wada, G.-H., Wada, Y., and Futai, M. (2003) From lysosomes to the plasma membrane: Localization of vacuolar-type H\textsuperscript{+}-ATPase with the a3 isofrom during osteoclast differentiation. J. Biol. Chem. 278, 22023–22030
15. Bexiga, M. G., and Simpson, J. C. (2013) Human diseases associated with form and function of the Golgi complex. Int. J. Mol. Sci. 14, 18670–18681
16. Guillard, M., Dimopoulou, A., Fischer, B., Morova, E., Lefeber, D. J., Kornak, U., and Wevers, R. A. (2009) Vacuolar H\textsuperscript{+}-ATPase meets glycosylation in patients with cutis laxa. Biochim. Biophys. Acta 1792, 903–914
17. Fischer, B., Dimopoulou, A., Egerer, J., Gardeitchik, T., Kidd, A., Jost, D., Kayserili, H., Alanay, Y., Tantcheva-Poor, I., Mangold, E., Daumer-Haas, C., Phadke, S., Peirano, R. I., Heusel, J., Desphande, C., Gupta, N., Nanda, A., Felix, E., Berry-Kravis, E., Kabra, M., Wevers, R., Van Maldergem, L., Mundlos, S., Morava, E., and Kornak, U. (2012) Further characterization of ATP6V0A2-related autosomal recessive cutis laxa. Hum. Genet. 131, 1761–1773
18. Kornak, U., Reynders, E., Dimopoulou, A., van Reeuwijk, J., Fischer, B., Rjab, A., Budde, B., Nürnberg, P., Foulquier, F., Dobyns, W. B., Quelhas, D., Vilarinho, L., Leao-Telas, E., Greally, M., Seemanova, E., Simandlova, M., Sali, M., Nanda, A., Basel-Vanagaite, L., Kayserili, H., Yuksel-Apak, M., Larregue, M., Vigneron, J., Giurgea, S., Lefeber, D., Urban, Z., Gruenewald, S., Annaert, W., Brunner, H. G., van Bokhoven, H., Wevers, R., Morava, E., Matthijis, G., an Maldergem, L. V., and Mundlos, S. (2008) Impaired glycosylation and cutis laxa caused by mutations in the vesicular H\textsuperscript{+}-ATPase subunit ATP6V0A2. Nat. Genet. 40, 32–34
19. Sobacchi, C., Schulz, A., Coxon, F. P., Villa, A., and Helfrich, M. H. (2013) Osteopetrosis: genetics, treatment and new insights into osteoclast function. Nat. Rev. Endocrinol. 9, 522–536

20. Bhargava, A., Voronov, I., Wang, Y., Glogauer, M., Kartner, N., and Manolson, M. F. (2012) Osteopetrosis mutation R444L causes ER retention and misprocessing of vacuolar H\(^+\)-ATPase a3 subunit. J. Biol. Chem. 287, 26829–26839

21. Stover, E. H., Borthwick, K. J., Bavalia, C., Eady, N., Fritz, D. M., Rungroj, N., Giersch, A. B. S., Morton, C. C., Axon, P. R., Akil, I., Al-Sabban, E. A., Baguley, D. M., Bianca, S., Bakkaloglu, A., Bircan, Z., Chauve, D., Clermont, M.-J., Guala, A., Hulten, S. A., Kroes, H., Li Volti, G., Mir, S., Mocan, H., Nayir, A., Ozen, S., Rodriguez Soriano, J., Sanjad, S. A., Tasic, V., Taylor, C. M., Topaloglu, R., Smith, A. N., and Karet, F. E. (2002) Novel ATP6V1B1 and ATP6V0A4 mutations in autosomal recessive distal renal tubular acidosis with new evidence for hearing loss. J. Med. Genet. 39, 796–803

22. Batlle, D., and Haque, S. K. (2012) Genetic causes and mechanisms of distal renal tubular acidosis. Nephrol. Dial. Transplant. 27, 3691–3704

