

Dominantly acting variants in *ATP6V1C1* and *ATP6V1B2* cause a multisystem phenotypic spectrum by altering lysosomal and/or autophagosome function

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Summary

The vacuolar H⁺-ATPase (V-ATPase) is a functionally conserved multimeric complex localized at the membranes of many organelles where its proton-pumping action is required for proper lumen acidification. The V-ATPase complex is composed of several subunits, some of which have been linked to human disease. We and others previously reported pathogenic dominantly acting variants in *ATP6V1B2*, the gene encoding the V1B2 subunit, as underlying a clinically variable phenotypic spectrum including dominant deafness-onychodystrophy (DDOD) syndrome, Zimmermann-Laband syndrome (ZLS), and deafness, onychodystrophy, osteodystrophy, intellectual disability, and seizures (DOORS) syndrome. Here, we report on an individual with features fitting DOORS syndrome caused by dysregulated *ATP6V1C1* function, expand the clinical features associated with *ATP6V1B2* pathogenic variants, and provide evidence that these *ATP6V1C1/ATP6V1B2* amino acid substitutions result in a gain-of-function mechanism upregulating V-ATPase function that drives increased lysosomal acidification. We demonstrate a disruptive effect of these *ATP6V1B2/ATP6V1C1* variants on lysosomal morphology, localization, and function, resulting in a defective autophagic flux and accumulation of lysosomal substrates. We also show that the upregulated V-ATPase function affects cilium biogenesis, further documenting pleiotropy. This work identifies *ATP6V1C1* as a new gene associated with a neurodevelopmental phenotype resembling DOORS syndrome, documents the occurrence of a phenotypic continuum between ZLS, and DDOD and DOORS syndromes, and classify these conditions as lysosomal disorders.

Introduction

The vacuolar H⁺-ATPase (V-ATPase) is an electrogenic proton pump located in the membranes of intracellular organelles, including secretory granules, endosomes, lysosomes, and Golgi cisternae. By its proton-pumping action, V-ATPase is required for proper lumen acidification¹ and is involved in several physiological processes, including

membrane trafficking, protein degradation, and pH homeostasis.^{2,3} This multimeric complex is highly conserved through evolution, and its presence in yeast indicates that the enzyme is required for basic cellular physiology. Consistently, functional dysregulation of the pump underlies a number of clinically heterogeneous human diseases.⁴ Loss-of-function (LoF) variants in genes encoding V-ATPase components (*ATP6V1B1* [MIM: 192132] and

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<https://doi.org/10.1016/j.xhgg.2024.100349>

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ATP6VOA4 [MIM: 605239]) underlie autosomal recessive distal renal tubular acidosis with or without sensorineural hearing loss (MIM: PS179800),⁵ biallelic variants in *ATP6V1A* (MIM: 607027) cause a recessive form of cutis laxa (MIM: 617403),³ and LoF variants in *ATP6VOA3* (MIM: 604592) cause osteopetrosis, a recessive disorder characterized by increased bone density due to impaired bone resorption (MIM: PS259700).⁶ Dysregulated function of these subunits have also been implicated in neurological diseases, including dominant developmental encephalopathy with epilepsy (MIM: 618012), which is caused by variants in *ATP6V1A* (MIM: 607027),⁷ a dominant early-onset form of epilepsy, with or without developmental delay (DD) (MIM: 620465),⁸ associated with variants in *ATP6VOC* (MIM: 108745), and X-linked parkinsonism with spasticity (MIM: 300911), congenital disorder of glycosylation (MIM: 301045), and intellectual developmental disorder, Hedera type (MIM: 300423), which are due to hemizygous variants in *ATP6AP2* (MIM: 300556).^{9–11}

V-ATPase is composed of a catalytic cytosolic V_1 region comprising eight subunits (stoichiometry A_3 , B_3 , C, D, E_3 , F, G_3 , and H), catalyzing ATP hydrolysis, and a membrane-embedded V_0 region made up of eight subunits (stoichiometry a, c_9 , c'' , d, e, f, ATP6AP1, and ATP6AP2) that is responsible for the proton transport from the cytoplasm to the lumen or extracellular space.^{4,12} The ATP6V1B2 and ATP6V1C1 subunits belong to the V_1 complex and are ubiquitously expressed. We and others reported that dominantly acting variants in *ATP6V1B2* are associated with a wide clinical spectrum, including dominant deafness-onychodystrophy (DDOD) syndrome (MIM: 124480), deafness, onychodystrophy, osteodystrophy, intellectual disability, and seizures (DOORS) syndrome (MIM: 220500), and Zimmermann-Laband syndrome (ZLS [MIM: PS135500]).^{13–18} Individuals with DDOD syndrome usually show normal development and cognitive function, while those with DOORS syndrome and ZLS present with intellectual disability (ID), with or without seizures. These disorders share hypoplasia/aplasia of nails and terminal phalanges; a recognizable craniofacial appearance, gingival overgrowth, and hypertrichosis also characterize ZLS. Of note, a recurrent truncating variant in *ATP6V1B2* (c.1516C>T, p.Arg506*) has been reported in both DDOD and DOORS syndromes,^{16–18} suggesting that these conditions are within a spectrum of a single disorder caused by altered V-ATPase function. No clinical phenotype has been reported to be caused by altered function of ATP6V1C1, to date.

Here, we causally link a *de novo* missense variant in *ATP6V1C1* to a neurodevelopmental phenotype with features resembling DOORS syndrome, and more accurately define the clinical spectrum of dominantly acting *ATP6V1B2* variants, which is consistent with a phenotypic continuum having DDOD syndrome and ZLS as extreme entities. We also provide evidence that all tested variants result in a functional increase in the V-ATPase pump activity, which has pleiotropic effects in cells, affecting lyso-

somal morphology and function, autophagy, and cilium biogenesis, defining a neurodevelopmental spectrum primed by generalized defective lysosome function.

Subjects and methods

Subjects

Affected subjects were identified through networking, including GeneMatcher and other professional links (e.g., European Reference Networks). Clinical data and DNA samples were collected, stored, and used under institutional review board-approved protocols. All procedures were conducted in accordance with the ethical standards for use of human participants in medical experimentation, after written informed consent was obtained from the parents or legal guardians of all subjects. The study was approved by the Ospedale Pediatrico Bambino Gesù Institutional Review Board (ref. 1702_OPBG_2018). DNA from leukocytes, hair bulb cells, and skin fibroblasts were extracted using standard protocols. Explicit permission was obtained to publish the photographs of the subjects shown in Figure 1.

Exome sequencing analyses

DNA of the affected subject and her parents was extracted from circulating leukocytes and sequenced using Illumina paired end technology coupled with the SureSelect AllExon V.5 (Agilent) enrichment kit. Exome sequencing (ES) raw data were processed and analyzed using an in-house implemented pipeline previously described,^{19,20} according to the GATK's Best Practices.²¹ The UCSC GRCh37/hg19 version of genome assembly was used as a reference for reads alignment by means of BWA-MEM tool,²² and the subsequent variant calling with HaplotypeCaller (GATK v3.7).²¹ Variants' functional annotation was made by SnpEff v.5.0 and dbNSFP v.4.2 tools.^{23,24} The most relevant *in silico* impact predictions were also evaluated, such as Combined Annotation Dependent Depletion v.1.6,²⁵ Mendelian Clinically Applicable Pathogenicity v.1.3, and InterVar v.2.0.1.^{26,27} By filtering against our population-matched database (approximately 3,000 whole ES [WES]) and public databases (dbSNP150 and gnomAD V.2.0.1), the analysis was focused on high-quality rare variants that affect coding sequences and splice site regions.

Constructs

The human *ATP6V1B2* (RefSeq: NM_001693.4) and *ATP6V1C1* (RefSeq: NM_001695.5) coding sequences were cloned in pCMV-Myc/HisA (Invitrogen) and in pFLAG-CMV (SIGMA) tagged expression vectors, respectively. The missense variants identified in two different subunits of ATP6V1 complex (p.Ala332Val, p.Gln376Lys, p.Tyr328His, p.Arg485Pro, ATP6V1B2; p.Glu289Lys, and ATP6V1C1) were introduced by site-directed mutagenesis using the QuikChange XL kit (Agilent Technologies) in accordance with the manufacturer's protocol. All generated constructs were checked by direct sequencing.

Cell cultures

Functional characterization of ATP6V1B2 and ATP6V1C1 mutants was carried out on cultured skin fibroblasts obtained from subcutaneous biopsies of enrolled individuals, as well as in transiently transfected cell lines. Primary human fibroblasts and 293T cell lines were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (EuroClone) and 1%

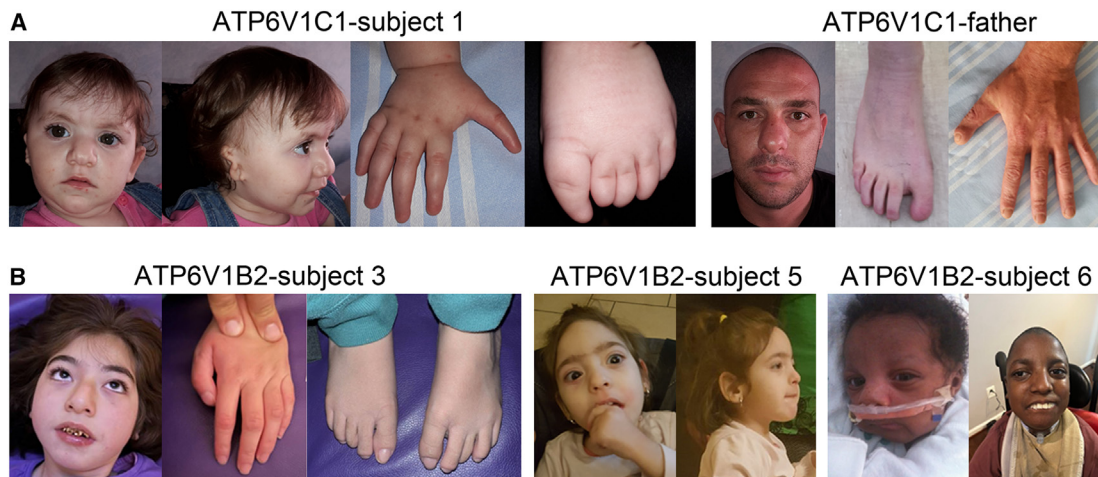


Figure 1. Craniofacial features of the individuals carrying dominant acting *ATP6V1C1/ATP6V1B2* variants

(A) Subject 1 at 2 years (left). Note the occurrence of bushy eyebrow, up-slanted palpebral fissures, epicanthal folds, microtia, broad nasal bridge, bifid nasal tip, deep philtrum, thin upper lip, prominent lower vermilion, nail aplasia of hands and feet, hypoplastic terminal phalanges and triphalangeal thumbs/big toes. Subject 1's father (right). Note nail aplasia of feet and hypoplastic terminal phalanges of hands and feet. Minor dysmorphism includes broad nasal bridge, bulbous nose, deep philtrum, and prominent upper and lower vermilion.

(B) Subject 3 at 8 years (left). Note facial hypotonia, straight eyebrow, hypertelorism, ptosis, long eyelashes, tubular nose with broad nasal base, short and deep philtrum and everted thin upper lip with prominent upper vermilion. Subject 5 at 4 years 7 months (middle panels). Note the high forehead, bitemporal narrowing, coarse face, bushy and straight eyebrows, long eyelashes, prominent upper and lower vermilion and large mouth. Subject 6 at 13 years (right). Note the bitemporal narrowing, coarse face with full-cheeks, thin upper lip, micrognathia/retrognathia, and short neck. A detailed clinical characterization of affected subjects is reported in [Tables 1](#) and [S3](#) and Supplemental Material (Clinical Reports).

penicillin-streptomycin, at 37°C with 5% CO₂. Specifically, 293T cells were seeded in six-well plates the day before transfection. Monolayer were transfected at 70%–80% confluency with wild-type (WT) or mutants C1-Flag-tagged or B2-Myc-tagged expression plasmids, using Fugene 6 transfection reagent (Promega). Forty-eight hours after transfection, cells were lysed and the level of ATP6V1B2 and ATP6V1C1 was assessed by immunoblotting with an anti-Myc (Cell Signaling) or anti Flag (SIGMA) antibodies respectively. ATP6V1B2 endogenous level was evaluated on fibroblasts lysates using a rabbit polyclonal anti-ATP6V1B2 antibody (Abcam). Membranes were probed with an anti-GAPDH antibody (Santa Cruz Biotechnology) for protein normalization.

Confocal analysis

Confocal analysis was performed on a Zeiss LSM 980 with Airyscan2, using the 63× oil objective and excitation spectral laser lines at 405, 488, 546, 594, and 633 nm. Quantitative analyses were performed using the Zen 3.3 software and mean fluorescence intensity (MFI) ± SEM of signals were determined in at least 3 independent experiment per marker (≥ 20 cells per experimental condition, in each repeat), and plotted following the formula: Final MFI = MFI (region of interest) – MFI (background). Signal colocalization was performed using the Pearson correlation coefficient.

Primary cilium staining

Cells were plated onto cover slips, maintained 24 h in low serum medium to promote emission of cilia and then fixed in 4% paraformaldehyde (PFA). Primary cilium was stained with a rabbit polyclonal anti-ARL13B antibody (Abcam) followed by goat anti-rabbit Alexa Fluor 594 (red), while basal bodies were stained with mouse monoclonal anti-pericentrin (Abcam) followed by goat anti-mouse Alex Fluor 488 (green) and nuclei with DAPI (blue).

Subcellular co-localization of ATP6V1B2 and lysosomes

For immunofluorescence, patients' and controls fibroblasts were seeded at the density of 20 × 10³, in 24-well cluster plates onto 12-μm cover glasses. After 24 h of culture in complete medium, cells were fixed with 3% PFA (30 min at 4°C). After permeabilization with 0.5% Triton X-100 (10 min at room temperature [RT]), fibroblasts were stained with mouse monoclonal anti-Lamp1 antibody (Cell Signaling), and rabbit polyclonal ATP6V1B2 antibody (Abcam), followed by the appropriate secondary antibody (Life Technologies) and DAPI.

