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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

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 (74^{th} centile, +0.64 SD), length 50 cm (87^{th} centile; + 1.12 SD), OFC 32.5 cm (25^{th} 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 gyrus). 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 heteroplasic 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 (9^{th} centile, -1.27 SD); height 117 cm (2^{nd} centile, -1.89 SD) OFC 49.9 cm (8^{th} 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 (36^{th} centile, -0.36 SD), height 51 cm (49^{th} centile, -0.02 SD). At the 10^{th} 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 ($<3^{rd}$ centile, -4.0 SD), height 90 cm ($<3^{rd}$ centile, -2.2 SD), OFC 46 cm ($<3^{rd}$ 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 spams 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 (49^{th} centile, -0.02 SD), length 50cm (34^{th} centile, -0.40 SD), and his OFC 33 cm (7^{th} 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, $<3^{rd}$ centile, -4.60 SD) with otherwise auxometric parameters in the normal range (height: 164 cm, 25^{th} centile, -0.52 SD; weight: 43.8 kg, 10^{th} 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

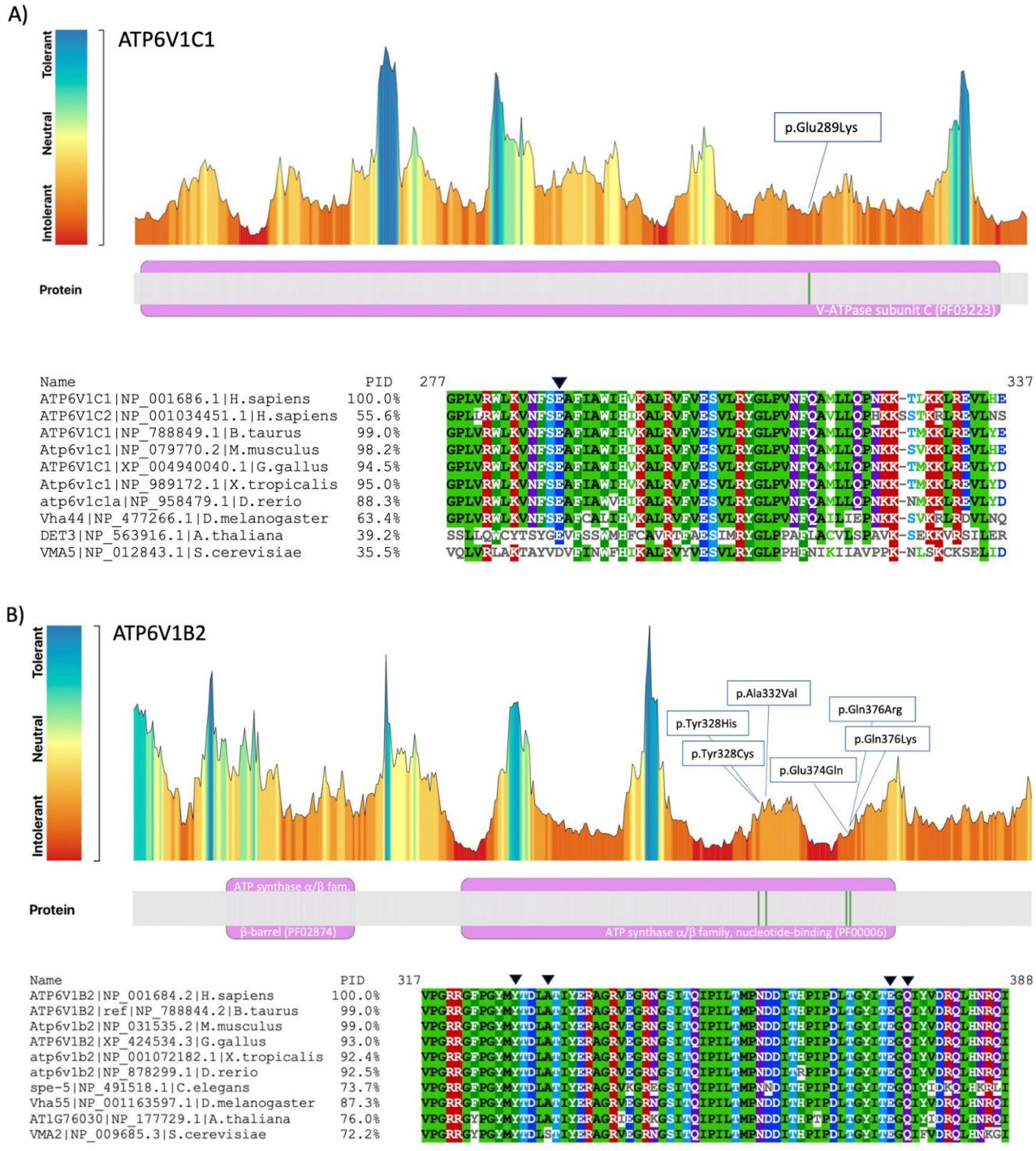
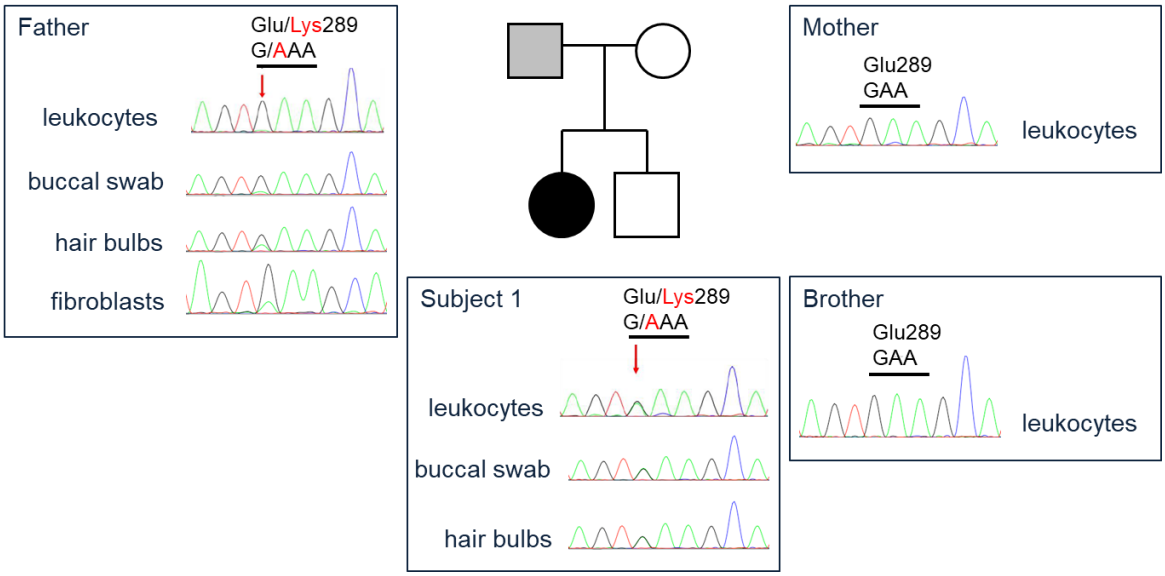