23. Schep, D. G., Zhao, J., and Rubinstein, J. L. (2016) Models for the a subunits of the Thermus thermophilus V/A-ATPase and Saccharomyces cerevisiae V-ATPase enzymes by cryo-EM and evolutionary covariance. Proc. Natl. Acad. Sci. U. S. A. 113, 3245–3250

24. Mazhab-Jafari, M. T., Rohou, A., Schmidt, C., Bueler, S. A., Benlekbir, S., Robinson, C. V., and Rubinstein, J. L. (2016) Atomic model for the membrane-embedded V\(\text{o}\) motor of a eukaryotic V-ATPase. Nature 359, 118–122

25. Esmail, S., Yao, Y., Kartner, N., Li, J., Reithmeier, R. A. F., and Manolson, M. F. (2016) N-linked glycosylation is required for vacuolar H\(^+\)-ATPase (V-ATPase) a4 subunit stability, assembly, and cell surface expression. J. Cell. Biochem. 117, 2757

26. Esmail, S., Kartner, N., Yao, Y., Kim, J. W., Reithmeier, R. A. F., and Manolson, M. F. (2018) N-linked glycosylation of a subunit isoforms is critical for vertebrate vacuolar H\(^+\)-ATPase (V-ATPase) biosynthesis. J. Cell. Biochem. 119, 861-875

27. Maley, F., Trimble, R. B., Tarentino, A. L., and Plummer Jr., T. H. (1989) Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. Anal. Biochem. 180, 195–204

28. Freeze, H. H., and Kranz, C. (2010) Endoglycosidase and glycoamidase release of N-linked glycans. Curr. Protoc. Mol. Biol.

29. Ramachandran, N., Munteanu, I., Wang, P., Ruggieri, A., Rilstone, J. J., Israeli, N., Naranian, T., Paroutis, P., Guo, R., Ren, Z.-P., Nishino, I., Charbrol, B., Pellissier, J.-F., Minetti, C., Udd, B., Fardeau, M., Tailor, C. S., Mahuran, D. J., Kissel, J. T., Kalimo, H., Levy, N., Manolson, M. F., Ackerley, C. A., and Mianssian, B. A. (2013) VMA21 deficiency prevents vacuolar ATPase assembly and causes autophagic vacuolar myopathy. Acta Neuropathol. (Berl). 125, 439–457

30. Ryan, M., Graham, L. A., and Stevens, T. H. (2008) Voa1p functions in V-ATPase assembly in the yeast endoplasmic reticulum. Mol. Biol. Cell 19, 5131–5142

31. Hill, K., and Cooper, A. A. (2000) Degradation of unassembled Vph1p reveals novel aspects of the yeast ER quality control system. EMBO J. 19, 550–561

32. Toei, M., Toei, S., and Forgac, M. (2011) Definition of membrane topology and identification of residues important for transport in subunit a of the vacuolar ATPase. J. Biol. Chem. 286, 35176–35186

33. Lemu, L., and Goder, V. (2014) Regulation of endoplasmic reticulum-associated protein degradation (ERAD) by ubiquitin. Cells 3, 824–847