Labeling lysosomes with DQ-BSA

To assess the autolysosome function, a red BODIPY dye-conjugated bovine serum albumin (DQBSA, Invitrogen) based assay was used. This BSA derivative is so heavily labeled that the fluorophore is self-quenched. Proteolysis of this compound results in de-quenching and release of brightly fluorescent fragments. Thus, the use of DQ-BSA is useful for the visualization of intracellular proteolytic activity. Control and patient's fibroblasts were seeded in 24-well cluster plates onto 12-mm cover glasses and maintained in culture complete medium for 24 h. After 24 h, cells were incubated with the BSA derivative (10 μg/mL) for 1 h at 37°C in complete culture medium, and then in starvation medium (Earle's balanced salt solution [EBSS]) for 16 h to induce the autophagic flux. After incubation, the coverslips were counterstained with DAPI and observed under confocal microscopy. Proteolysis of this BSA derivative results in the release of fluorescence (590 nm).

LysoSensor staining

For CLSM analyses patients' and controls fibroblasts were seeded on coverslips at a density of 300 × 10³. After 24 h of culture in

complete medium, cells were rinsed with an isotonic buffer pH 7.4 (105 mM NaCl, 5 mM KCl, 6 mM HEPES-acid, 4 mM HEPES-Na, 5 mM NaHCO₃, 60 mM mannitol, 5 mM glucose, 0.5 mM MgCl₂, 1.3 mM CaCl₂) and then incubated with 2 μM LysoSensor Yellow/Blue DND-160 (Thermo Fisher Scientific) for 3' at RT. Then cells were extensively rinsed with the isotonic buffer and observed on a Zeiss LSM 980. Images were then acquired using a 63× oil objective, laser and filter settings were adjusted according to the fluorescence excitation and emission requirements of the reagent: excitation 365 nm and dual emission at 440 nm (neutral pH 5–7, blue) and 540 nm (acidic pH 3–5, yellow).

Endo-lysosomal pH analysis by LysoSensor yellow/blue dextran

To measure the pH of endo-lysosomal compartments, 2×10^5 fibroblasts were plated on a 35-mm imaging dish with a glass bottom (Ibidi), after 48 h of culture in complete medium were incubated overnight with 0.1 mg/mL LysoSensor Yellow/Blue dextran (#L22460, Thermo Fisher Scientific) in complete medium at 37°C in a humidified atmosphere with 5% CO₂. The loading solution was then removed, cells were washed and live imaged in complete medium (without phenol red) with excitation at 360 nm and double emission at 450–470 nm (blue) and 520–540 nm (yellow) on a Zeiss LSM980 confocal microscope. The pH calibration curve was generated using an isotonic buffer (105 mM NaCl, 5 mM KCl, 6 mM HEPES-acid, 4 mM HEPES-Na, 5 mM NaHCO₃, 60 mM mannitol, 5 mM glucose, 0.5 mM MgCl₂, 1.3 mM CaCl₂) supplemented with 20 μM nigericin. Each buffer solution was adjusted to the appropriate final pH, ranging from 2.5 to 6.5, using 1 N NaOH or 1 N HCl.

Cells (>50) were analyzed both in fluorescence and in bright field with the Zeiss Zen 3.3 software. ROIs within cells that were reflective of LysoSensor Yellow/Blue dextran-labeled organelles were selected and the fluorescence intensity values of such regions were recorded for emissions at both wavelengths (450 and 540 nm). Additionally, ROIs outside of cells (background) were selected and the background fluorescence intensity values were recorded for each emission wavelength.

After subtracting the background fluorescence intensity values for each emission wavelength from the corresponding fluorescence intensity values relating to LysoSensor Yellow/Blue dextran-labeled organelles, the ratio of intensity 450 nm:540 nm was calculated for each region of interest and for each pH calibration curve buffer. Next, the data for the pH calibration curve were fitted to a linear regression using GraphPad Prism5 software and the data of experimentally measured ratios in samples were converted into absolute pH values by interpolation in the pH calibration curve.

Filipin fluorescence staining of free cholesterol

Filipin dye is used as a pro-fluorescence probe to stain the cholesterol in cells or tissues. Filipin binds to model crystalline phase lipid bilayers containing cholesterol, and to liquid phase bilayers with or without cholesterol, in an orientation that forms membrane pores. Due to its fluorescence shift upon binding to cholesterol, filipin is visible under simple UV *trans*-illumination. Briefly, control and patient fibroblasts were seeded in 24-well cluster plates onto 12-mm cover glasses and maintained in culture complete medium for 24 h. After 24 h, cells were rinsed twice with PBS, fixed in with 3% PFA, washed twice with PBS, and incubated with 1 mL of 1.5 mg glycine/mL PBS for 10 min at RT to quench

the PFA. Fixed cells were washed three times and labeled with the Filipin staining solution for 2 h at RT, followed by three washes before imaging. Cells were observed in PBS by fluorescence microscopy using a UV filter set (340–380 nm excitation, 40 nm dichroic, 430-nm-long pass filter).

Evaluation of LC3I/II levels by western blot analysis

To monitor the function of the autophagic pathway, primary control and patients' fibroblasts were incubated in EBSS (Gibco) medium for 2, 4, and 8 h with and without addition of the inhibitor bafilomycin A1 (200 nM for 4 h, Sigma). LC3I/II expression level was assessed by western blot analysis and immunofluorescence microscopy. For western blot analyses, cell pellets were washed twice in PBS and lysed in RIPA buffer (20 min, 4°C). Lysates were centrifuged at 10,000×g (15 min, 4°C), and 20 μg cell extracts were separated by 15% SDS-PAGE, and transferred to PVDF membranes (Bio-Rad). Blots were incubated with rabbit polyclonal anti-LC3 antibody (Cell Signaling). Membranes were re-probed with an anti-GAPDH antibody (Santa Cruz Biotechnology) to normalize protein content. For immunofluorescence, patients' and controls fibroblasts were seeded at the density of 20×10^3 in 24-well cluster plates onto 12-μm cover glasses. After 24 h of culture in complete medium, fibroblasts were treated with EBSS medium to promote the autophagosomes formation, for 4 and 8 h with or without bafilomycin (4 h). Then were fixed with absolute chilled methanol for 10 min at –20°C. Cells were stained with a rabbit polyclonal anti-LC3I/II antibody (Cell Signaling) only or co-stained with mouse monoclonal anti-Lamp1 antibody (Cell Signaling) followed by the appropriate secondary antibodies (Life Technologies) and DAPI.

Electron microscopy

Control and patients-derived fibroblast were fixed in 1% glutaraldehyde and 4% PFA in 0.1 M sodium cacodylate buffer (pH 7.2), overnight, at 4°C, and processed as described previously.²⁸ Cells were washed in cacodylate buffer and post-fixed with 1% OsO₄ in 0.1 M sodium cacodylate buffer for 1 h at RT, treated with 1% tannic acid in 0.05 M cacodylate buffer for 30 min, and rinsed in 1% sodium sulfate in 0.05 M cacodylate buffer for 10 min. Post-fixed specimens were washed, dehydrated through a graded series of ethanol solutions (30%–100% ethanol) and embedded in Agar 100 (Agar Scientific). Ultrathin sections, obtained by an UC6 ultramicrotome (Leica Microsystems), were stained with uranyl zeresolution (Agar Scientific) and Reynolds' lead citrate and examined at 100 kV with a FEI/Philips EM 208S Transmission Electron Microscope equipped with acquisition system/Megaview SIS camera (Olympus).

Structural analyses

Structures with pdb codes 6wm2 and 3vr6 were used in our analysis. In 6wm2, the A and B subunits are present as trimers with chain identifiers A, B, and C for the subunits A, while D, E, and F identify the subunits B. To avoid confusion with the letters identifying the subunits, we refer to these chains as α, β, γ, δ, ε, and ζ, respectively. The β/ε and the α/δ interfaces represent the open and semi-open configurations, while the γ/ζ is the only one bound to ADP and is representative of the closed state. The C subunit is present as a monomer (with chain identifier O). All the figures were obtained by using the software Chimera.²⁹ The same software package was used to carry out the structural analysis.

Results

Genetic and clinical findings

In the context of an intramural research program dedicated to individuals affected by undiagnosed diseases, trio-based ES was performed to reach diagnosis in an individual with a phenotype partially overlapping DOORS syndrome and ZLS, who was negative for mutations in the previously identified disease genes. The main clinical features of the individual included DD/ID, electroencephalogram abnormalities, a bulbous nose with a bifid nasal tip and additional minor dysmorphism, microtia with malformation of inner and outer ear, profound sensorineural deafness, short stature, nail aplasia of hands and feet, hypoplastic terminal phalanges, and triphalangeal thumbs/big toes (Figure 1A; Supplemental Material, Clinical Reports). Of note, occurrence of minor digit anomalies (i.e., hypoplastic nails and terminal phalanges of hands and feet) was also observed in her father, who however did not show any additional feature reported in his daughter (Figure 1A). Sequencing statistics and data output are reported in Table S1. ES data analysis allowed us to identify a previously unreported, apparently *de novo*, missense variant, c.865G>A (p.Glu289Lys [C1^{E289K}]), affecting *ATP6V1C1*. No functionally relevant variants compatible with known Mendelian disorders based on the inheritance model and clinical presentation were identified. *ATP6V1C1* was considered as an excellent candidate based on its functional link with *ATP6V1B2*, the high degree of conservation of the affected residue, and the predicted damaging consequences of the amino acid substitution on protein function (Figure S1A; Table S2). The heterozygous variant was validated via Sanger sequencing, which confirmed its occurrence in all tested tissues (Figure S2). The variant was not present in the healthy brother and mother, while testing multiple paternal tissues (i.e., leukocytes, fibroblasts, hair bulb cells, and oral mucosal epithelial cells) documented a low level of mosaicism for the variant in hair bulb and buccal cells, supporting a post-zygotic onset of the variant in the father, in line with the clinical findings (Figure S2). Based on these findings, manual inspection of the WES data in the father confirmed a low-level mosaicism in the sample (estimated as 7%, 6 reads out of 87).

Networking failed to identify additional cases with putative pathogenic *ATP6V1C1* variants. Based on the clinical overlap and functional link, phenotypic and molecular data on six unrelated subjects with *bona fide* pathogenic heterozygous *ATP6V1B2* variants were collected to more precisely characterize the clinical and molecular spectra of the disorder, and investigate the mechanism of disease. All variants were missense (NM_001693.4:c.995C>T, p.Ala332Val [B2^{A332V}]; c.982T>C, p.Tyr328His [B2^{Y328H}]; c.983A>G, p.Tyr328Cys; c.1120G>C, p.Glu374Gln; c.1126C>A, p.Gln376Lys [B2^{Q376K}]; and c.1127A>G, p.Gln376Arg), occurred *de novo*, had not previously been reported in public databases (e.g., gnomAD), involved conserved residues

located within regions of the protein that were intolerant to variation (Figure S1B), and were predicted to have a disruptive impact on protein function (Table S2). The clinical phenotype of the subjects was variable but invariably characterized by dysmorphic features, DD with impact both on cognitive and motor functions, ID, and seizures. A subset of individuals showed horizontal nystagmus/visual impairment, growth delay, brain abnormalities (e.g., brain atrophy and several degrees of myelination delay), deafness, gingival overgrowth, and minor skeletal abnormalities (e.g., scoliosis, pes planus, joint hyperextensibility). No individuals had onychodystrophy, osteodystrophy, or skeletal dysplasia. The severity of the condition was fatal in one individual (subject 2) (Supplemental Material, Clinical Reports). The observed clinical spectrum was tentatively associated with DOORS, DDOD, and/or ZLS without reaching the diagnostic criteria for none of these disorders (Table 1; Figure 1B).

Functional and structural analyses

We investigated the functional consequences of the identified *ATP6V1B2* and *ATP6V1C1* variants, together with the p.Arg485Pro change in *ATP6V1B2* [B2^{R485P}], which had previously been associated with ZLS.¹³ To assess whether the identified variants impact protein stability, the protein levels of all *ATP6V1B2*/*ATP6V1C1* mutants were evaluated in transiently transfected 293T cells, documenting a negligible effect of each of the tested variants (Figure S3). Consistently, similar levels of endogenous *ATP6V1B2* were also found in primary fibroblasts obtained from affected (B2^{A332V}, B2^{Q376K}, and B2^{R485P}) and control individuals (Figure S3). Assessment of the endogenous level of the *ATP6V1C1* protein was not possible due to the poor sensitivity of all tested commercially available antibodies.