Figure S1. MetaDome analyses and multiple protein sequence alignments for ATPase H+ transporting V1 subunits. (A) MetaDome tolerance landscape for ATP6V1C1 (top), and protein multiple alignment for *ATPV1C1* homologs (bottom). (B) MetaDome tolerance landscape for ATP6V1B2 (top) and protein multiple alignment 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



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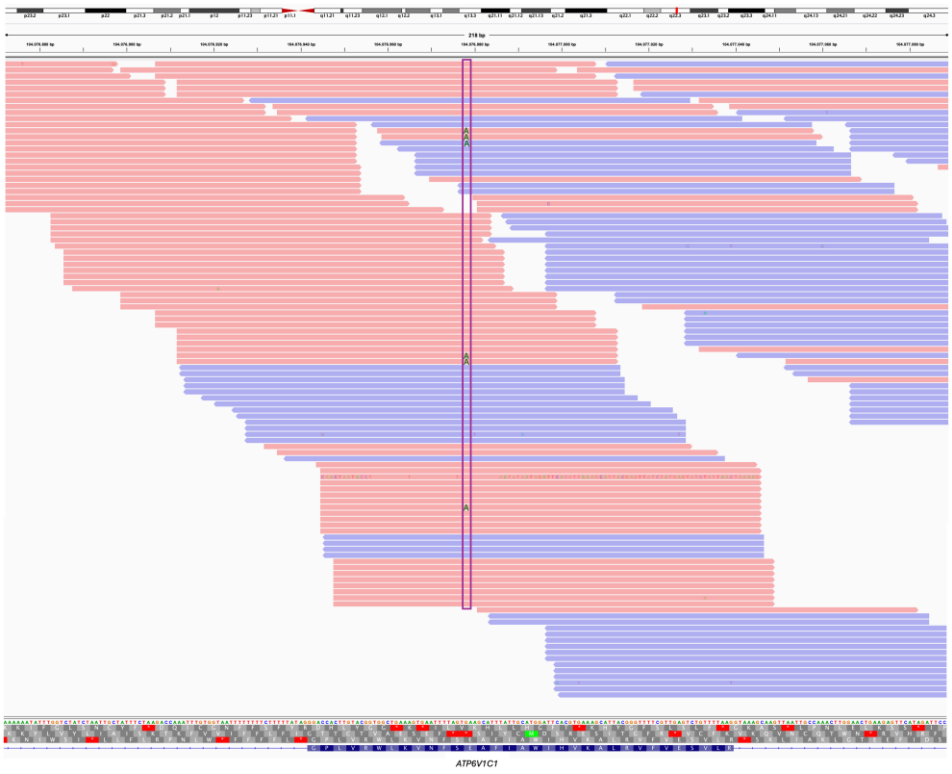

Figure S2. *ATP6V1C1* variant validation and segregation analysis. (**A**) The heterozygous occurrence of the c.865G>A substitution (p.Glu289Lys) in Subject 1 was validated *via* Sanger sequencing using different tissues/cell types. The variant is not observed in leukocytes from the healthy brother and 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.

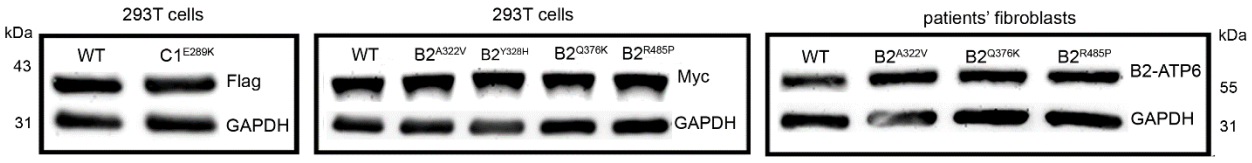

Figure S3. Disease-associated *ATP6V1B2*/*ATP6V1C1* variants do not affect B2 and C1 proteins stability. Western blot (WB) analysis showing comparable level of B2 and C1 proteins in transiently transfected 293T cells and primary fibroblasts from affected individuals and control cells (WT). Membrane were 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.

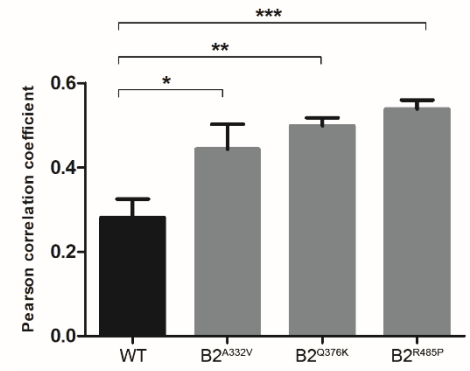

Figure S4. Pearson correlation coefficient of Lamp1 co-localization with B2 subunits. The coefficient was calculated using the Zen 3.3 software. Histograms indicate mean ± SEM; p values were calculated by One way ANOVA with Tukey's correction for multiple testing.