34. Hurtado-Lorenzo, A., Skinner, M., Annan, J. E., Futai, M., Sun-Wada, G.-H., Bourgoin, S., Casanova, J., Wildeman, A., Bechoua, S., and Ausiello, D. A. (2006) V-ATPase interacts with
ARNO and Arf6 in early endosomes and regulates the protein degradative pathway. Nat. Cell Biol. 8
35. Seol, J. H., Shevchenko, A., Shevchenko, A., and Deshaies, R. J. (2001) Skp1 forms multiple protein complexes, including RAVE, a regulator of V-ATPase assembly. Nat. Cell Biol. 3
36. Kawasaki-Nishi, S., Nishi, T., and Forgac, M. (2001) Yeast V-ATPase Complexes Containing Different Isoforms of the 100-kDa a-subunit Differ in Coupling Efficiency and in Vivo Dissociation. J. Biol. Chem. 276, 17941–17948
37. Malkus, P., Graham, L. A., Stevens, T. H., and Schekman, R. (2004) Role of Vma21p in assembly and transport of the yeast vacuolar ATPase. Mol. Biol. Cell 15, 5075–5091
38. Su, Y., Blake-Palmer, K. G., Sorrell, S., Javid, B., Bowers, K., Zhou, A., Chang, S. H., Qamar, S., and Karet, F. E. (2008) Human H^{+}ATPase a4 subunit mutations causing renal tubular acidosis reveal a role for interaction with phosphofructokinase-1. Am. J. Physiol. Renal Physiol. 295, F950–F958
39. Guevara-Coto, J., Schwartz, C. E., and Wang, L. (2014) Protein sector analysis for the clustering of disease-associated mutations. BMC Genomics 15, 1–7
40. Valastyan, J. S., and Lindquist, S. (2014) Mechanisms of protein-folding diseases at a glance. Dis. Model. Mech. 7, 9–14
41. Okiyoneda, T., Veit, G., Dekkers, J. F., Bagdany, M., Soya, N., Xu, J., Verkman, A. S., Kurth, M., Simon, A., Hegedus, T., Beekman, J. M., and Lukacs, G. L. (2013) Mechanism-based corrector combination restores ΔF508-CFTR folding and function. Nat. Chem. Biol. 9, 1–27
42. Cheng, S. H., Gregory, R. J., Marshall, J., Paul, S., Souza, D., White, G. A., O’Riordan, C. R., and Smith, A. E. (1990) Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell 63, 827–834
43. Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Cassarino, T. G., Bertoni, M., Bordoli, L., and Schwede, T. (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res. 42, W252–W258
44. Van Durme, J., Delgado, J., Stricher, F., Serrano, L., Schymkowitz, J., and Rousseau, F. (2011) A graphical interface for the FoldX forcefield. Bioinformatics 27, 1711–1712
Figure legends

FIGURE 1. Glycosylation and stability of a\textsuperscript{2P405L}, a\textsuperscript{4R449H} and a\textsuperscript{4G820R}. Sequence alignments show a high degree of conservation of residues affected by cutis laxa and dRTA mutations in V-ATPase a subunit proteins. The mutant proteins are all glycosylated, but stability is variably affected. A, amino acid (a.a.) sequence alignments from end of NTa domain to end of TM3 of human a1–4 (H1–4), mouse a1–4 (M1–4) and yeast Vph1p (YV). Domains are indicated below alignments, in bold: CL, cytoplasmic loop; EL, extracellular (luminal) loop; NTa, N-terminal cytoplasmic domain; TM, transmembrane helix. Cyan highlights extrapolated from studies done in Vph1p indicate TM predictions (12). Red highlights indicate a.a. affected by human disease-causing mutations (noted above alignments). Yellow highlights indicate a.a. corresponding to the human mutations, within the subunit isoforms and species shown. B, alignments as in A, but of sequences from the end of TM8 to the C-terminus, encompassing the cytoplasmic (C-terminal) tail domain (CTD). C, HEK293 cells were transfected with either WT a2-2FLAG (WT a2), or mutant a\textsuperscript{2P405L}-2FLAG (a\textsuperscript{2P405L}), and lysates treated with (+) or without (-) Endo H. D, Same constructs as in C, but treated with 10 \mu M CHX for time indicated; plots show band intensities quantified from immunoblots of post-CHX chase. Data were normalized to GAPDH and zero time control. E, same as C, except HEK 293 cells were transfected with either WT a4-2FLAG (WT a4), mutant a\textsuperscript{4R449H}-2FLAG (a\textsuperscript{4R449H}), or mutant a\textsuperscript{4G820R}-2FLAG (a\textsuperscript{4G820R}), and lysates treated with or without PNGase F or Endo H. F, same as D, except HEK 293 cells were expressing WT a4, mutant a\textsuperscript{4R449H}, or mutant a\textsuperscript{4G820H}. Data are representative of three independent biological experiments; error bars indicate ± SD.