The vacuolar V-ATPase is present in the membranes of many organelles, where its proton-pumping action is required to maintain their acidic luminal pH, especially in lysosomes. Using patient and control fibroblasts, we first analyzed the subcellular localization of B2 subunits. By immunofluorescence confocal microscopy analysis, we observed proper localization of WT and all tested mutant B2 subunits (B2^{A332V}, B2^{Q376K}, and B2^{R485P}) to lysosomes, as indicated by their co-localization with LAMP1 (Figures 2A, 2B, and S4). Notably, a marked increase in the size and number of lysosomes in cells endogenously expressing each of the tested B2 and C1 mutants was observed by immunofluorescence analysis and western blot analyses (Figures 2C and 2D), as observed in lysosomal storage disorders. Then, we evaluated a possible impact of altered ATPase pump function on lysosome function by assessing the accumulation of ceramide and cholesterol as representative of lysosomes substrates. Analyses performed using specific probes (i.e., BTR ceramide and Filipin) showed a significantly increased storage of both substrates in all tested patients' cell lines compared with controls cells (Figures 3A-3D), suggesting a generalized defective

Table 1. Major clinical features of the identified subjects with *ATP6V1B2/ATP6V1C1* variants, and comparison with DDOD syndrome, DOORS syndrome, and ZLS

	DD	ID	Seizures	Hypoplasia/aplasia terminal phalanges and/or onychodystrophy	Hypertrichosis	Gingival overgrowth	Facial dysmorphisms	Deafness	Osteodystrophy
DDOD	+/-	+	-	+	-	-	+/-	+	-
DOORS	+	+	+	+	+/-	-	+	+	+
ZLS	+	+	+/-	+	+/-	+	+	+/-	-
<i>ATP6V1B2</i> (p.Arg506*)	8/20 (40%)	8/20 (40%)	7/20 (35%)	20/20 (100%)	1/20 (5%)	1/20 (5%)	18/18 (100%)	20/20 (100%)	0/20 (0%)
<i>ATP6V1B2</i> (missense changes)	5/11 (45%)	9/11 (81%)	9/11 (81%)	3/10 (30%)	4/11 (36%)	5/11 (45%)	11/11 (100%)	1/10 (10%)	0/11 (0%)
S1 (<i>ATP6V1C1</i>)	+	+	+	+	-	-	bushy eyebrow, up-slanted palpebral fissures, epicanthal folds, microtia, broad nasal bridge, "bifid" nasal tip, deep philtrum, thin upper lip, prominent lower vermillion	+	-
S2 (<i>ATP6V1B2</i> , p.Ala332Val)	+	+	+	-	-	+	bitemporal narrowing, long eyelashes, deep philtrum, everted upper lip, micrognathia, full cheeks, high-arched palate, blue sclerae	NA	-
S3 (<i>ATP6V1B2</i> , p.Tyr328His)	+	+	+	-	-	-	facial hypotonia, straight eyebrow, eyes appear wide set, ptosis, long eyelashes, tubular nose with broad nasal base, decreased facial movement/wrinkles, short and deep philtrum, everted thin upper lip with prominent upper vermillion, mouth open at rest	-	-
S4 (<i>ATP6V1B2</i> , p.Tyr328Cys)	+	+	+	-	-	-	high forehead, dolichocephaly, bitemporal narrowing, straight eyebrows, long eyelashes, short philtrum, slight micrognathia, posteriorly rotated ears	-	-
S5 (<i>ATP6V1B2</i> , p.Gln376Lys)	+	+	+	-	-	-	high forehead, dolichocephaly, bitemporal narrowing, coarse face, brushy and straight eyebrows, long eyelashes, prominent upper and lower vermillion, large mouth	-	-
S6 (<i>ATP6V1B2</i> , p.Gln376Arg)	+	+	+	-	-	-	dolichocephaly, bitemporal narrowing, coarse face with full-cheeks, thin upper lip, micrognathia/retrognathia, glossoptosis, high arched palate, and short neck	-	-
S7 (<i>ATP6V1B2</i> , p.Glu374Gln)	+	+	+	-	-	-	dolichocephaly, bitemporal narrowing, mildly coarse face, short philtrum, everted upper lip, thin upper lip, prominent upper and lower vermilions, large mouth	-	-
NA, not available.									

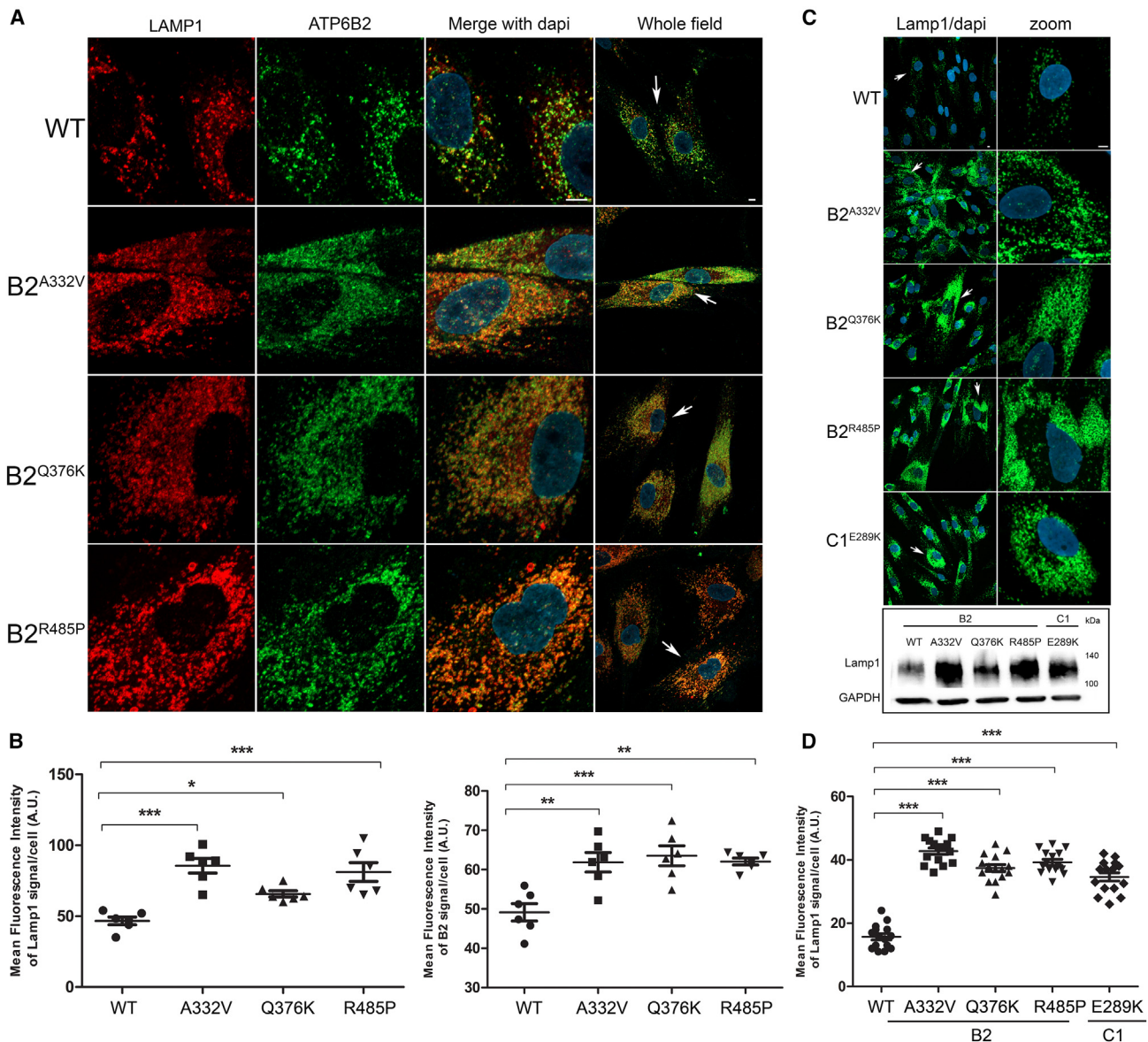


Figure 2. Disease-causing *ATP6V1B2* and *ATP6V1C1* variants affect lysosome morphology but maintain a proper lysosomal localization

(A and B) Confocal laser scanning microscopy analysis shows a proper *ATP6V1B2* subcellular localization with lysosomes. Cells were stained with Lamp1 (lysosome marker, red) and *ATP6V1B2* (B2 subunit marker, green) antibodies, and DAPI (DNA marker, blue). Representative images are shown (A) together with the quantitative data and statistical analysis (B). Arrows indicate the selected cells reported in the enlarged images. Scale bar, 5 μ m. MFI \pm SEM of B2 and Lamp1 signal per cell was quantified (≥ 20 cells per experimental condition, in six independent experiments per marker), and plotted in the corresponding graphs, defining final MFI as MFI (region of interest [ROI]) – MFI (background). Bars indicate mean \pm SEM, *p* values were calculated by one-way ANOVA with Tukey's correction for multiple testing.

(C and D) Representative images reporting the altered morphology, distribution, and number of lysosomes in cells from individuals carrying heterozygous *ATP6V1B2* missense variants (p.Ala332Val [A332V], p.Gln376Lys [Q376K], and p.Arg485Pro [R485P]) compared with control cells are shown (C). Arrows indicate the selected cells reported in the enlarged images. Scale bar is 5 μ m. In the same panel, western blot analysis shows the higher expression level of lysosomes marker in patients' fibroblasts than control cells. GAPDH was used to normalize the experiments. Quantitative data and statistical analysis are also shown (D). MFI and SEM were calculated as above.

hydrolytic activity of lysosomal enzymes possibly as a result of an altered vacuolar ATPase proton function. To evaluate the lysosomal acidification status, we first used LysoSensor Yellow/Blue DND-160 as a dye (Figure 4A). Cells were treated with this probe at steady state condition, after 4 h of EBSS treatment to induce autophagy, and after 4 h of treatment with EBSS together with bafilomycin, the

latter used to inhibit the V-ATPase function.³⁰ We observed a significant higher acidity of lysosomes in all patients' cell lines compared with control cells, indicating a hyperactive behavior of the proton pumps assembling the mutant B2/C1 subunits. As expected, bafilomycin treatment promoted the complete loss of staining indicating the efficient inhibition of both mutant and WT proton-pumping

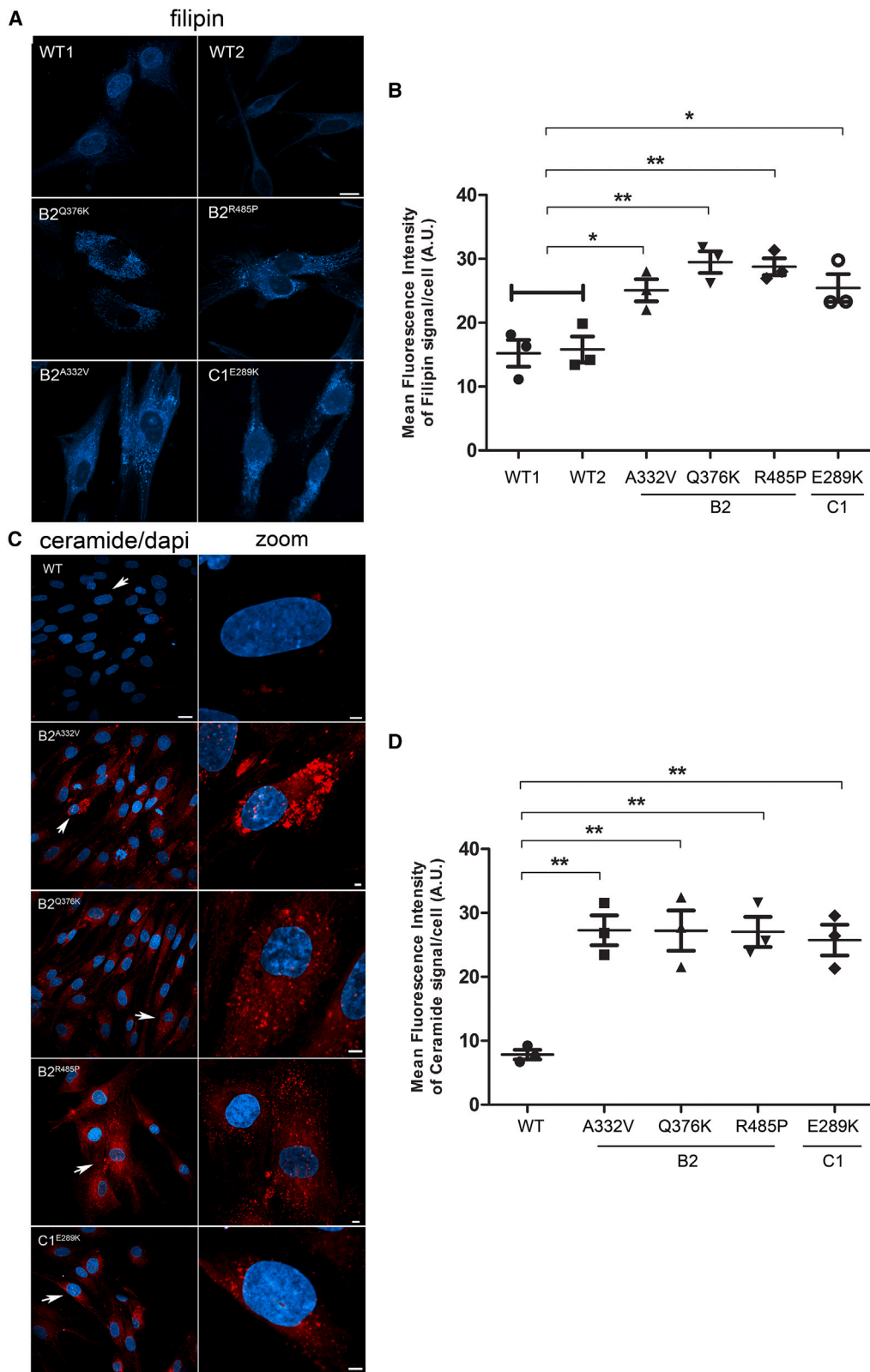


Figure 3. Effects of autophagic flux dysregulation promoted by ATP6V1B2 and ATP6V1C1 mutants

Confocal laser scanning microscopy analyses show a significant storage of cholesterol (A and B) and ceramide (C and D) on patients' fibroblasts compared with control cells. Cells were stained with filipin (cholesterol) (A) and BTR-ceramide (C) probes, respectively. Scale bars are 10 μm (A), 20 μm (C, left), and 2 μm (C, right). Arrows indicate the selected cells reported in the enlarged images. MFI \pm SEM of filipin (B) and ceramide (D) signal per cell, was quantified (3 independent experiments, ≥ 20 cells per condition, in each repeat), and plotted in the corresponding graphs, defining Final MFI as MFI (region of interest) - MFI (background). WT1 and WT2 were pooled together vs. patient's samples. Bars indicate mean \pm SEM, *p* values were calculated by One way ANOVA with Tukey's correction for multiple testing.

