ATP6V0C variants impair V-ATPase function causing a neurodevelopmental disorder often associated with epilepsy

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Heterozygous ATP6V0C Variants Cause a Neurodevelopmental Disorder Strongly Associated with Epilepsy and Impair Vacuolar H+-ATPase Function


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ABSTRACT
The vacuolar H⁺-ATPase (V-ATPase) is an enzymatic complex that that functions in an ATP-dependent manner to pump protons across membranes and acidify organelles, thereby creating the proton/pH gradient required for membrane trafficking by several different types of transporters. We describe heterozygous point variants in ATP6V0C, encoding the c-subunit in the membrane bound integral domain of the V-ATPase, in 26 patients with neurodevelopmental problems with or without epilepsy. Corpus callosum hypoplasia and cardiac abnormalities, such as pulmonary valve stenosis, were also present in some patients. In silico modeling suggested that the patient variants interfere with the interactions between the ATP6V0C and ATP6V0A subunits during ATP hydrolysis. Functional analysis conducted in the Saccharomyces cerevisiae ortholog VMA3 revealed decreased V-ATPase function when measured by LysoSensor fluorescence and reduced growth in media containing varying concentrations of CaCl₂, suggesting decreased V-ATPase function. Selected patient variants in C. elegans led to reduced growth and motor dysfunction in regular media and reduced lifespan under osmotic stress. In summary, this study establishes ATP6V0C point variants as a cause of human neurodevelopmental disorder, describes the clinical phenotype of the condition and provides insights into disease mechanism.

INTRODUCTION
The vacuolar H⁺-ATPase (V-ATPase) is a highly conserved enzymatic complex that functions in an ATP-dependent manner to pump protons across membranes and acidify organelles. The V-ATPase is comprised of a peripheral V₁ domain and an integral V₀ domain. The V₁ domain is responsible for the hydrolysis of ATP creating the necessary energy to translocate protons.
through the V₀ domain via a rotational mechanism (Figure 1). The V-ATPase plays a crucial role in many cellular processes involving membrane trafficking by creating a proton/pH gradient used by several different types of transporters.

The human V-ATPase, comprised of 13 different subunits in humans, is encoded by 23 genes (Table S1). This genetic redundancy allows for the formation of tissue-specific V-ATPase complexes, including in synaptic vesicles (SVs) where it creates the necessary proton/pH gradient to load various neurotransmitters. To date, eleven genes corresponding to seven different subunits of the V-ATPase have been associated with human disease (Table S1).

Early-onset epilepsy has been observed in patients with variants in either ATP6V1A or ATP6V0A1, with heterozygous, de novo variants leading to less severe presentations when compared to patients with biallelic variants. Mutations in ATP6V1B2 can cause epileptic conditions such as Zimmerman-Laband (OMIM*) and DOORS (OMIM*) syndromes, or deafness and nail dysplasia without epilepsy (OMIM*). An accessory protein to the V-ATPase, encoded by ATP6AP2, is associated with X-linked syndromic intellectual disability that can present with or without epilepsy (OMIM*).

ATP6V0C, a three-exon gene located on human chromosome 16p13.3, encodes the 155 amino acid long c-subunit of the V₀ domain which along with the c'' subunit (encoded by ATP6V0B) forms the intramembrane c-ring that facilitates the movement of protons across the membrane. The process of proton translocation is reliant on a glutamate residue at position 139 (p.E139) in ATP6V0C as well as an arginine residue (p.R735) in ATP6V0A.

Previously, we described patients with developmental delay, intellectual disability, microcephaly, and seizures with 16p13.3 microdeletions encompassing a minimal overlapping region that included TBC1D24, ATP6V0C, and PDK1. By reviewing the known function(s)
and expression patterns of genes in the minimal overlapping region, we proposed haploinsufficiency of \textit{ATP6V0C} as the primary contributor to the clinical features of 16p13.3 microdeletion syndrome.\textsuperscript{23} However, we did not provide any functional evidence to support our claim.