FIGURE 2. a\textsuperscript{2P405L} is degraded in the proteasomal pathway with some lysosomal contribution, while a\textsuperscript{4R449H} is degraded only in the proteasomal pathway. A, plot of quantified bands from anti-FLAG antibody-probed immunoblots of whole-cell lysates from WT a2-2FLAG and a\textsuperscript{2P405L}-2FLAG-transfected HEK 293 cells. Cells were treated with CHX (10 \mu g/ml) for the times indicated, with and without proteasome inhibitor (designated ‘MG’) as indicated. B, same as panel A, but cells were transfected with WT a4-2FLAG and a\textsuperscript{4R449H}-2FLAG constructs. C, same as panel A, but cells were treated with lysosomal inhibitor (designated ‘Am’) rather than proteasomal inhibitor. D, same as panel B, but cells were treated with lysosomal inhibitor. Data were normalized to GAPDH and zero time control and are representative of three independent biological experiments; error bars indicate ± SD.

FIGURE 3. Localization of mutant a subunit proteins in the secretory pathway. A, representative confocal fluorescence images of empty vector-transfected HEK 293 cells (left panel), cells transiently transfected with WT a2-2FLAG (middle panel), or with a\textsuperscript{2P405L}-2FLAG (right panel). All panels show cells stained with anti-calnexin (red) and anti-FLAG (green). Nuclei are counter-stained with DAPI (blue). B, same as A, except cells were stained with anti-syntaxin 6 (red) and anti-FLAG (green). C, fluorescence images of empty vector-transfected HEK 293 cells (left-most panel), cells transiently transfected with WT a4-2FLAG (2\textsuperscript{nd} from left), with a\textsuperscript{4R449H}-2FLAG (2\textsuperscript{nd} from right), or with a\textsuperscript{4G820R}-2FLAG (right-most panel). All panels show cells stained with anti-calnexin (red) and anti-FLAG (green). D, same as C, except cells were stained with anti-syntaxin 6 (red) and anti-FLAG (green). E, quantitative colocalization analysis of data in panels A and B. Ordinate is Pearson’s correlation coefficient (r). Results show that a\textsuperscript{2P405L} colocalized with calnexin at a rate similar to that of WT a2, but there was significantly less colocalization with syntaxin 6 compared to WT a2. F, quantitative colocalization analysis of data in panels C and D. Results show that a\textsuperscript{4R449H} is mostly retained in the ER. Images are representative of 20 images (10-15 cells/image) each from three independent biological experiments.

FIGURE 4. Defective cell-surface expression of a\textsuperscript{4R449H}. Fluorescence photomicrograph images show DAPI nuclear staining (blue) of HEK 293 cells transfected as indicated (left-most panels), fluorescent double antibody staining, first with anti-HA on non-permeabilized cells (2\textsuperscript{nd} from left panels), followed by anti-FLAG after cell permeabilization (2\textsuperscript{nd} from right panels), and merged images (right-most panels). Staining of non-permeabilized cells with anti-HA antibody indicated cell-surface accessibility of the epitope tag. A, empty vector-transfected (control) cells stained with anti-FLAG (green) and anti-HA (red). B, same as A, but cells were transfected with WT a4-3HA-2FLAG. C, same as A, but cells were transfected with WT a\textsuperscript{4R449H}-3HA-2FLAG. D, same as A, but cells were transfected with WT a\textsuperscript{4G820R}-3HA-2FLAG. The scale bar in the bottom
right panel (bottom right) is 5 µm; all panels are of the same magnification. Each panel is representative of 20 micrographs obtained from 3 independent experiments. E, surface proteins of intact transfected cells were subjected to biotinylation followed by streptavidin affinity purification (a.p.) and immunoblotting; blots were probed with anti-FLAG antibody (three independent experiments); lane 1 (from left), whole-cell lysate from WT a4-transfected HEK 293 cells (lysate); lane 2, surface protein biotinylation showing surface protein fraction from cells transfected with WT a4 (a.p.); lanes 3 and 4, same as lanes 1 and 2, except cells were transfected with a4Gb20R; lanes 5 and 6, same as lanes 1 and 2, except cells were transfected with a4R449H. Blot is representative of three independent biological experiments.