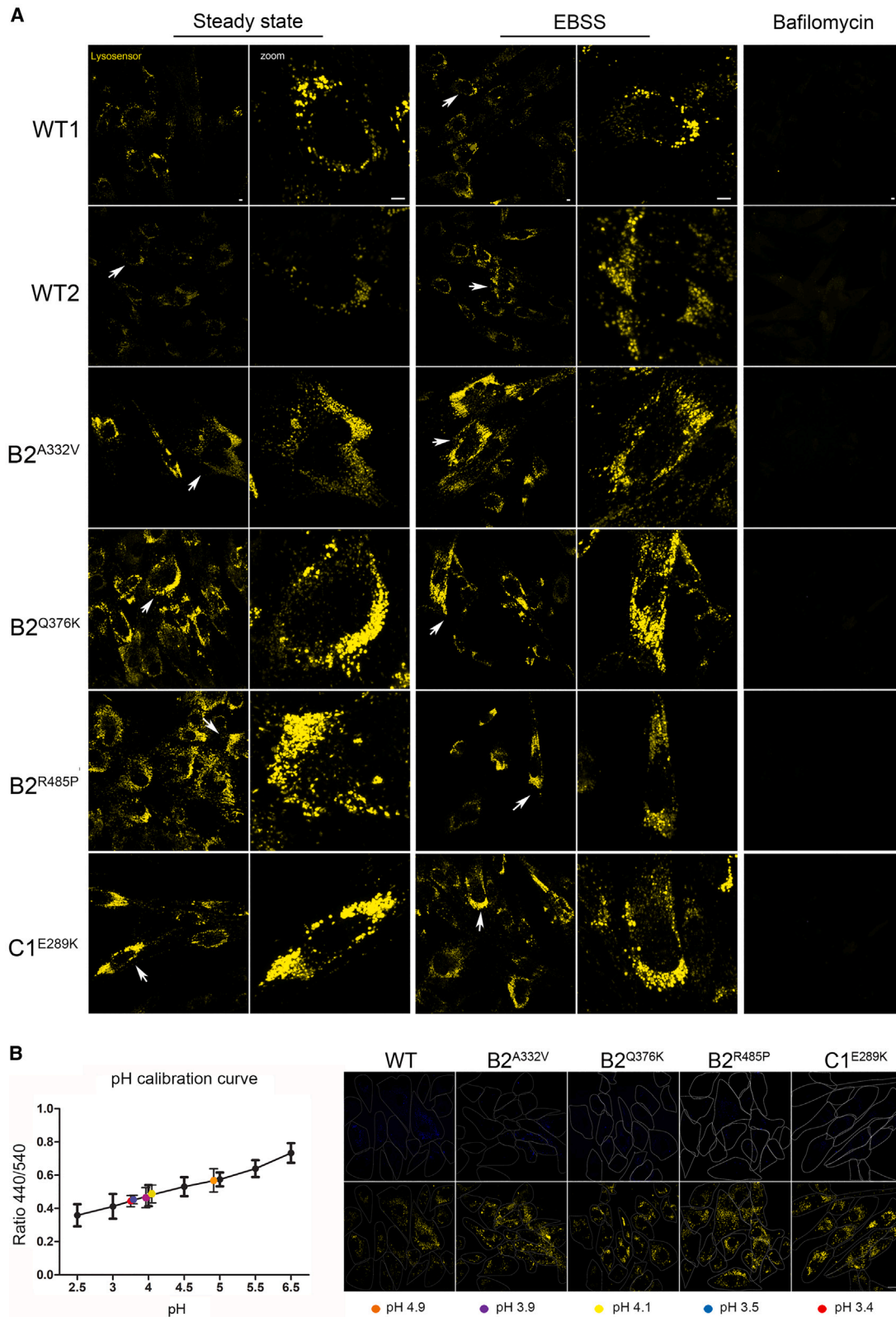


Figure 4. Expression of ATP6V1B2 and ATP6V1C1 mutants is associated with increased acidification of lysosomes
 (A) Patients' and control cells were incubated with 2 μ M LysoSensor Yellow/Blue DND-160 (Thermo Fisher Scientific) and observed on a Zeiss LSM 980 confocal microscope. Images were acquired using a 63 \times oil objective, laser and filter settings were adjusted according to the fluorescence excitation and emission requirements of the reagent. Scale bar, 5 μ m.
 (B) Evaluation of lysosomal pH values in patient-derived fibroblasts. The pH calibration curve performed using WT1 cells, and obtained by plotting the fluorescence intensity 450/540 ratios as a function of pH, was fitted with linear regression using GraphPad Prism5

(legend continued on next page)

function. To further validate this finding, we performed experiments to measure the intraorganellar pH in patient-derived fibroblasts by treating cells with the ratiometric LysoSensor Yellow/Blue dextran (Figure 4B). These assays revealed a substantial decrease of intra-organellar pH in patients' cells compared with control cells, further supporting an increase of proton-pumping activity in the former.

Ultrastructural analyses were performed to better characterize the effects of aberrant V-ATPase functioning. Control fibroblasts showed identifiable cytoplasmic components, including well-organized mitochondria, slightly dilated endoplasmic reticulum, and lysosomes that were filled with typical multilamellar and electron-dense material (Figures 5A–5D). Remarkably, compared with control cells, patients' fibroblasts showed a high number of enlarged vesicles containing lighter granular material, which resembled early lysosomes, and vacuoles containing osmiophilic material with different electron densities, reminiscent of lysosomes. In particular, in fibroblasts expressing B2^{A322V} and B2^{R485P} many of these vesicles seemed to be partially fused (Figures 5E and 5F) or in close proximity to each other (Figures 5I–5L). Cells endogenously expressing B2^{Q376K} showed accumulation of large heterogeneous vacuoles filled with osmiophilic material and substances with different electron densities (Figures 5G and 5H), while cells expressing the C1^{E289K} variant showed only vesicles uniform in size (Figures 5M and 5N). Dilated endoplasmic reticulum and lipid droplets were also visible in the cytoplasm of the cells. Overall, these findings were suggestive of aberrant lysosome maturation and functioning, as commonly observed in lysosomal storage diseases,^{31,32} in line with the data collected by immunofluorescence analysis.

Since proper acidification of lysosomes is required for autophagosome-lysosome fusion, we hypothesized that the altered lysosomal function might also result in an impairment of the autophagic flux. Immunofluorescence and western blot analysis using an anti LC3I/II antibody as probe showed a significant accumulation of autophagosomes at steady state condition as well as after autophagy flux induction with EBSS in all tested patients' fibroblasts compared with control cells (Figures 6A–6C, S5, and S6). Remarkably, these organelles, which appeared as enlarged vesicles mainly accumulated in the perinuclear region, did not colocalize with lysosomes after autophagic flux induction and bafilomycin treatment (Figures 6B and S6). To further assay the extent of dysregulation of the autophagic flux and/or lysosomal function, we evaluated the fluorescence emission of a BSA derivative (DQ-BSA) conjugated to a self-quenched fluorophore in patients' fibroblasts and control cells after starvation with EBSS for 16 h to induce autophagy. We did not observe dequenched

DQ-BSA in patients' cells over the time period, further supporting a defective autophagosome-lysosomal function (Figure S7).

Increasing evidence testifies the interplay between cilia and autophagy.^{33–35} On one hand, the autophagy core machinery is known to localize at ciliary compartments; on the other, the primary cilium, representing a sensorial antenna of cells, promotes the activation of the autophagic flux in response to diverse stimuli. Autophagy can also control the level of positive or negative regulators of cilium biogenesis, influencing cilia formation and elongation. Based on these considerations, we investigated the effect of altered B2 and C1 subunit function on primary cilium biogenesis and morphology in primary fibroblasts from affected subjects. The analysis performed in starved fibroblasts revealed the presence of aberrant primary cilium formation in all cell lines (Figure 7). Specifically, the cilium was either shorter compared with control cells or absent (dot cilium), documenting a pleiotropic effect of each of the tested variants also involving cilium biogenesis.

Recently, the cryoelectron microscopy structures of the human V-ATPase in three rotational states representing the sequential conformational changes after ATP hydrolysis and release were solved.¹² We used these structures to explore the possible structural and dynamics effects of the identified amino acid substitutions. In general, ATP hydrolysis in the V₁ catalytic subcomplex drives a general rearrangement of the pump, leading to proton translocation (Figure 8A). The ATP binding site is located at the A/B interface, in the hexamer formed by subunits A and B, though only the A subunits are directly involved in ATP hydrolysis. The C subunit interacts with two E/G heterodimers and is part of the “stator” ensuring the reciprocal stationary configuration of the V_O and V₁ subcomplexes (Figure 8A).³⁶ The mutated Tyr³²⁸, Ala³³², and Gln³⁷⁶ residues of the B2 subunit are located at the interface with the A subunit, where the ATP/ADP binds to the complex (Figure 8B). In particular, Tyr³²⁸ is close to multiple residues of the A subunit that undergo significant conformational transformations in the transition between the open and closed states (Figure 8C), suggesting that the p.Tyr328Cys and p.Tyr328His substitutions could affect the ADP/ATP exchanges and, in turn, the transition between the open and closed states. Ala³³² interacts very tightly with Ser³¹⁶ and Asn³¹⁷ in the A subunit. The p.Ala332Val substitution would introduce steric clashes (Figure 8D). The proximity to the ATP/ADP binding region suggests a possible effect of the substitution on the ATP/ADP binding and exchange; however, in this case, a non-specific perturbation of the A/B interface cannot be ruled out. Gln³⁷⁶ is quite close to ADP (minimum distance below

software (left). Data are means \pm SEM from >50 cells analyzed for each pH value. Colored filled circles represent the experimentally measured ratios converted into absolute pH values by interpolation in the pH calibration curve. Representative images of fibroblasts incubated with LysoSensor Yellow/Blue dextran and visualized in live at 450 nm (top) and 540 nm (bottom) emission wavelengths (right). The cell contours recognition was performed in bright field images and used to effectively quantify fluorescence intensity. Scale bar, 20 μ m. All panels are identical in scale.

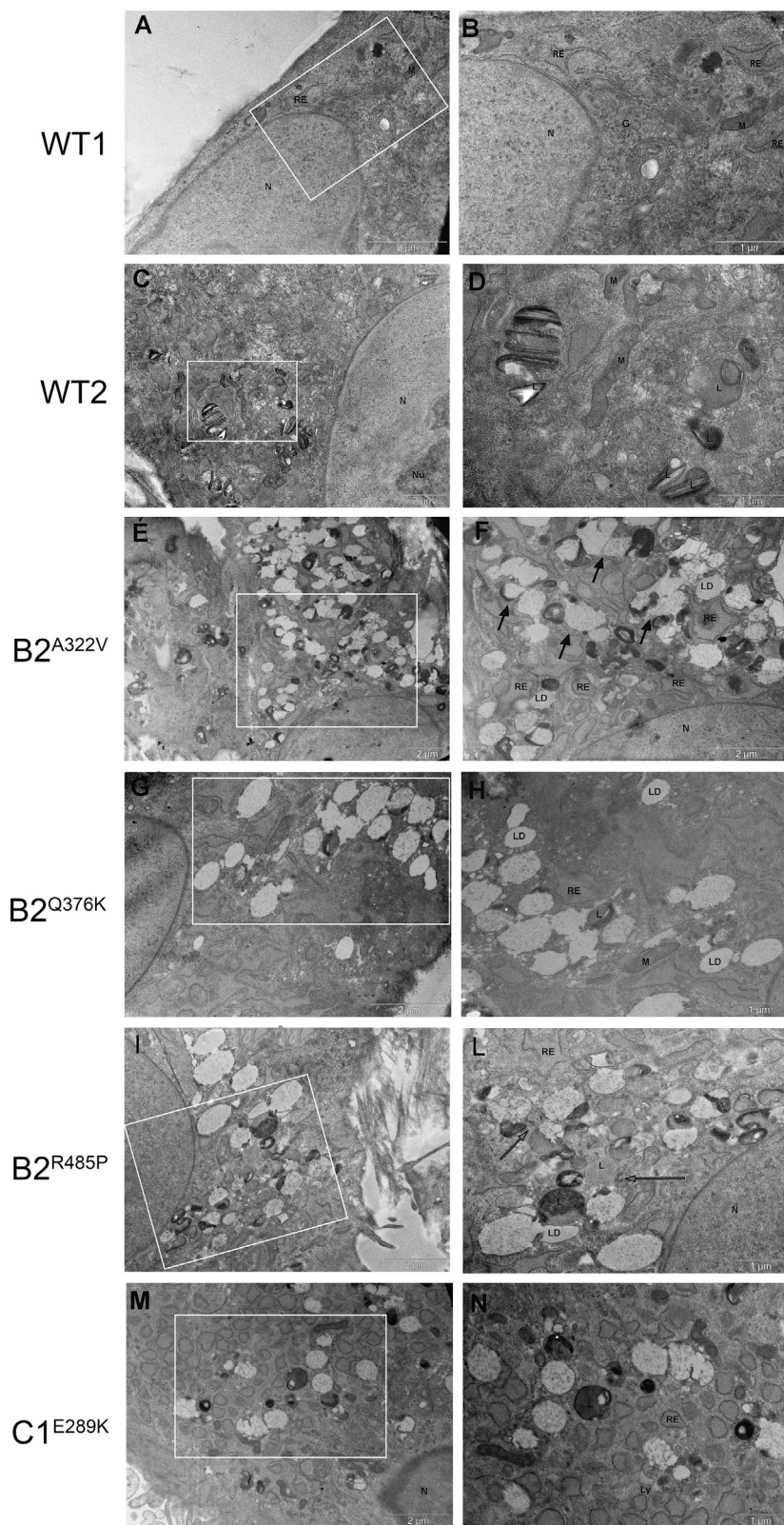


Figure 5. Electron microscopy analysis

Electron micrographs showing the ultrastructure of control (A–D) and patient-derived fibroblasts (E–N).

(E and F) Fibroblasts from subjects B2^{A322V} and (I–L) B2^{R485P} showed vesicles partially fused to each other (black arrows) and in close proximity (gray arrows).

(G–H) B2^{Q376K} patients' cells showed accumulation of large heterogeneous vacuoles whereas the cells carrying the C1^{E289K} mutant presented vesicles uniform in size.