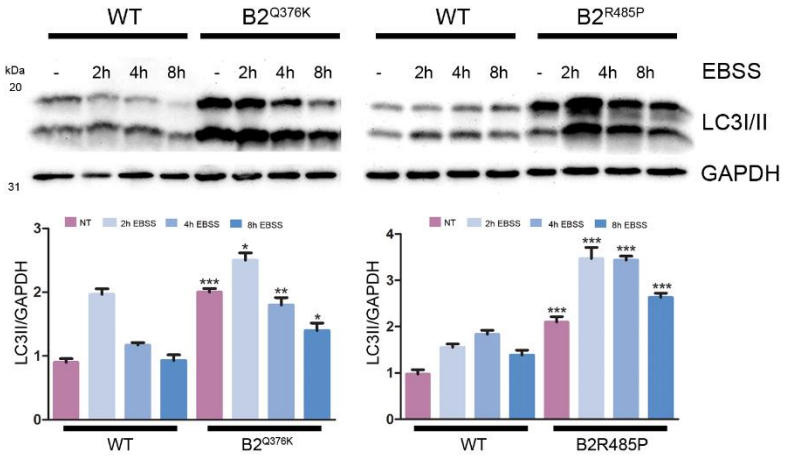

Figure S5. Variants identified in *ATP6V1B2* gene promote an altered autophagic flux. WB analyses performed on patients' fibroblasts carrying the ATP6V1B2$^{Q376K/R485P}$ variants confirm the data obtained with immunofluorescence showing an anomalous accumulation of LC3I/II in the steady state condition 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 (-).

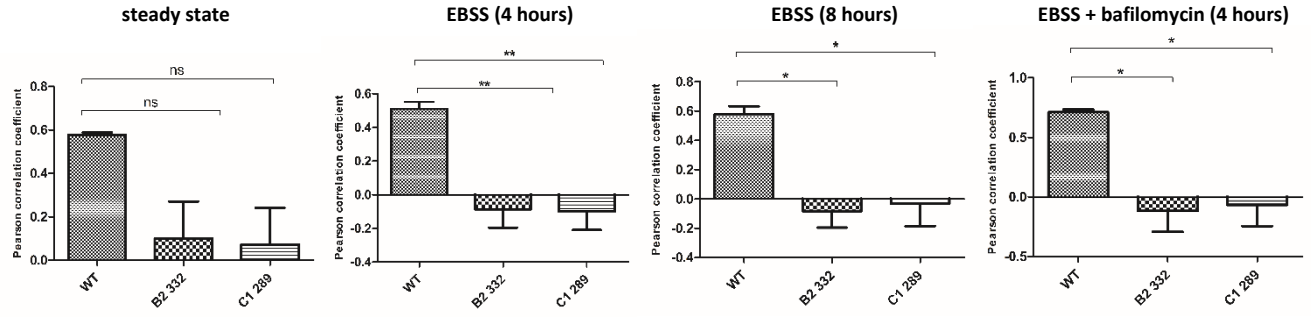

Figure S6. Pearson correlation coefficient of Lamp1 co-localization with LC3I/II. The coefficient was calculated using the Zen 3.3 software and relative histograms are reported. Histograms indicate mean ± SEM, p values were calculated by One way ANOVA with Tukey's correction for multiple testing.