FIGURE 5. Association of a subunit with V-ATPase assembly chaperone, VMA21, and V1 marker, ATP6V1B1. HEK 293 cells were transfected with WT and mutant FLAG-tagged constructs. After 24 h expression, whole-cell lysates were immunoprecipitated with anti-FLAG antibody. A–C, immunoprecipitates were blotted and probed with anti-FLAG (A), anti-B1 (B) or anti-VMA21 (C) antibodies. D, quantification of WT and mutant a2 associations with VMA21 and B1 in blots shown in panels A–C. No significant differences were observed between WT and mutant a2 associations with either VMA21 or B1. E, quantification of WT and mutant a4 associations with VMA21 and B1 in blots shown in panels A–C. Results showed significantly higher association of a4R449H with VMA21 (p < 0.05), and reduced association with B1 (p < 0.05), compared with WT a4. No significant difference was seen for a4Gb20R (p = 0.064 for association with VMA21, p = 0.090 for association with B1). Images are representative of 20 images (10-15 cells/image) each from three independent biological experiments; error bars are ± SD.

FIGURE 6. a4 G820 resides within the putative proton translocation pathway. A, homology model for C-terminal integral membrane (CtA) domain of the human a4 subunit. This model was constructed based on the recent high-resolution cryo-EM structure and evolutionary covariance analysis for the yeast a subunit, Vph1p (23). The indicated residues are highly conserved and essential for proton translocation. The red dashed line shows the hypothetical proton channel from the cytoplasmic side of the membrane to the luminal space. Cyan dashed box indicates the sub-region where amino acid residue G820 is located. B, shows the close proximity of G820 and the highly conserved E729, a residue thought to be key in proton translocation (10,32,38). C, illustrates how the G820R mutation may result in a salt-bridge interaction (red asterisk) between E729 and R820, possibly distorting or blocking the proton channel, resulting in inhibition of proton translocation, and providing a causative explanation for the role of the a4Gb20R mutation in dRTA.

FIGURE 7. Model for human a4 trafficking in the secretory pathway and to the plasma membrane. Steps 1–7 and 6′–8′ suggest two putative mechanisms of mammalian V-ATPase assembly. Firstly, within the ER, the VMA21 assembly factor facilitates the assembly of subunit a into the V0 subcomplex (steps 1-3). The assembled V0 subcomplex is subsequently trafficked to Golgi (step 4), assembled with the V1 subcomplex within the Golgi (step 5 and 6), with the fully assembled V-ATPase complex targeted to the plasma membrane (step 7). Alternatively, the V0 subcomplex itself could traffic to the plasma membrane (7′) and only assemble with the V1 subcomplex at the plasma membrane (steps 6′–8′). A, an unglycosylated mutant, a4R449D, which was described in a previous study (25), is unable to assemble into a V0 subcomplex. It is retained in the ER and is targeted to the ERAD pathway for proteolysis. In contrast, the glycosylated a4R449H mutant described here assembles within the V0 complex; however, it is ultimately degraded in the proteasome, and thus also fails to reach the plasma membrane. B, the glycosylated a4Gb20R assembles within the V1:V0 complex and is trafficked to the plasma membrane but, unlike the wild type complex, functional proton translocation appears to be inhibited by the mutation. Red asterisks symbolize the a4 G820R mutation; a dark green circle symbolizes the a4 R449H mutation; red bars indicate blockade of a pathway; V1 subunits are indicated by upper case letters; V0 subunits are indicated by lower case italic letters.
Figure 1

A

| Protein | WT a2 | a2-P405L |
|---------|-------|----------|
| Endo H  | -     | +        |
| M_r (kDa) | 120  |          |