(M and N) (white asterisks) Heterogeneous substances and osmiophilic material. G, Golgi apparatus; N, nucleus; n, nucleolus; L, lysosome; LD, lipid droplets; M, mitochondria; RE, rough endoplasmic reticulum.

charge, very likely perturbs the correct dynamics of the A/B interface, and the interactions with the ATP/ADP phosphate. It is worth mentioning that variants introducing a change in the net charge of the side chain of Glu³⁷⁴, which is only two residues apart from Gln³⁷⁶, have already been associated with ZLS (p.Glu374Gln)¹⁴ and an epileptic neurodevelopmental phenotype (p.Glu374Gly).³⁷ The possible effects of the p.Arg485Pro substitution have been discussed previously,¹³ based on a homology model. Overall, the recently reported structures confirm the previously reported analyses. Arg⁴⁸⁵ is far from the ADP/ATP-binding site, making a significant impact of its substitution with proline on the ATP binding unlikely. In contrast, the substitution is predicted to destabilize the helical tract encompassing residue 485 and weakens a possible salt bridge with Asp⁵⁰⁷, likely perturbing the interactions within the V₁ subcomplex. Finally, Glu²⁸⁹ is located within the so-called neck domain of boot-shaped subunit C. It does not interact with residues of other subunits but is involved in an ion pair interaction with Lys¹¹¹ (Figure 8F). This electrostatic interaction, stabilizing the peculiar topology of the C subunit, is strongly perturbed by the charge inversion caused by the p.Glu289Lys substitution, likely destabilizing the correct interaction

8 Å from the phosphate groups). Similar to Tyr³²⁸, the residues in the A subunit surrounding Gln³⁷⁶, including Lys⁴³⁷, display significant variations when examining the different states of the complex (Figure 8E). The p.Gln376Arg substitution, introducing a positive net

with the E/G heterodimers, and in turn perturbing the correct transduction of the signal after ATP release. Overall, coherently with the observed effects on the pumping activity, all the identified substitutions were expected to perturb the transduction of the signal caused by ATP

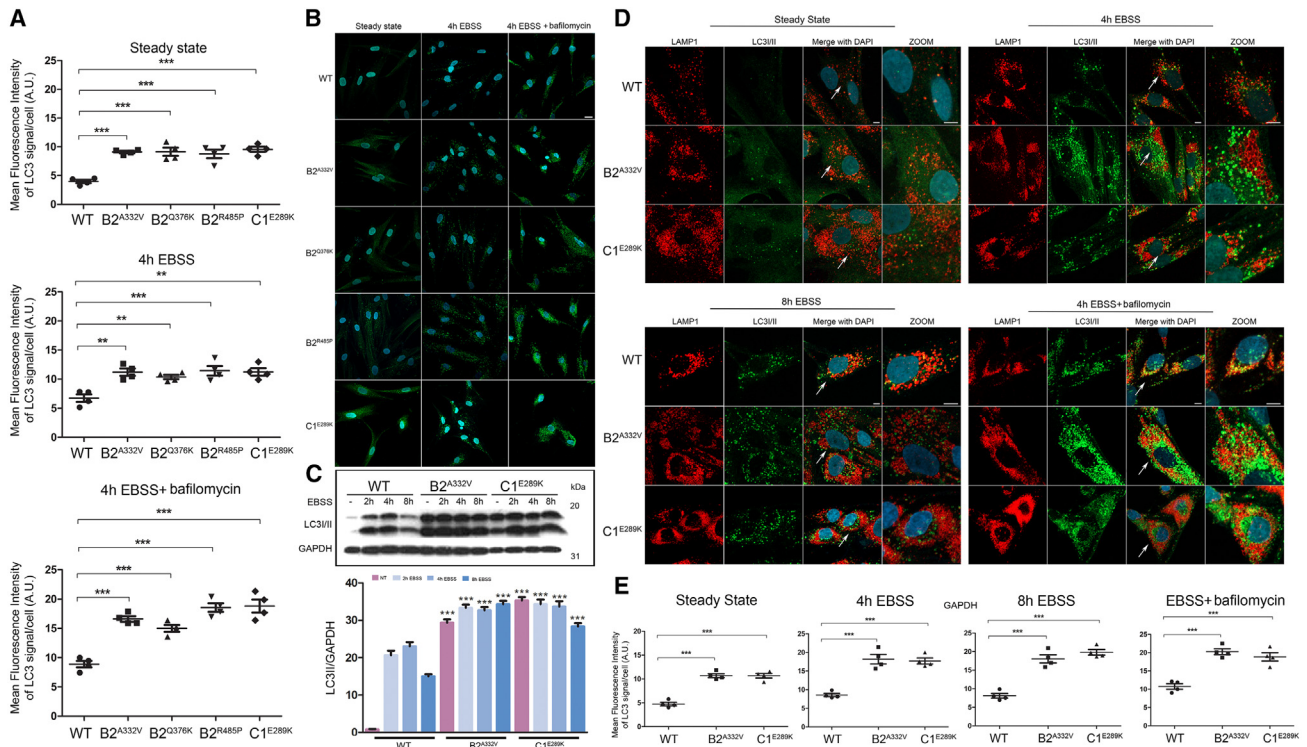


Figure 6. An impaired autophagic flux in patients' fibroblasts promotes LC3I/II proteins accumulation and affects autophagosomes number and subcellular localization

(A–C) Confocal laser scanning microscopy (CSLM) analysis performed on patients' fibroblasts show a significant high level of LC3I/II proteins already in the steady state condition, which remains constant after treatment with EBSS for 4 h without bafilomycin, to indicate an autophagic flux impairment. Graphs reporting MFI \pm SEM of LC3I/II (A) and representative images (B) are shown. Fixed cells were stained with rabbit monoclonal anti-LC3I/II antibody followed by rabbit anti-goat Alexa Fluor 488 (green). Nuclei are visualized by DAPI staining (blue). Scale bar, 20 μ m. Western blot analyses performed on patients' fibroblasts (C) confirm the data obtained by immunofluorescence, showing an anomalous accumulation of LC3I/II in the steady state condition and after autophagic flux induction with EBSS for 2, 4, and 8 h. Equal amounts of cell lysates were resolved by 15% polyacrylamide gel electrophoresis. Membranes were probed with an anti-LC3I/II antibody and then re-probed with an anti-GAPDH antibody for data normalization. Non-treated cells (i.e., cells cultured in steady state conditions) were reported as internal controls (–). Bars indicate mean \pm SEM, *p* values were calculated by one-way ANOVA with Tukey's correction for multiple testing. MFI \pm SEM signals per cell were quantified (4 independent experiments per marker, \geq 20 cells per experimental condition, in each repeat), and plotted in the corresponding graphs, defining Final MFI as MFI (region of interest [ROI]) – MFI (background). Bars indicate mean \pm SEM, *p* values were calculated by one-way ANOVA with Tukey's correction for multiple testing.

(D and E) CSLM analysis shows an unusual subcellular localization of autophagosomes in patients' cells compared with control cells (D). In particular, these organelles localized near the nucleus and a few number colocalized with lysosomes residing in the peripheral region of the cell in the steady state condition as well as after treatment with EBSS without bafilomycin. Fixed cells were stained with rabbit monoclonal anti-LC3I/II antibody followed by anti-rabbit Alexa Fluor 488 (green). The lysosomes were stained with an anti-mouse Lamp1 followed by anti-mouse Alexa Fluor-546 (red). Nuclei are visualized by DAPI staining (blue). Scale bar, 5 μ m. Quantitative data assessing the LC3I/II levels and statistical analysis are also shown (D). MFI and SEM were calculated as above.

hydrolysis. However, the predicted mechanism of perturbation seems to be different for the various substitutions. Specifically, those involving the B subunit are predicted to affect the dynamics of the A_3B_3 hexamer, whose conformational change leads to hydrogen pumping and/or exert a direct effect on the ATP/ADP binding site, while the p.Glu289Lys substitution in the C subunit is predicted to perturb the correct orientation of the E/G heterodimers.

Discussion

Here we provide evidence that *ATP6VIC1* variants can underlie a neurodevelopmental phenotype with features

resembling DOORS syndrome, and report that dominantly acting *ATP6V1B2* variants are causally associated with a phenotypic continuum having DDOD syndrome and ZLS as extreme phenotypes. We show that variants involving these two subunits of the V-ATPase pump promote enhanced function of the complex, which affects lysosomal morphology and function, autophagy and cilium biogenesis.

By mediating proton transport in intracellular compartments, V-ATPases play a central role in regulating electrochemical gradients between membranes, which is crucial in a variety of cellular processes. In addition, V-ATPases might also localize at the plasma membrane of a number of cells, mediating their specialized functions, including

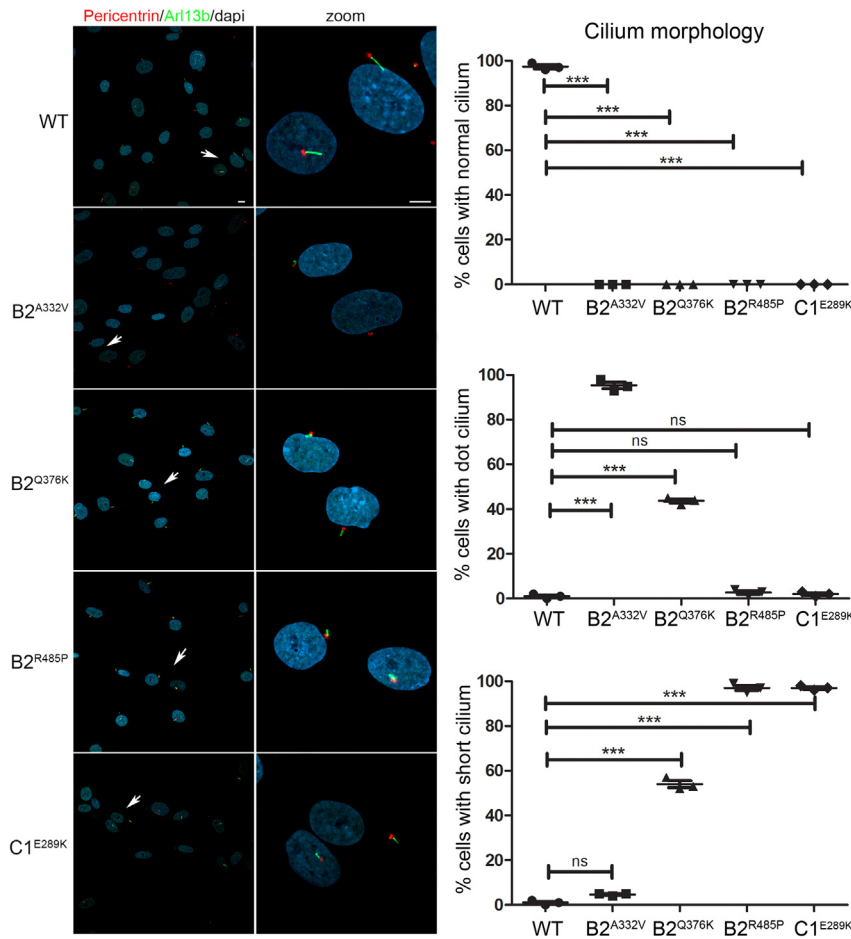


Figure 7. *ATP6V1B2* and *ATP6V1C1* variants are associated with an aberrant primary cilium morphology

Representative confocal images showing altered primary cilium morphology in patients' fibroblasts compared with control cells. Specifically, cilia with altered morphology (either short or characterized by a basal body in absence of any visible cilium [dot cilium], zoomed images) were invariably observed in fibroblasts heterozygous for the all variants studied. Primary cilia are labeled with ARL13B (green), basal bodies and nuclei are labeled with pericentrin (red) and DAPI (blue), respectively. Scale bars, 10 μ m (left) and 2 μ m (right). Cells were analyzed for each line over three independent experiments (50 cells/line each) for a total of 150 cells/line scored. *p* values were calculated by one-way ANOVA with Tukey's correction for multiple testing. Graph bars show mean \pm SEM.

renal acidification, bone resorption, homeostasis of cytoplasmic pH and sperm maturation.⁴ V-ATPase functional pleiotropy, the tissue/cell-specific expression pattern of the different subunits, as well as the gain-of-function (GoF) vs. LoF effect of individual disease-causing variants explain the striking phenotypic heterogeneity associated with dysregulated V-ATPase function. Exemplarily, biallelic LoF variants in *ATP6VOA4* cause a form of renal tubular acidosis,³⁸ which is characterized by a derangement of acid-base balance due to an inability to secrete acid in the distal nephron. The physiological impact of these LoF variants is related to the specific expression of the encoded accessory subunits at the level of the proton-secreting cells in the distal nephron.³⁸ A strikingly different clinical phenotype (neurodevelopmental disorder with epilepsy and brain atrophy [MIM: 619971]) is associated with biallelic LoF variants in *ATP6VOA1*,³⁹ which encodes a different "a" subunit of the V_O subcomplex that is highly expressed in the central nervous system. Remarkably, dominantly acting variants in the same gene causing LoF of the pump have been reported to underlie a severe form of developmental and epileptic encephalopathy (MIM: 619970).³⁹ In the present series, the multisystemic involvement of the phenotypic spectrum resulting from the dysregulated function of the B2 and C1 subunits is in line with the wide expression pattern of both sub-

units. Of note, two distinct classes of dominant *ATP6V1A* mutations were recently identified to cause a developmental encephalopathy with distinct pathomechanisms implicating both GoF and LoF converging to an altered lysosomal homeostasis.⁴⁰ Our findings provide a new example of clinical phenotypes resulting from GoF mutations enhancing V-ATPase function.

Pathogenic dominantly acting variants in *ATP6V1B2* have been associated with different phenotypes clinically fitting DOORS and DDOD syndromes, and ZLS, indicating that these conditions represent a variable expression of a single disorder caused by altered V-ATPase function (Table 1). Specifically, individuals with DDOD syndrome primarily share sensorineural deafness and onychodystrophy with digital anomalies characterized by bulbous fingertips of digits, and finger-like thumbs/triphalangeal thumb. Some affected individuals may also show tooth abnormalities (e.g., oligodontia, conical teeth, and late dentition). DOORS syndrome shares sensorineural hearing loss, onychodystrophy, and digital abnormalities (i.e., triphalangeal thumb and hypoplastic/absent distal phalanges) with DDOD syndrome. Osteodystrophy is reported in most cases as well as a neurodevelopmental/neurological involvement, which includes seizures. Facial dysmorphisms are also reported (e.g., bitemporal narrowing, coarse facies, ear abnormalities, broad nasal bridge, large nose, bulbous nasal tip, anteverted nares, long philtrum, thick everted lower lip, downturned corners of the mouth, and high-arched palate). Facial features of DOORS syndrome have a significant overlap with ZLS, a condition also associated with organomegaly, gingival hypertrophy, and hypertrichosis, which are not usually reported in DDOD and DOORS syndromes. ZLS is more frequently