Most recently, Itiwut \textit{et al.} reported a \textit{de novo} stop-loss variant in \textit{ATP6V0C} in an individual with epilepsy and intellectual disability.\textsuperscript{24} Analysis of RNA derived from the patient’s leukocytes revealed that, as expected, the mutant transcript escaped nonsense mediated decay (NMD). The authors proposed haploinsufficiency as the likely pathomechanism given the observed decrease in mRNA levels; however, a dominant negative effect is also possible given the transcript escape from NMD. Hence, thus far it is unclear if \textit{ATP6V0C} missense variants are a cause of human disease. Furthermore, the functional impact and mechanistic basis of \textit{ATP6V0C}-associated human disease remain unclear.

In this study, we report the identification of heterozygous \textit{ATP6V0C} missense variants in patients with a novel syndrome of intellectual disability, developmental delay, and epilepsy. We present multiple lines of computational and functional analyses to demonstrate that these variants disrupt \textit{V}-ATPase activity.

\textbf{MATERIALS AND METHODS}

\textbf{Identification of Individuals with \textit{ATP6V0C} Variant}

Informed consent for patients was obtained through protocols approved by the institutional review boards at each site of patient recruitment. Patients with \textit{ATP6V0C} variants were identified via GeneMatcher, and by interrogating the 100,000 Genomes database, the Deciphering Developmental Disorders (DDD) study, and ClinVar.\textsuperscript{25-27} We also screened whole-exome
sequencing (WES) data from epilepsy patients referred for genetic testing at EGL Genetics. Three patients (Patients 13, 2 and 26) were previously reported in other publications.\textsuperscript{24,25,28} Patient 5 was reported in ClinVar and available clinical information was provided by the depositing organization (Table S2). Clinical information for all patients was collected using a custom form provided to each site.

**Sequencing and Analysis of Sequence Data**

Whole-exome or whole-genome sequencing was performed on patient DNA extracted through standard protocols. All libraries were sequenced on Illumina HiSeq systems. Sequences were aligned using BWA, GATK, or PEMapper software. The variants were called using GATK, SAMtools, or PECaller. Further details are provided in Table S2. When possible, \textit{ATP6V0C} variant segregation was confirmed with Sanger sequencing using standard protocols.

**Lollipop and Missense Tolerance Ratio Diagram**

The Lollipop diagram was created as previously described using the UniProt accession number, P27449.\textsuperscript{29,30} Non-synonymous and synonymous population variants were downloaded from gnomAD (v.2.1.1).\textsuperscript{31} Resulting diagrams of gnomAD and patient variants were merged into a single image for ease of visualization, and the locations of the transmembrane domains were superimposed over the resulting image. A Missense tolerance ratio (MTR) plot was generated using MTR-Viewer v2 on the ENST00000330398 transcript.\textsuperscript{32} Codon window size was set to 21.

**Drosophila Studies**

The Drosophila ortholog, Dmel\textbackslash Vha16-3 (CG32090), was knocked-down using pan-neuronal (elaV-GAL4) expression of a gene-specific RNAi construct (102067), provided by the Vienna Drosophila Resource Center. As a control, an RNAi to GFP was used. Seizure activity was elicited using electroshock of wall-climbing third instar larvae as described in Marley and Baines
Drugs were fed to larvae by mixing (at 2mM) in molten fly food which was allowed to cool and set before being seeded with first instar larvae. Drugs were solubilized in DMSO, which was also included in the ‘no drug’ control (0.66%).

In silico Variant Modeling

The amino acid sequence of ATP6V0C was submitted to ConSurf with default settings. A score of 1 to 9 was returned from least to most conserved, and converted into a heat map. A binary heat map was used to note whether a given amino acid substitution was present or absent in the ConSurf multiple sequence alignment. Patient and gnomAD variants were displayed in the context of a structure for the V₀ domain of human V-ATPase (PDB: 6wlw). PyMOL and Swiss PDB Viewer were used for visualization of protein structure.

Saccharomyces cerevisiae Strains and Plasmids

_E. coli_ and yeast manipulations were done following standard molecular biology protocols. The vma3::kanMX yeast strain (vma3Δ, Cat No. YSC6273-201929081) was obtained from GE Dharmacon and is isogenic with BY4741 (MATa his3Δ leu2Δ met15Δ ura3Δ). Plasmids are listed in Table S3. A plasmid (pKM16) containing the promoter and wild type ORF for VMA3 was generated by amplifying a 924bp fragment from _S. cerevisiae_ gDNA and cloning it into pRS316 using _BamHI_ and _SacI_ (Forward cloning primer: 5’ taacgcagagctcctgaaatagttggg cacgcctgattgctctac
 Reverse cloning primer: 5’ taacgcagagctcctgaaatagttggg). pKM16 was used as the backbone to generate all variants via conventional cloning techniques or the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies). Sanger sequencing was performed to confirm the presence of each variant as well as the absence of any unwanted substitutions.
Plasmids were transformed into the *vma3Δ* strain and selected on plates containing synthetic minimal media plus dextrose supplemented to select for a *URA3* plasmid (SD-ura). Selected transformants were maintained on SD-ura throughout the course of experiments.