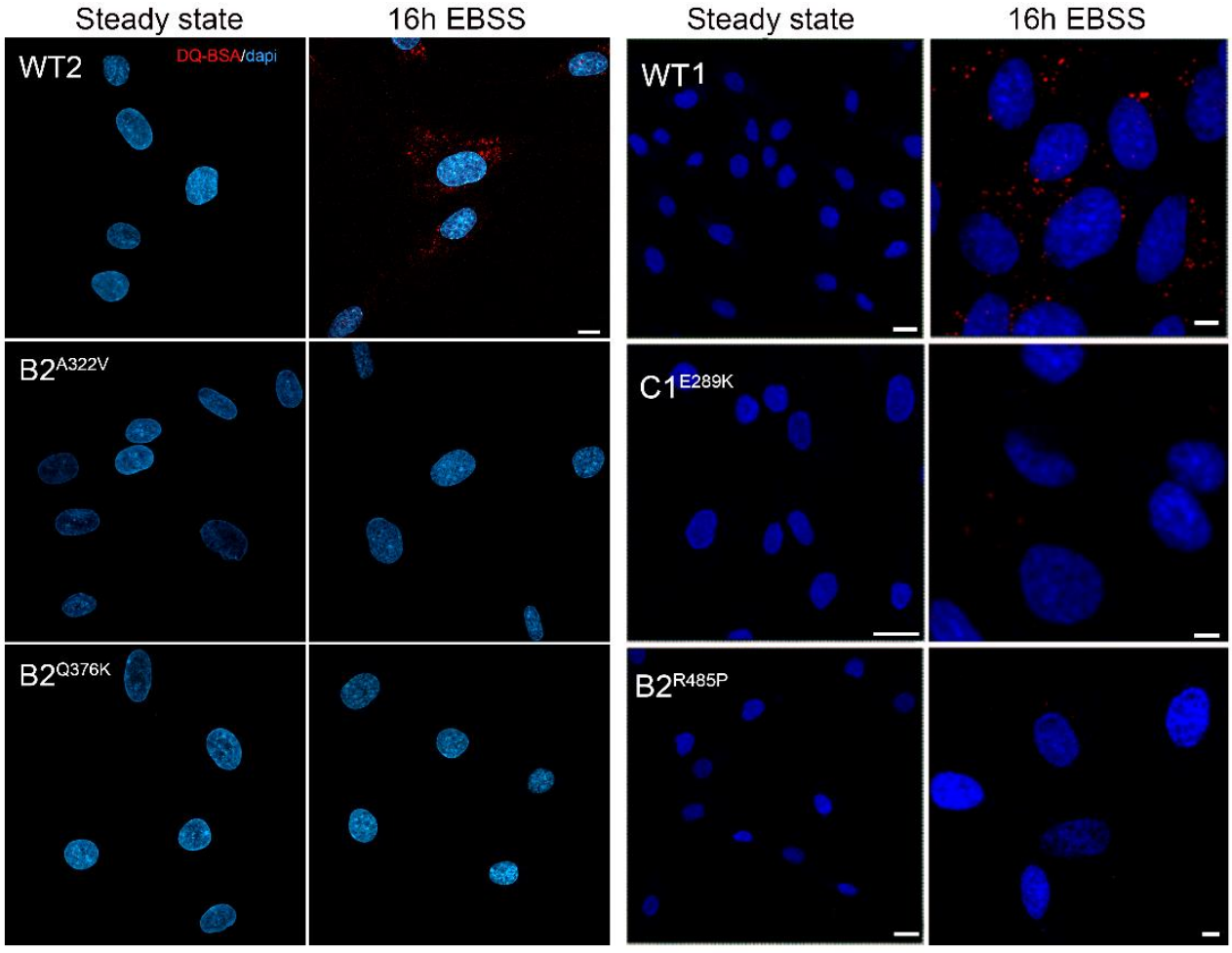

Figure S7. Analysis of autophagic flux with DQ-BSA. Primary fibroblasts were incubated with bovine serum albumin derivative (dye-conjugated [DQ] BSA) conjugated to a self-quenched fluorophore (10 μg/ml) for 1 h at 37 °C in complete culture medium and then left in a starvation medium for 16 h to induce 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 μm and 5 μm (zoom) (left) and 10 μm and 5 μm (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, *ATP6V1C1*
	3[4]
Putative disease genes (Autosomal Recessive trait)[2]	0
- candidate genes	

[1]High-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).

[2]Functional 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)
ATP6V1C1 (NM_001695.5): c.865G>A (p.Glu289Lys)	n.r.	32.0	0.092	0.71	0.001	0.95/0.66
ATP6V1B2 (NM_001693.4): c.982T>C (p.Tyr328His)	n.r.	29.5	0.294	0.93	0	1/0.99
ATP6V1B2 (NM_001693.4): c.983A>G (p.Tyr328Cys)	n.r.	30.0	0.375	0.93	0.009	1/1
ATP6V1B2 (NM_001693.4): c.995C>T (p.Ala332Val)	n.r.	29.0	0.358	0.89	0.001	0.95/0.67
ATP6V1B2 (NM_001693.4): c.1120G>C (p.Glu374Gln)	n.r.	29.6	0.132	0.75	0	1/0.94
ATP6V1B2 (NM_001693.4): c.1126C>A (p.Gln376Lys)	n.r.	25.0	0.481	0.85	0	0.28/0.2
ATP6V1B2 (NM_001693.4): c.1127A>G (p.Gln376Arg)	n.r.	29.1	0.456	0.89	0	0.96/0.88