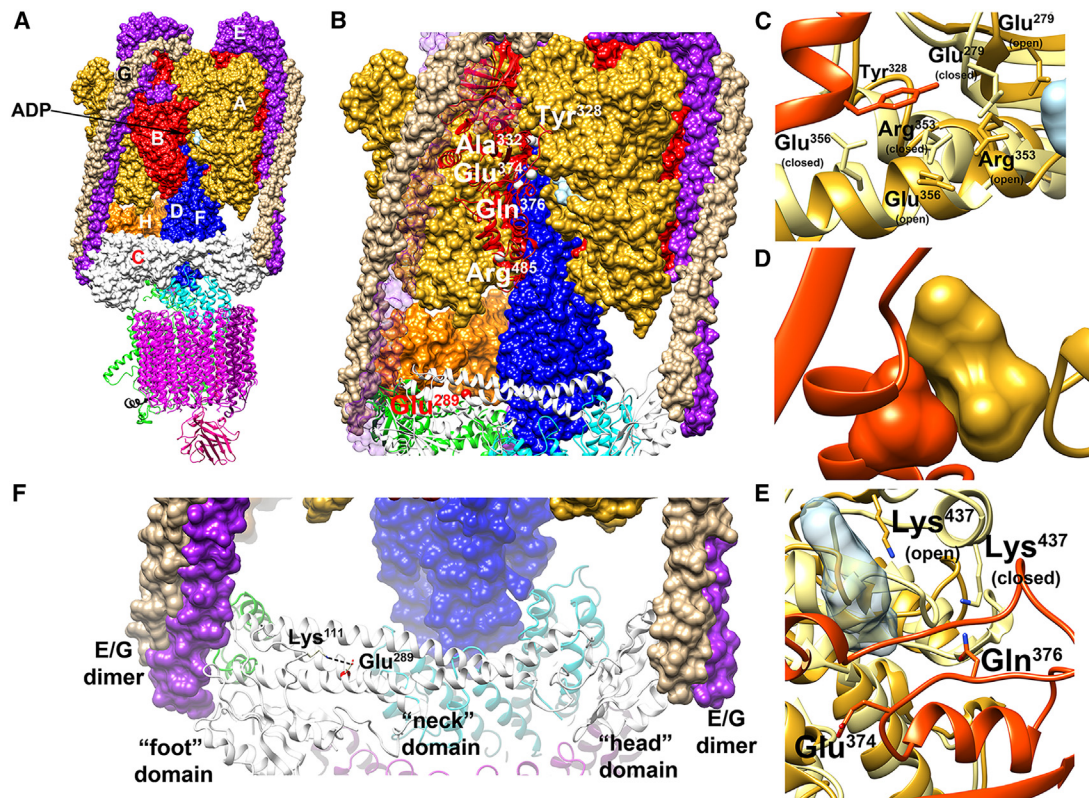


Figure 8. Location of the mutated residues of the B2 and C1 subunits in the cryo-EM structures of human V-ATPase

(A) Side view of the of human V-ATPase complex (PDB: 6wm2). The subunits belonging to the V_1 and V_O regions are reported as surface and ribbon, respectively. The B and C subunits in the V_1 region are colored red and white, respectively. ADP is reported as a light blue surface in the V_1 region at the interface between the A and B subunits.

(B) The V-ATPase complex in the region containing the residues discussed in the present study. The C subunit and one of the three B subunits are reported as ribbons. The C_α atoms of the muted residues are reported as white and red spheres for the B and C subunits, respectively. For the sake of clarity, one of the E subunits is not reported.

(C) p.Tyr328Cys and p.Tyr328His substitutions (B2 subunit). The helix tract in the B subunit comprising Tyr³²⁸ is reported as a red ribbon (chain ζ), Tyr³²⁸ is represented as red sticks. Two different conformations of the A subunit are reported as ribbons, corresponding to the closed (chain β , light yellow) and open (chain γ , gold) states, respectively. The residues in the A subunit in contact with Tyr³²⁸ (minimum distance less than 0.5 nm) and populating different conformations in the two states are indicated and reported as sticks.

(D) p.Ala332Val substitution (B2 subunit). Sketch of the interface comprising Ala³³² in one of the three interfaces (chains γ/ζ in the PDB file); in the sketch, Ala³³² (in the B subunit) and Ser³¹⁶ and Asn³¹⁷ (in the A subunit) are reported as red and gold surfaces, respectively.

(E) p.Glu374Gln, p.Gln376Arg and p.Gln376Lys substitutions (B2 subunit). Interfaces between the A and B subunits in the region surrounding Gln³⁷⁶ and Glu³⁷⁴ in the B subunits from the 6wm2 structure. The backbone of the strand comprising Glu³⁷⁴ and Gln³⁷⁶ is reported as red ribbon; Glu³⁷⁴ and Gln³⁷⁶ are represented as sticks. Two different conformations of the A subunit are reported as ribbons, corresponding to the closed (chain β , light yellow) and open (chain γ , gold) states, respectively. Lys⁴³⁷ in chain A is reported as sticks and colored by atoms, with the carbons colored as the corresponding ribbon. The ADP molecule in the γ chain is reported as a semi-transparent light blue surface.

(F) p.Glu289Lys substitution (C1 subunit). The C subunit is reported as a white ribbon. Glu²⁸⁹ is reported as sticks, and the backbone is colored red. Lys¹¹¹ is also reported as white sticks.

caused by GoF variants in the potassium transporters KCNH1 and KCNN3,^{13,41} which are not related to lysosomal acidification; the different pathways involved might explain phenotypic differences. The present findings further support these observations, documenting the presence of a phenotypic continuum among these disorders. Notwithstanding the relatively small number of affected individuals reported thus far, first genotype-phenotype correlations are emerging. Specifically, sensorineural hearing loss and onychodystrophy appear to invariably occur in individuals carrying the recurrent truncating variant (p.Arg506*) in *ATP6V1B2*, with or without neurodevelopmental involvement. Moreover, the mutation cluster

affecting residues located close to the ADP/ATP binding site (Gly³²⁵, Tyr³²⁸, Ala³³², Glu³⁷⁴, Gln³⁷⁶, and Leu³⁹⁸) seems to be associated with a phenotype principally characterized by DD affecting both cognitive and motor functions, ID, seizures and distinctive facial features (high forehead, bitemporal narrowing, coarse face, bushy and straight highbrows, deep and short philtrum, prominent lower vermillion, and large mouth), with onychodystrophy, gingival overgrowth, and hypertrichosis affecting just a minority of affected individuals. A subset of individuals shows brain abnormalities (e.g., brain atrophy and several degree of myelination delay). Consistent with these findings, the clinical features observed in the subject with

the p.Glu289Lys substitution in ATP6V1C1 show a substantial overlap with DOORS syndrome, further supporting the occurrence of a phenotypic continuum characterizing DOORS syndrome, DDOD syndrome, and ZLS, which should be considered as a single clinically variable phenotypic spectrum/nosologic entity.

In conclusion, we show a continuum in the clinical spectrum associated with dominant variants affecting the ATP6V1C1 and ATP6V1B2 subunits causing upregulation of the V-ATPase function. Notwithstanding the multiple processes that are altered in cells expressing these variants, increased lysosomal acidification seems to be the driver event of such pleiotropy, indicating that these disorders can be considered as lysosomal diseases. These findings provide a rationale for the use of molecules targeting the upregulated V-ATPase function to ameliorate evolutive features in these subjects.

Data and code availability

WES datasets have not been deposited in a public repository due to privacy and ethical restrictions but will be made available on request. The *ATP6V1C1* c.865G>A (p.Glu289Lys; NM_001695.5) has been submitted to ClinVar (SCV004814160).

Acknowledgments

The authors thank the participating families, Kerstin Kutsche (University Medical Center Hamburg-Eppendorf, Hamburg, Germany) for providing primary fibroblasts carrying the heterozygous ATP6V1B2 Arg485Pro amino acid substitution, and Serenella Venanzi (Istituto Superiore di Sanità, Rome) for technical assistance. This work was supported by the Italian Ministry of Health (RCR-2022-23682289 and PNRR-MR1-2022-12376811 to M.T.), Italian Ministry of University and Research (PNRR_M4C2I1.3-HEAL ITALIA PE 00000019 to L.S., FOE_2020 to M.T.), and Fondazione Bambino Gesù (Vite Coraggiose), and the Telethon Undiagnosed Diseases Program (GSP15001). J.S.C., A.H., and C.S. receive support from the NIH National Institute of Child Health and Human Development (P50 HD103538). J.S.C. additionally receives support from the NIH National Institute of Neurologic Disorders and Stroke (1U24NS131172-01) and U.S. Department of Health and Human Services, Health Resources and Services Administration (MCH T7317245). This work has been carried out in the frame of the ERN-ITHACA research activities.

Declaration of interests

The authors declare no competing interests.

Supplemental information

Supplemental information can be found online at <https://doi.org/10.1016/j.xhgg.2024.100349>.

Web Resources

Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org/>;

gnomAD, <http://gnomad.broadinstitute.org>;
ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>;
MetaDome, <https://stuart.radboudumc.nl/metadome/>;
GeneMatcher, <https://genematcher.org/>;
CADD, <https://cadd.gs.washington.edu/>;
M-CAP, <http://bejerano.stanford.edu/mcap/>;
InterVar, <http://wintervar.wglab.org>.

Received: June 6, 2024

Accepted: August 27, 2024

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Supplemental information

Dominantly acting variants in *ATP6V1C1* and *ATP6V1B2* cause a multisystem phenotypic spectrum by altering lysosomal and/or autophagosome function

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Clinical reports

Figure S1. MetaDome analyses and multiple protein sequence alignments for ATPase H⁺ transporting V1 subunits.

Figure S2. *ATP6V1C1* variant validation and segregation analysis.

Figure S3. Disease-associated *ATP6V1B2/ATP6V1C1* variants do not affect B2 and C1 proteins stability.

Figure S4. Pearson correlation coefficient of Lamp1 co-localization with B2 subunits.

Figure S5. Variants identified in *ATP6V1B2* gene promote an altered autophagic flux.

Figure S6. Pearson correlation coefficient of Lamp1 co-localization with LC3I/II.

Figure S7. Analysis of autophagic flux with DQ-BSA.

Table S1. Exome sequencing statistics and data output (Subject 1).

Table S2. *In silico* prediction scores for the identified *ATP6V1C1* and *ATP6V1B2* variants.

Clinical reports

Subject 1 (ATP6V1C1, p.Glu289Lys)

The 8-year-old girl is the daughter of unrelated parents; the child's father has nail agenesis of the feet and nail hypoplasia of the hands, triphalangeal thumb. She was born at 37 weeks of pregnancy complicated by placental abruption in the first month and oligohydramnios. The mother denies taking medication during pregnancy. At birth her weight was 3120 g (74th centile, +0.64 SD), length 50 cm (87th centile; + 1.12 SD), OFC 32.5 cm (25th centile, -0.50 SD), Apgar score 1'9 and 5'10, she also presented right microtia and agenesis of all fingernails and toenails.

Psychomotor development was delayed with sitting position acquired at 2 years of age, standing position at 2.5 years of age, no language acquired. The child presents hyperactivity and her nonverbal IQ, tested at age 7 years, was 58, consistent with mild intellectual disability. The girl has profound sensorineural deafness, and a cochlear implant was placed at 4 years of age. CT scan of the petrous spools showed atresia auris, associated with malformation of the posterior labyrinth (hypoplasia of the right lateral semicircular canal, bilateral agenesis of the crus commune) with normal cochlae and acoustic nerves. She also presented with a distinctive nose with broad nasal bridge, bifid nasal tip, broad columella, a groove between tip and alae of the nose, deep philtrum and thin upper lip. Similar nasal features were, in part, present also in the father.

Brain MRI showed an area of inhomogeneous signal alteration with "target" appearance (variably hyperintense in T2w images, hypointense in T1w images, and weakly hypointense with major AP axis with epicenter in the left frontal radiation of the corpus callosum extending anteriorly to affect the white matter of the ipsilateral I and II frontal gyri). There were no other signal changes in the remaining brain, cerebellar and brainstem parenchyma. Ventricular system within limits. No obvious areas of endocranial pathologic impregnation after mdc. No expansions in the cisternae of the ponto-cerebellar angles. The most likely etiologic hypothesis is a leukopathic focus (Von Balo type) in chronic phase, alternatively a low-grade heteroplastic formation (but against this hypothesis the MRS showing a finding of normality) or dysplasia (against this hypothesis the absence of adjacent cortical changes). Although the child never presented with seizures, she performed EEG that showed altered brain electrical activity due to the presence of epileptiform abnormalities prevalent at the parietotemporal center that are activated during sleep.

The growth parameters recorded at age 8 years were: weight 21.1 kg (9th centile, -1.27 SD); height 117 cm (2nd centile, -1.89 SD) OFC 49.9 cm (8th centile, -1.37 SD).

At the last clinical evaluation, the following was appreciated: grade 3 microtia according to Mauerman on the right side with atresia auris, telecanthus, wide nasal bridge, nose with a widened tip and bifid at the level of the passage with the columella, groove between the tip and nasal nostrils, well-defined philtrum, thin upper lip, nail agenesis on all fingers and toes, finger-like toes, hypoplasia of the distal phalanx of the big toe.

Subject 2 (ATP6V1B2, p.Ala332Val)

The patient, a male, was the first child of a healthy French-Chinese couple. Polyhydramnios was discovered during the pregnancy. The patient was born at 41 WA with a birth weight of 3400 g (36th centile, -0.36 SD), height 51 cm (49th centile, -0.02 SD). At the 10th hour of life, he had seizures and was referred to the neonatal intensive care unit. Seizures were frequent during the first months of life, mainly tonic, evolving to infantile epileptic spasm syndrome associated with a developmental regression, at 3 months. Seizures stopped at 6 months and anti-epileptic drugs were being withdrawn at the time of the consultation.

The child had feeding difficulties from birth, needed a feeding tube until the age of 6 months-old. He never achieved sitting up, never spoke and did not use his hand. His eye contact was poor, he had a horizontal nystagmus. Fundus oculi and electroretinogram were normal.

At examination, the patient was 3.5 years-old, he had a severe scoliosis, thoracic deformation due to scoliosis, clenched fists with thumbs in hands. His weight was 10.3 kg (<3rd centile, -4.0 SD), height 90 cm (<3rd centile, -2.2 SD), OFC 46 cm (<3rd centile, -3.0 SD). Marked amyotrophy, blue sclerae, gingival hypertrophy and high-arched palate were noted. The patient died at 3 years 8 months.

Subject 3 (ATP6V1B2, p.Tyr328His)

The patient is a 9-year-old female. She was the product of a normal pregnancy with an uncomplicated birth history. At 3 months of age, she developed infantile spasms which were difficult to treat and progressed to intractable epilepsy. Seizures management has included multiple antiseizure medications, vagal nerve stimulator, and the ketogenic diet which decreases seizure frequency but she still has daily seizures. Her brain MRI at 23 months demonstrated volume loss and white matter T2 hyperintensity.