**Serial Dilution Spotting Assay**

Liquid cultures of transformants were grown at 30°C overnight in SD-ura adjusted to a pH of 5.5. 1 OD<sub>600</sub> per ml of cells was collect and suspended in dH<sub>2</sub>O. Serial 10-fold dilutions were spotted onto SD-ura plates. Plates were imaged after 48 hours incubation at 30°C.

**LysoSensor Uptake and Confocal Imaging**

Cells were collected at OD<sub>600</sub> 0.6-1.0 and incubated with LysoSensor Green DND-189 (Invitrogen, Cat# L7535) as previously described. Cells were resuspended in 1X PBS (pH 7.6) to an OD<sub>600</sub> of 0.6, deposited on 1.5% agarose pads, and visualized immediately. Images were taken at room temperature using a confocal laser scanning microscope (A1R HD25, Nikon) with an Apo TIRF 60x 1.49 NA oil immersion lens, WD 0.12mm (Nikon). Images were acquired using NIS-Elements (AR 5.21.02, Nikon). Images were processed using FIJI. Briefly, all cells visible in the DIC channel were selected, the selection was copied to the FITC channel, and the mean grey area was calculated for each cell. Measurements were corrected for background signal by subtracting the mean grey area of a background only selection from each image. Two biological replicates totaling 71-132 cells for each variant were analyzed.

**Generation of Growth Curves**

Liquid cultures of transformants were grown at 30°C overnight in SD-ura adjusted to a pH of 5.5. Overnight cultures were diluted 1:20 in dH<sub>2</sub>O and further diluted 1:10 in YPD, pH 5.5, with 5mM, 100mM or 200mM CaCl<sub>2</sub>. OD<sub>600</sub> measurements were taken every 15 minutes for 30 hours at 30°C with shaking using an ELx808 plate reader (BioTek). Three independent transformants
were assayed in triplicate for each variant. The R package, GrowthCurver, was used to calculate the empirical area under the curve (eAUC) from the OD\textsubscript{600} measurements for each replicate.\textsuperscript{40}

\textit{Caenorhabditis elegans} Studies

All \textit{C. elegans} strains were cultured and handled as per standard methods. All experiments were carried out at 20°C. Mutations were knocked in via CRISPR/Cas9, and homozygous worms were studied. The strains used in this project are described in Table S4.

\textbf{Paralysis assays}

In 3 separate experiments, 30-40 L4 worms (in triplicate) were picked to standard NGM plates either with 51mM NaCl (physiological conditions), or with 200mM or 300mM NaCl, and scored daily for paralysis starting the day after they were picked. A total of 270-360 worms were tested per condition. Worms were counted as paralyzed if they failed to move their body upon prodding with a platinum wire and were considered dead if they failed to move their head and showed no pharyngeal pumping when prodded. Dead or lost animals were censored from statistical analyses. Worms were transferred every 2 days to avoid progeny.

\textbf{Lifespan assays}

Lifespan experiments were conducted similarly to paralysis assays. Two separate experiments were performed using 35-40 worms in triplicate, leading to a total 315-360 worms tested per condition. Worms were counted every second day from day 1 of adulthood until death. Lost animals were censored from statistical analyses, paralyzed worms were not censored and kept until death.

\textbf{Liquid culture motility assays}
Synchronized day 1 adult worms were transferred into 200uL of M9 buffer with or without 350mM NaCl in each well of a 96 well plate, to a density of 30 worms per well. Motility was automatically analyzed for 4 hours using a WMicrotracker-One plate reader (PhylumTech).