B

| Protein | WT a4 | a4-R449H | a4-G820R |
|---------|-------|----------|----------|
| PNGase F | -     | -        | -        |
| Endo H  | -     | -        | -        |
| M_r (kDa) | 120  |          |          |

C

| Protein | WT a2 | a2-P405L |
|---------|-------|----------|
| Endo H  | -     | +        |
| M_r (kDa) | 120  |          |

D

| Protein | WT a2 | a2-P405L |
|---------|-------|----------|
| Endo H  | -     | +        |
| M_r (kDa) | 120  |          |

E

| Protein | WT a4 | a4-R449H | a4-G820R |
|---------|-------|----------|----------|
| PNGase F | -     | -        | -        |
| Endo H  | -     | -        | -        |
| M_r (kDa) | 120  |          |          |

F

| Protein | WT a4 | a4-R449H | a4-G820R |
|---------|-------|----------|----------|
| PNGase F | -     | -        | -        |
| Endo H  | -     | -        | -        |
| M_r (kDa) | 120  |          |          |
Figure 2

A

B

C

D

Esmail et al.

Functional domains of ATP6V0A2 and ATP6V0A4
Figure 3

A. Calnexin control, a2/calnexin, a2\textsuperscript{G405L}/calnexin

B. Syntaxin 6 control, a2/syntaxin 6, a2\textsuperscript{G405L}/syntaxin 6

C. Calnexin control, a4/calnexin, a4\textsuperscript{G405L}/calnexin

D. Syntaxin 6 control, a4/syntaxin 6, a4\textsuperscript{G405L}/syntaxin 6

E. Correlation (r) of WT a2 and a2\textsuperscript{G405L}

F. Correlation (r) of WT a4, a4\textsuperscript{G405L}, a4\textsuperscript{G405L/R}

\textsuperscript{WT} = wild type

\textsuperscript{G405L} = G405L mutation

\textsuperscript{G405L/R} = G405L/R mutation
Figure 4

A

Control | Permeabilized | Non-permeabilized | MERGE

Empty vector

B

a4-3HA-2FLAG

C

a4^{GR420E}_{-3HA-2FLAG}

D

a4^{GR420E}_{3HA-2FLAG}

DAPI | Anti-FLAG | Anti-HA | Anti-HA/Anti-FLAG

E

| Protein | WT a4 | a4^{GR420E} | a4^{GR444H} |
|--------|-------|-------------|-------------|
| Biotinylation | - a.p. | - a.p. | - a.p. |
| lysate | 1 2 | 3 4 | 5 6 |

M: (kDa)

Anti-FLAG
Figure 5

A Protein

\( \text{a}^2_{\text{a}^4\text{G50L}} \)  \( a^2 \)  \( a^4_{\text{G820R}} \)  \( a^4_{\text{E449H}} \)  \( a^4 \)

\( M_r \) (kDa)

95 120

FLAG IP / anti-FLAG

B

55 43

FLAG IP / anti-B1

C

15 8

FLAG IP / anti-VMA21

D

Relative protein ratio

WT a2  \( a^2_{\text{a}^4\text{G50L}} \)

E

Relative protein ratio

WT a4  \( a^4_{\text{G820R}} \)

\( \text{VMA21:a}^2 \)  \( \text{B1:a}^2 \)  \( \text{VMA21:a}^4 \)  \( \text{B1:a}^4 \)
Figure 6
Molecular mechanisms of cutis laxa and distal renal tubular acidosis-causing mutations in V-ATPase α subunits, ATP6V0A2 and ATP6V0A4
Sally Esmail, Norbert Kartner, Yeqi Yao, Joo Wan Kim, Reinhart A.F. Reithmeier and Morris F. Manolson

*J. Biol. Chem.* published online January 8, 2018

Access the most updated version of this article at doi: [10.1074/jbc.M117.818872](http://dx.doi.org/10.1074/jbc.M117.818872)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/) to choose from all of JBC's e-mail alerts