She experienced a developmental regression at 4 months of age and has continued to have global delays. She smiled at 2 months but did not roll over until after her first birthday. At age 5, she was able to sit without support and learned to kneel while grabbing onto objects at age 8. She currently uses her whole hand to rake objects and recently started putting her hands in her mouth. She is non-verbal and does not understand commands. She communicates by smiling, laughing, groaning, and crying. She does show preference for some toys and recognizes parents. She does not track objects due to cortical visual impairments. She has not had an audiology exam but responds to sounds.

She had a normal cardiac echocardiogram and a normal abdominal ultrasound. She was diagnosed with obstructive sleep apnea requiring CPAP support initially and is currently requiring just 0.5 L O₂ at night. She has a history of chronic lung disease due to aspiration history and bronchiectasis due to poor airway clearance. She required G-tube placement as an infant due to aspiration and does not feed orally. She also developed precocious puberty with onset of pubertal development at age 7 thought to be related to her seizure and medication history.

Subject 4 (ATP6V1B2, p.Tyr328Cys)

The patient, a female, born after uneventful twin pregnancy of a healthy German couple. The patient was born at 37+3 WA with a birth weight of 2720 g (36th percentile, -0.36 SD), height 47 cm (32nd percentile; -0.46 SD), OFC 35 cm (94th centile, +1.53 SD). Perinatal period was complicated by feeding difficulties. She experienced neonatal hypotonia and global developmental delay. Seizures onset in childhood and treated with valproate.

At examination, the patient was 2 years-old, she had craniofacial dysmorphisms (*i.e.*, high forehead, dolichocephaly, bitemporal narrowing, straight eyebrows, long eyelashes, short philtrum, slight micrognathia and posteriorly rotated ears), late dentition and pes planus with extreme pes valgus. No onychodystrophy nor digital abnormalities and no obvious deafness was reported but narrow auditory canals.

Subject 5 (ATP6V1B2, p.Gln376Lys)

The patient is a female born at term of gestation to healthy non-consanguineous parents. At birth she had jaundice treated with phototherapy but otherwise normal perinatal events. At 3 months of age, she did not have eye tracking and at 5 months she was noted to have nystagmus. At 7 months of age, she developed infantile spasms that were treated with ACTH and valproate. She was also noted to have hypotonia and she was not able to sit independently by 2 years of age. At 7 months she performed a brain MRI that showed severe hypomyelination and a repeated MRI at 4 years showed mild improvement in myelination. She acquired the sitting and standing positions, but she has not acquired independent walking up to the age of 4 years and 7 months. She developed few episodes of generalized seizures.

She performed extensive testing including standard chromosome analysis, array CGH and metabolic screening tests with plasma amino acids, VLCFA, acylcarnitine profile, urinary organic acids, urinary glycosaminoglycans, very long fatty acids (VLCFA), enzyme assays for galactocerebrosidase and arylsulfatase A (ARSA) that were all unremarkable. Sequencing of a panel of leukodystrophy genes was also negative.

At her last examination at the age of 4 years and 7 months, her weight was 13.9 Kg (5th centile, -1.68 SD), length 100 cm (15th centile, -1.06 SD) and head circumference 46.8 cm (<3rd centile, -2.5 SD). She was noted to have sloping forehead, brushy and straight eyebrows with synophrys, but neither gingival hypertrophy nor nail dystrophy were noted. She had generalized hypotonia.

Subject 6 (ATP6V1B2, p.Gln376Arg)

The patient, a 13-year-old male, was born at term (39 weeks 4 days) following a pregnancy complicated by polyhydramnios. He was delivered by Cesarean section due to fetal decelerations and breech presentation. Birth weight was 2790 g (4th centile, -1.71 SD). APGAR scores were 6 and 8 at 1 and 5 minutes. At birth he was apneic, hypertonic, and noted to have severe micro/retrognathia. His neonatal course was complicated by airway and swallowing difficulties, glossoptosis, tone abnormalities, and proximal humerus fracture. He had tracheostomy, Nissen

fundoplication, and gastrostomy during his 2 month NICU stay. He developed infantile spasms at 3 months, which resolved after 1 month of steroid treatment.

He had history of global developmental delay, nystagmus, undescended testes, and otitis media in childhood. He had mandibular distraction at approximately 3 years of age. Brain MRI at approximately 3.5 years of age revealed thin corpus callosum, diffusely reduced cerebral white matter, enlarged ventricles, and prominent sulci. He developed multifocal epilepsy at the age of 8 years. He has dysmorphic craniofacial features, coarse hair, misaligned teeth, short neck, and scoliosis. He is currently G-tube dependent and incontinent. He is non-verbal and non-ambulatory and has spastic quadriplegia cerebral palsy (GMFCS-V) and profound intellectual disability.

Subject 7 (ATP6V1B2, p.Glu374Gln)

The male individual was born after uncomplicated pregnancy at term (APGAR 10/10/10). He was the first child of healthy non-consanguineous parents. At birth his weight was 3460 g (49th centile, -0.02 SD), length 50cm (34th centile, -0.40 SD), and his OFC 33 cm (7th centile, -1.51 SD). Psychomotor development was delayed with sitting age of 2 years without muscular hypotonia. He was able to speak simple words, but not forming sentences. Speech comprehension was reported to be in the lower normal range.

At age 14 years and 8 months, when first presented, he showed pronounced microcephaly (49 cm, <3rd centile, -4.60 SD) with otherwise auxometric parameters in the normal range (height: 164 cm, 25th centile, -0.52 SD; weight: 43.8 kg, 10th centile, -1.27 SD). At that age, starting with puberty, he developed hyperactive, aggressive behavior. Beside a mild scoliosis and a tendency to vomiting, no organic abnormalities were observed. He was noted to have dolichocephaly, mild bitemporal narrowing, a short philtrum, everted upper lip and a large mouth.

Generalized seizure were first noted at 13 years and successfully treated with oxcarbazepine. Brain MRI scan at age 14 years was normal.

Supplemental Figures

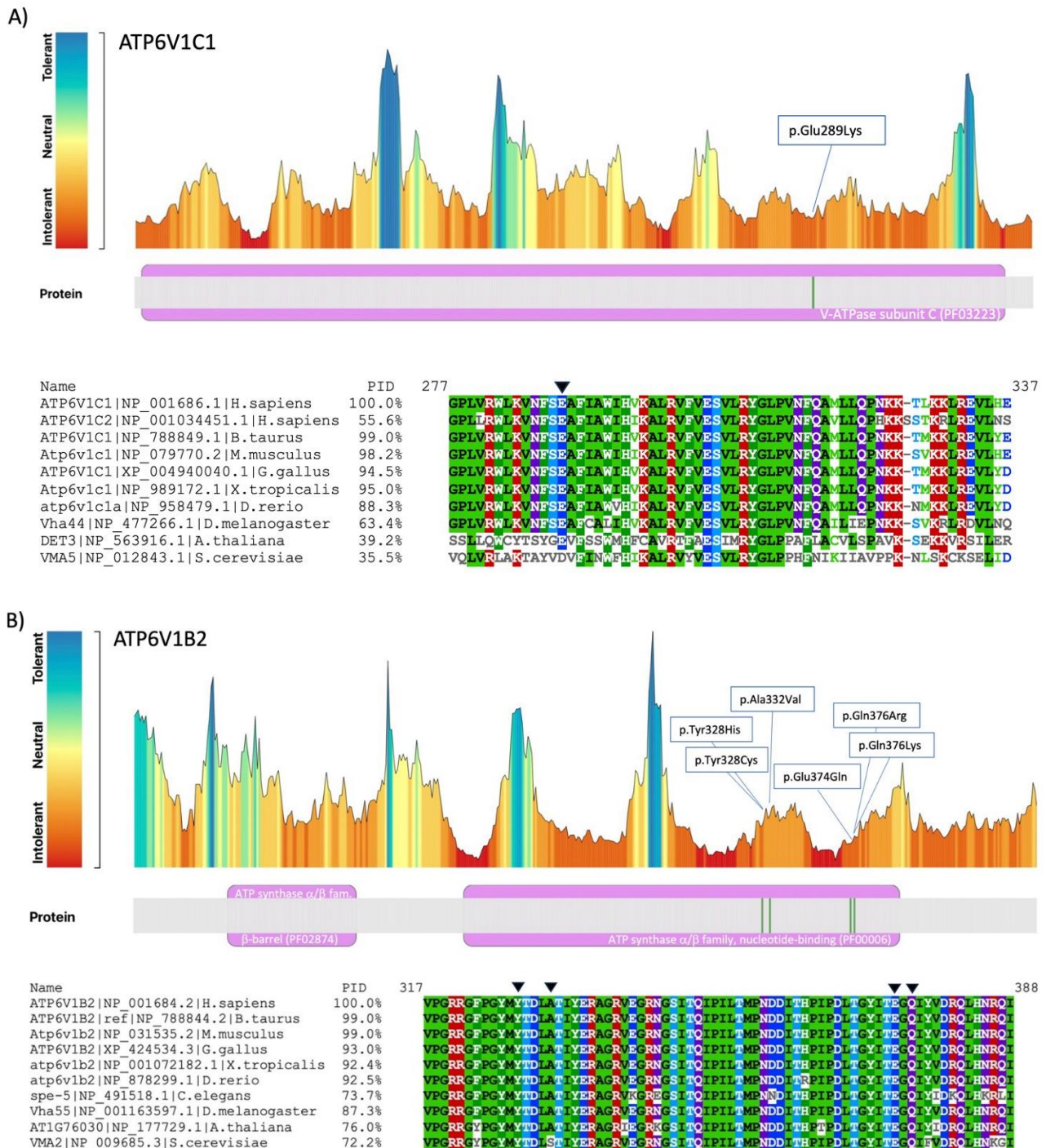


Figure S1. MetaDome analyses and multiple protein sequence alignments for ATPase H⁺ transporting V1 subunits. (A) MetaDome tolerance landscape for ATP6V1C1 (top), and protein multiple alignment for ATPV1C1 homologs (bottom). (B) MetaDome tolerance landscape for ATP6V1B2 (top) and protein multiple alignment for ATPV1B2 homologs (bottom). Protein sequences from ATP6V1C1, ATP6V1B2, and their orthologs were obtained from NCBI database (HomoloGene), aligned by means of Muscle (v. 3.8) and visualized with MView (v.1.63). Background in consensus positions (identity > 70%) was colored according to amino acid biochemical properties. PID=percent of identity. Variant positions are highlighted by black arrows.

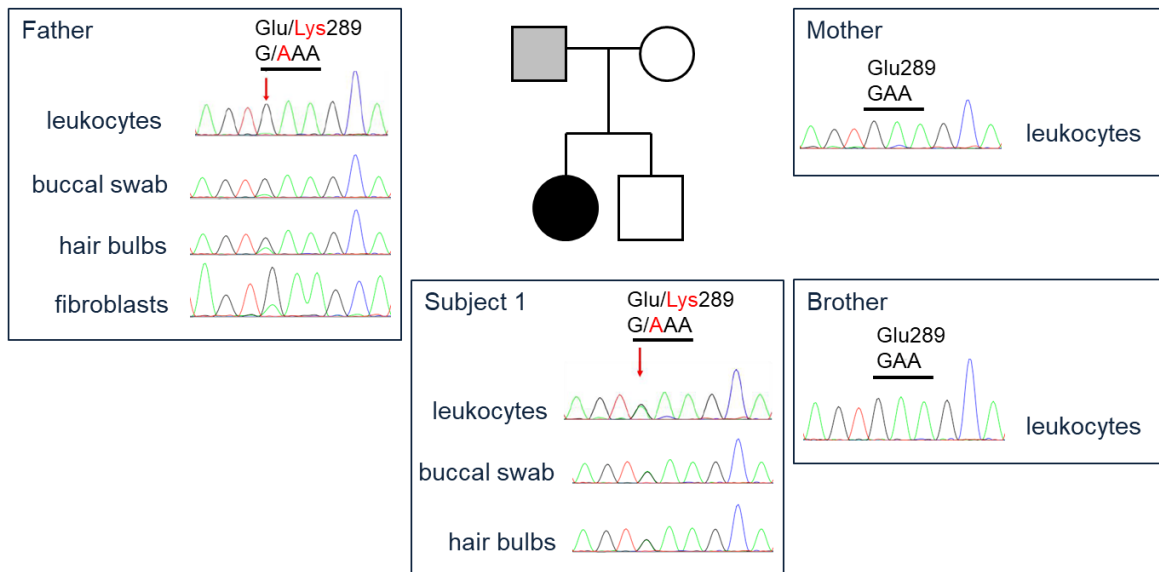
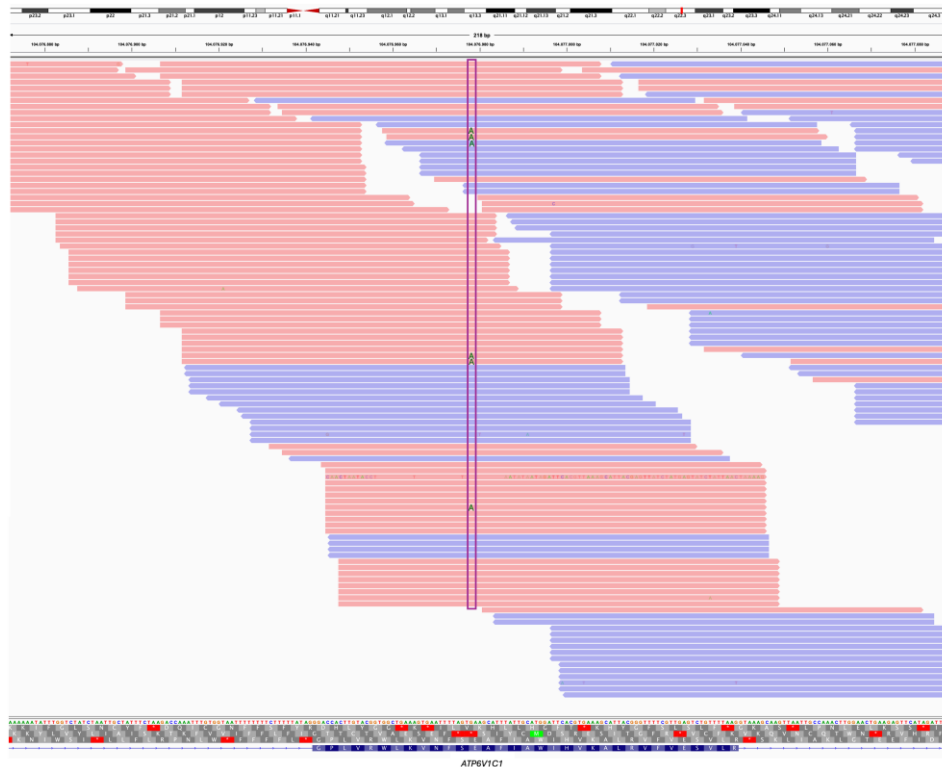
A**B**

Figure S2. *ATP6V1C1* variant validation and segregation analysis. (A) The heterozygous occurrence of the c.865G>A substitution (p.Glu289Lys) in Subject 1 was validated *via* Sanger sequencing using different tissues/cell types. The variant is not observed in leukocytes from the healthy brother and mother, while was detected with a low level of mosaicism in paternal hair bulbs, fibroblasts, and mucosal epithelial cells, supporting its postzygotic onset. (B) Reads alignment of the relevant coding region (chr8:104,076,872-104,077,089; hg19) obtained from WES is shown. Position of the variant (NM_001695.5:c.865G>A) is highlighted by the purple frame. While GATK's HaplotypeCaller did not correctly call the variant, a low-level mosaicism is visible (7%, 6 reads out of 87). Reads colored in red are mapped on the forward strand, those mapped on the reverse strand are in blue.