**WormLab analysis**

Synchronized day 1 adult worms were video recorded for 30-33 seconds using a Leica Stereomicroscope S9i. Automated movement and worm size analyses were conducted using WormLab software (MBF Biosciences) which tracks individual worms from the recorded videos. Activity index is defined by the brush stroke (area "painted" by the animal's body in a single complete stroke) normalized by the time taken to perform two strokes. Wave initiation rate is defined as the number of body waves initiated from either the head or the tail per minute. Swimming speed was measured over a two-stroke interval.\textsuperscript{41} For body size analysis, worms were recorded on bacteria free NGM plates. For swimming parameters, worms were placed in M9 with 500mM NaCl and recorded 30 minutes later. At least 50 worms were recorded in two independent experiments.

**Aldicarb sensitivity assays**

Worms were grown on standard NGM plates until day 1 of adulthood, when they were transferred to NGM plates containing 1mM aldicarb. Paralysis was assayed every 30 minutes for 2 hours. Animals were counted as paralyzed if they failed to move upon prodding with a platinum wire.

**Statistical Analyses**

Drosophila recovery time was analyzed using one-way ANOVA with Dunnett’s post-hoc test for multiple comparisons. A Fisher’s Exact test was used to demonstrate the presence of a variant hot-spot in the fourth transmembrane domain (TM4, $\alpha = 0.05$). For LysoSensor, fluorescence
values for each cell measured were normalized to the mean fluorescence of the wild type rescue. For the growth rate assay, eAUC values were normalized to the mean eAUC of the wild type rescue within each plate. A one-sample t-test was used to compare the mean fluorescence and eAUC for each variant to a hypothetical mean of 100 (representing the wild type rescue). Significance levels were corrected for multiple comparisons using a Bonferroni correction (LysoSensor and 5mM, \( \alpha = 0.0003125 \); 100mM, \( \alpha = 0.00714 \); 200mM, \( \alpha = 0.00833 \)). A two sample t-test (two-tailed) was used to compared p.G103S to p.F137L at 200mM (\( \alpha = 0.05 \)). Paralysis curves and lifespan assays were compared using the log-rank (Mantel–Cox) test (\( \alpha = 0.05 \)). The Liquid culture motility assays (WormTracker) results were analyzed using two-way ANOVA to compare each variant to the wild type N2 strain. WormLab results were analyzed using a one-way ANOVA with a Dunnett’s post-hoc test for multiple comparisons to compare each variant to the wild type N2 strain. The WormLab data is presented as box and whisker plots indicating minimal and maximal data points. Normalization and statistical analyses were carried out using Prism 9.0 (GraphPad Software Inc.). All data is presented as mean ± SEM.

**Reagent Availability**

All strains and plasmids (Tables S3 and S4) used in this study are available upon request.

**RESULTS:**

**Identification of ATP6V0C Variants in Patients**

Through the use of GeneMatcher, 100,000 Genomes database, the DDD study, ClinVar, and EGL Genetics we identified 26 patients with heterozygous ATP6V0C variants. Of the 26 patients, 21 had missense substitutions with 18 being unique, 4 had frameshifting variants, and 1 had a stop-loss variant (Table 1). Three variants p.A138P, p.A149T, and p.L150F were
recurrent, each being seen in two unrelated individuals. Different substitutions at p.A95 and p.L150 were also observed. Multiple lines of evidence supported the pathogenicity of these variants. Firstly, all variants were absent from the Genome Aggregation Database (gnomAD, v.2.1.1) which is a population database comprised of approximately 200,000 alleles from WES and whole-genome sequencing of individuals without severe pediatric neurological disorders. Secondly, in 18 patients the variants were shown to have occurred de novo. Thirdly, all missense substitutions affected highly conserved residues (Figure 2A), with 17 out of the 18 unique missense variants having CADD scores that placed them in the top 1% of predicted deleterious variants. Lastly, ATP6V0C has a predicted intolerance to missense variation with only 21 missense variants observed in gnomAD compared to a predicted 108 (observed/expected = 0.19) and all population variants being see at a low frequency (three times or less) (Figure 2B-C, Table 1).42

**ATP6V0C Variants Cause a Human Syndrome of Developmental Delay, Intellectual Disability and Epilepsy**

The primary clinical presentation of identified patients was early-onset epilepsy with intellectual disability (Table 1). The mean age of seizure onset was 21.6 ± 8.5 months, with 12 of 17 patients for whom this information was available having onset prior to 24 months. Based on clinical information from 16 patients, the most common seizure types observed were generalized tonic-clonic (9/16), focal (7/16), atonic (4/16), and myoclonic seizures (5/16). Intellectual disability, ranging from mild to severe, was seen in 16/16 patients who were old enough for a formal diagnosis and for whom this information was available. Development delay was seen in 18/22 patients. Some patients (3/22) experienced regression in abilities after seizure onset. Nineteen patients had MRIs with thirteen showing abnormalities (Table 1). MRIs in patients 5, 6, 18, 20
and 25 showed agenesis/hypoplasia of the corpus callosum or cerebellar vermis (Figure 3), as well as delayed myelination in patients 6, 18, 22 and 24. Four patients were reported to have cardiac abnormalities: Patient 3 had pulmonary valve stenosis, patient 6 had a thickened left ventricular wall, patient 7 had a heart murmur, and patient 13 exhibited several cardiac defects including hypertrophic cardiomyopathy, mitral valve prolapse, and mild to moderate mitral valve regurgitation. Patients 5 and 24 both showed dental enamel defects, with patient 24 lacking dental enamel. Patient 13 also has biallelic variants in \textit{LZTR1} which has been associated with Noonan-like syndrome (OMIM*).\textsuperscript{43}

Collectively, these data show that \textit{ATP6V0C} variants cause a human syndrome of developmental delay, intellectual disability, and epilepsy. Furthermore, as most individuals were ascertained on the basis of genotype (i.e., having a variant in \textit{ATP6V0C}), their phenotypic convergence on reverse phenotyping further supports the pathogenicity of the variants described in this study.\textsuperscript{44}

\textbf{\textit{ATP6V0C} Knockdown in Drosophila Results in Seizure Phenotype}

Drosophila is an excellent model to study the genetic basis of epilepsy.\textsuperscript{45} Based on our hypothesis that haploinsufficiency of \textit{ATP6V0C} drives the neurological phenotype of 16p13.3 microdeletion syndrome and identification of patients with frameshift predicted loss of function point variants, we first tested the consequences of \textit{ATP6V0C} knockdown in Drosophila.\textsuperscript{23} The orthologous protein in \textit{Drosophila}, (Dmel\textbackslash Vha16-3, CG32090), shows 78\% amino acid identity to \textit{ATP6V0C}. CG32090 was knocked-down via pan-neuronal expression of a gene-specific RNAi construct (VDRC-102067). Controls of the same neuronal driver line (elaV-GAL4) driving expression of GFP RNAi, and the homozygous 102067 RNAi line without the elaV-GAL4 driver were used. Following knock-down of CG32090, wall climbing third instar larvae
showed a significant increase in recovery time (i.e., increased seizure duration) following electroshock-induced seizure \( (p < 0.0001, \text{one-way ANOVA, Figure 4A}) \). Pre-treatment of larvae \( (\text{elaV}>102067 \text{RNAi}) \) with a range of established antiepileptic drugs show that significant reduction of recovery time (i.e., reduction in seizure duration) was achieved with levetiracetam and topiramate \( (p < 0.001) \), and to a lesser extent with lamotrigine and valproate \( (p < 0.01 \text{ and } < 0.05, \text{respectively, Figure 4B}) \). Phenytoin, at the same concentration \( (2\text{mM in fly food}) \) did not significantly alter recovery time. These results are consistent with the hypothesis that haploinsufficiency of \( \text{ATP6V0C} \) contributes to seizure.

\textit{ATP6V0C Variants Are Predicted to Interfere with V-ATPase Rotary Mechanism}

The majority of identified \( \text{ATP6V0C} \) variants in patients were missense, and three out of four frameshifting variants are predicted to escape NMD. Taken together with the previous demonstration that the stop loss variant in patient 26 escapes NMD, this suggests that a dominant negative or gain of function effect is the likely mechanism.\(^{24}\) To understand the basis of pathogenicity of the missense variants, we first turned to \textit{in silico} modelling. Higher conservation across \( \text{ATP6V0C} \) orthologs was seen at sites of patient variants compared to gnomAD variants \( (\text{Figure 5A}) \). Interestingly, the majority of gnomAD variants resulted in changes to amino acid residues that are considered wild type in orthologs whereas this was only seen for two patient variants \( (\text{p.S98R and p.G42D, Figure 5A}) \).