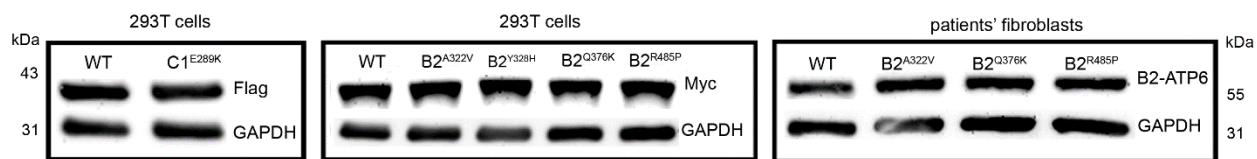


Figure S3. Disease-associated *ATP6V1B2/ATP6V1C1* variants do not affect B2 and C1 proteins stability. Western blot (WB) analysis showing comparable level of B2 and C1 proteins in transiently transfected 293T cells and primary fibroblasts from affected individuals and control cells (WT). Membranes were immunoblotted with a mouse monoclonal anti-Flag or anti mouse monoclonal Myc antibodies for the transfected cells and with a rabbit polyclonal anti ATP6V1B2 antibody for the fibroblasts. For protein normalization was used the mouse monoclonal GAPDH antibody.

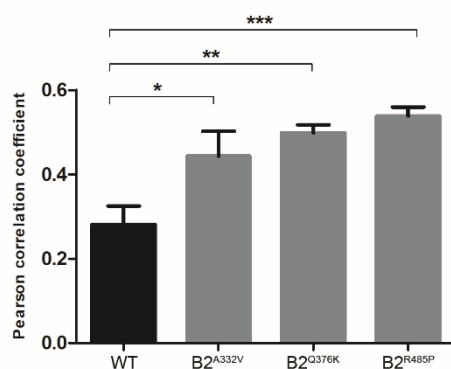


Figure S4. Pearson correlation coefficient of Lamp1 co-localization with B2 subunits. The coefficient was calculated using the Zen 3.3 software. Histograms indicate mean \pm SEM; p values were calculated by One way ANOVA with Tukey's correction for multiple testing.

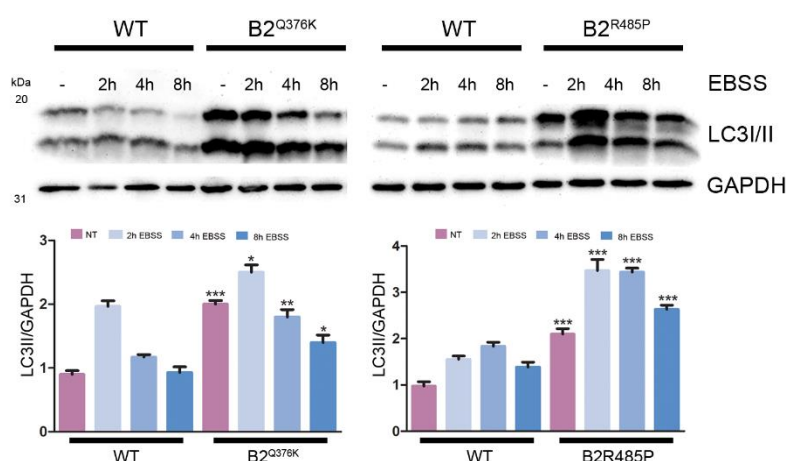


Figure S5. Variants identified in *ATP6V1B2* gene promote an altered autophagic flux. WB analyses performed on patients' fibroblasts carrying the ATP6V1B2^{Q376K/R485P} variants confirm the data obtained with immunofluorescence showing an anomalous accumulation of LC3I/II in the steady state condition and after autophagic flux induction with EBSS for 2, 4 and 8 h. Equal amounts of cell lysates were resolved by 15% polyacrylamide gel electrophoresis. Membranes were probed with an anti-LC3I/II antibody and then re-probed with an anti-GAPDH antibody for data normalization. Non-treated cells (*i.e.*, cells cultured in steady state conditions) were reported as internal controls (-).

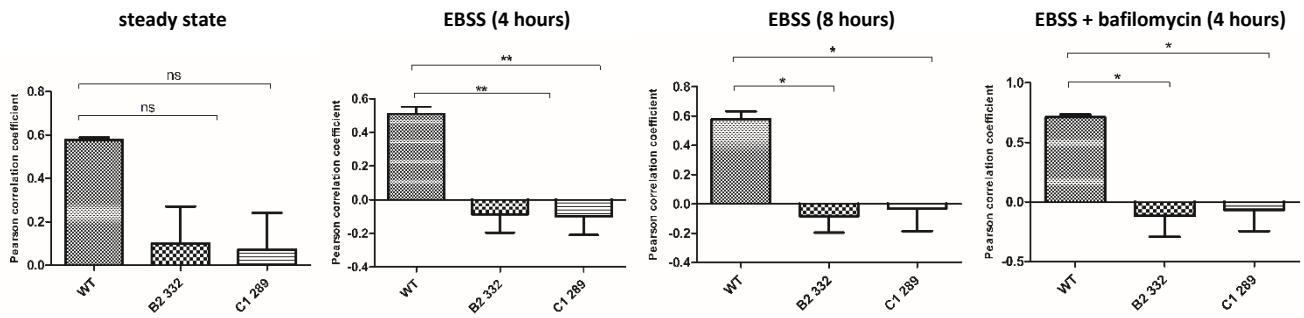


Figure S6. Pearson correlation coefficient of Lamp1 co-localization with LC3I/II. The coefficient was calculated using the Zen 3.3 software and relative histograms are reported. Histograms indicate mean \pm SEM, p values were calculated by One way ANOVA with Tukey's correction for multiple testing.

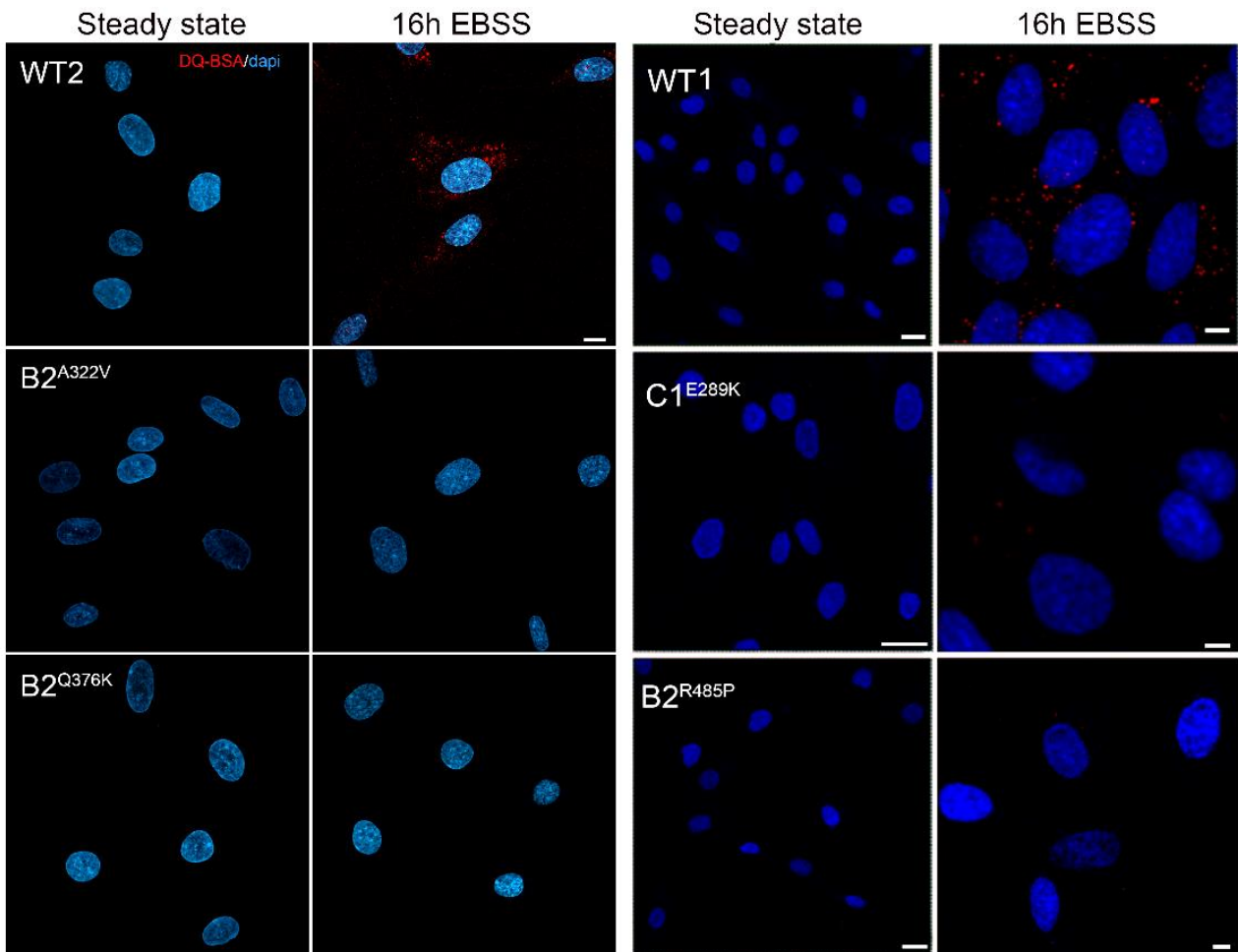


Figure S7. Analysis of autophagic flux with DQ-BSA. Primary fibroblasts were incubated with bovine serum albumin derivative (dye-conjugated [DQ] BSA) conjugated to a self-quenched fluorophore (10 μ g/ml) for 1 h at 37 $^{\circ}$ C in complete culture medium and then left in a starvation medium for 16 h to induce autophagy. Cells were mounted on coverslips and immediately analyzed by confocal microscopy. Dequenched DQ-BSA was not observed over the time period of 16 h in patient cells. Scale bars are 20 μ m and 5 μ m (zoom) (left) and 10 μ m and 5 μ m (right).

Table S1. Exome sequencing statistics and data output (Subject 1).

	Subject 1
Enrichment kit	SureSelect AllExon V5
Total reads	87,453,409
Target regions coverage >10x	96.8%
Target regions coverage >20x	93.5%
Average sequencing depth on target	110x
Private/low frequency variants with effect on CDS or affecting splice region	272
Putative disease genes (Autosomal Dominant trait) ²	3 ³
- candidate genes	1, <i>ATP6V1C1</i>
	3 ⁴
Putative disease genes (Autosomal Recessive trait) ²	0
- candidate genes	

¹High-quality non-synonymous single nucleotide variants plus indels within coding exons and splice regions (-3/+8): functionally relevant variants and either unknown, private or low frequency variants are reported (gnomAD MAF<0.1% and recurrency <1% within our ~3,000 exomes database).

²Functional impact assessed by Combined Annotation Dependent Depletion (CADD) v.1.6 (<http://cadd.gs.washington.edu/>), Mendelian Clinically Applicable Pathogenicity (M-CAP) v.1.3 (<http://bejerano.stanford.edu/mcap/>) and InterVar (<http://wintervar.wglab.org>) v2.0.1. Variants predicted as benign or likely benign by InterVar were discarded and only those with CADD score>15 or M-CAP score>0.025 were retained.

³*ATP6V1C1* (c.865G>A; p.Glu289Lys), *TMEM247* (c.380G>T; p.Arg127Leu); *TMEM66* (c.280-4A>C)

⁴*DNAH11* (c.5665T>A; p.Ser1889Thr, c.12691A>G; p.Thr4231Ala), *KIAA1875* (c.982G>A; p.Val328Met, c.2081-5G>A), *SERPINA10* (c.580C>T; p.Arg194Cys, c.367G>; p.Gly123Arg)

Table S2. *In silico* prediction scores for the identified *ATP6V1C1* and *ATP6V1B2* variants.

Variant	gnomAD	CADD	M-CAP	REVEL	SIFT	PP2 (HumDiv/HumVar)
<i>ATP6V1C1</i> (NM_001695.5): c.865G>A (p.Glu289Lys)	n.r.	32.0	0.092	0.71	0.001	0.95/0.66
<i>ATP6V1B2</i> (NM_001693.4): c.982T>C (p.Tyr328His)	n.r.	29.5	0.294	0.93	0	1/0.99
<i>ATP6V1B2</i> (NM_001693.4): c.983A>G (p.Tyr328Cys)	n.r.	30.0	0.375	0.93	0.009	1/1
<i>ATP6V1B2</i> (NM_001693.4): c.995C>T (p.Ala332Val)	n.r.	29.0	0.358	0.89	0.001	0.95/0.67
<i>ATP6V1B2</i> (NM_001693.4): c.1120G>C (p.Glu374Gln)	n.r.	29.6	0.132	0.75	0	1/0.94
<i>ATP6V1B2</i> (NM_001693.4): c.1126C>A (p.Gln376Lys)	n.r.	25.0	0.481	0.85	0	0.28/0.2
<i>ATP6V1B2</i> (NM_001693.4): c.1127A>G (p.Gln376Arg)	n.r.	29.1	0.456	0.89	0	0.96/0.88