Upon hydrolysis of ATP, the c-ring \( (\text{comprised of 9 copies of ATP6V0C and one copy of ATP6V0B}) \) rotates within the membrane delivering protons to the \( \text{ATP6V0A} \) subunit \( (\text{encoded by ATP6V0A}) \) for transport across the membrane \( (\text{Figure 1}) \).\(^{21}\) Transmembrane (TM) domains 2 and 4 of \( \text{ATP6V0C} \) are outward facing and interact with \( \text{ATP6V0A} \) during this rotational mechanism.\(^{46,47}\) The location of patient variants shows an enrichment within TM domains and
the presence of a ‘hot-spot’ in the fourth TM of ATP6V0C (p = 0.006, Fisher’s Exact test, Figure 2B-C). When viewed structurally, some patient and gnomAD variants are located at sites of packing between c-ring subunits; however, many more patient variants are located outward-facing from the c-ring so as to potentially interfere with interactions between mutant ATP6V0C subunits and the ATP6V0A subunit (Figure 5B-C). These data indicate a dominant negative effect as the likely underlying mechanism for most missense variants in ATP6V0C.

**ATP6V0C Patient Variants are Deleterious in Yeast**

Budding yeast, *S. cerevisiae*, possesses an ATP6V0C ortholog, VMA3, which shares a 72% amino acid identity and a conserved four transmembrane protein structure. *S. cerevisiae* has been previously used to study the effect of variants within different subunits on V-ATPase function. Given that all identified missense patient variants affected residues that are conserved between human and yeast, we expressed 12 of the patient variants in VMA3 using a centromeric plasmid in a vma3Δ yeast strain (Figure 2, Figure S1, Table S2). Six variants were identified after completion of these experiments and, therefore, were not modeled in yeast.

We also examined the functional effects of three population variants in ATP6V0C from gnomAD (p.R48W, p.G103S, p.M131I, Table 2) on V-ATPase function. p.R48W was chosen as it effects the same residue as the p.R48P patient variant. p.G103S and p.M131I were chosen as they have the highest CADD scores for variants seen twice and once, respectively, in gnomAD. The altered residues are also conserved between human and yeast (Figure S1). All ATP6V0C variants in gnomAD (21 total) are rare, being seen no more than three times out of approximately 200,000 alleles. In addition, we generated and tested p.E139A, which removes the glutamate residue necessary for V-ATPase function.
To first establish the ability of the vma3Δ strain to grow when transformed with a plasmid containing a patient or population variant, a serial dilution spot assay was performed on SD-ura plates. We confirmed the ability of all transformants to grow under no selective V-ATPase pressure, thereby allowing us to examine V-ATPase function in our yeast model (Figure S2).

LysoSensor Green DND-189 is an acidotropic dye that accumulates in the membranes of vacuoles. Upon protonation, quenching of the fluorescent probe is relieved and fluorescent intensity increases in a pH dependent manner. It has been previously demonstrated that V-ATPase activity in yeast and fluorescent intensity of LysoSensor Green are correlated. We looked for rescue of V-ATPase function by transformation of each patient or population variant into the vma3Δ strain. Nine patient variants and p.E139A showed little to no fluorescence (Figure 6). Two patient variants (p.G63A and p.L150F) and two gnomAD variants (p.R48W and p.G103S) showed intermediate levels of fluorescence intensity compared to the wild type rescue. One gnomAD variant (p.M131I) and one patient variant (p.F137L) show levels of fluorescence intensity that were comparable to wild type rescue. Overall, 10 of 12 patient variants and 2 of 3 gnomAD variants elicited significant decreases in fluorescence intensity when compared to the wild type rescue (p < 0.0001, one sample t-test, Figure 6).

V-ATPase, and therefore ATP6V0C, function is required for yeast to grow at increased CaCl₂ concentrations. To further examine the impact of patient ATP6V0C variants on V-ATPase function, transformants with patient or population variants were used to inoculate YPD with 5mM CaCl₂ and growth curves were generated for each variant (Figure 7A-D). Eight patient variants and p.E139A showed no growth at 5mM CaCl₂. The remaining four patient variants and the three gnomAD variants showed varying degrees of growth at 5mM CaCl₂. A significant decrease compared to wild type rescue was seen for 11 of 12 patient and the three
gnomAD variants tested (p < 0.0001, one sample t-test, Figure 7E). The results seen at 5mM CaCl$_2$ mirrored those seen with LysoSensor uptake.

Next, variants that grew at 5mM CaCl$_2$ along with all three gnomAD variants were also tested at 100mM and 200mM CaCl$_2$ to determine whether a higher concentration of calcium would provide further separation of variants relative to the wild type rescue. At 100mM CaCl$_2$, p.L150F showed almost no growth compared to wild type (p < 0.0001, one sample t-test, Figure 8A and C) while the three other patient variants (p.G63A, p.V74F, p.F137L) showed intermediate growth relative to the wild type rescue. One gnomAD variant (p.M131I) showed no difference in growth relative to wild type while p.R48W and p.G103S both showed decreased growth relative to the wild type rescue. The three patient variants and two gnomAD variants with intermediate growth were further tested at 200mM CaCl$_2$ (Figure 8B and D). Further separation between the patient variants and two gnomAD variants (p.R48W and p.M131I) compared to wild type rescue was observed. p.G103S showed less growth relative to the other gnomAD variants (p.R48W and p.M131I), but still yielded a significantly larger eAUC compared to the best growing patient variant at 200mM CaCl$_2$, p.F137L (59.22 ± 2.278 vs. 45.63 ± 2.243, p = 0.0006, two sample t-test).

**Assessment of Three Patient Variants in C. elegans**

Next, we assessed the effects of patient ATP6V0C variants on neurological function using C. elegans. Worms express three orthologous genes to ATP6V0C in neurons, vha-1, vha-2 and vha-3. VHA-2 and VHA-3 have identical amino acid sequences and share 66.7% amino acid identity with ATP6V0C while VHA-1 has slightly less homology to ATP6V0C at 63% amino acid identity. The patient variants that were selected for further analysis were distributed throughout the protein, showed tolerance to 5mM CaCl$_2$ in the yeast growth assay, and were
associated with severe neurocognitive deficits and poly-medicated epilepsy in patients. Specifically, we studied p.F137L (corresponding to p.F143L in VHA-2) and p.G63A and p.L150F (corresponding to p.G69A and p.L156F, respectively, in VHA-3). A fourth strain carrying the p.A95T variant (corresponding to p.A101T in VHA-2) was generated but caused sterility in homozygous worms and was not studied further.

Mutants were shorter and smaller than N2 controls, indicating a morphological delay even in ideal conditions (p < 0.05, one-way ANOVA, Figure 9A-B). When tested in liquid physiological M9 culture over a 4 hour period, movement of day 1 young adult worms, each mutation was comparable to wild type N2 worms (Figure 9C). However, when motor function was examined under osmotic stress conditions (350mM NaCl), mutants expressing p.G63A and p.F137L exhibited significantly reduced movement scores when compared to N2 worms (p < 0.01, two-way ANOVA Figure 9D). Although mutants expressing p.L150F also exhibited less movement than N2 worms, this difference was not statistically significant (p = 0.0869).

We next compared paralysis and lifespan of each strain with N2 worms when maintained on NGM plates under physiological conditions or exposure to osmotic stress (200mM and 300mM NaCl). All mutants showed greater levels of paralysis when compared to N2 worms over a 14-day period, and these differences were strongly exacerbated in the presence of osmotic stress (p < 0.0001, log-rank (Mantel–Cox) test, Figure 9E-G). Lifespans of the mutant strains were also reduced when compared to N2 worms when maintained under physiological conditions and osmotic stress (p < 0.0001, log-rank (Mantel–Cox) test, Figure 9H-J).

To compare fine motor phenotypes, we exposed the mutant strains and N2 worms to liquid M9 with 500mM NaCl for 30 minutes and analyzed movement using WormLab. Activity index and wave initiation were significantly increased in the mutant strains when compared to
N2 worms (p < 0.05), but swimming speeds were not significantly altered, suggesting increased but uncoordinated movement (p > 0.05, one-way ANOVA, Figure 10A-C).

To test if the mutant strains have an impairment in nervous system signaling, we added an acetylcholinesterase inhibitor, aldicarb, to NGM plates and scored the number of paralyzed worms over 2 hours. Aldicarb causes an accumulation of acetylcholine in the neuromuscular junctions resulting in muscular hypercontraction and acute paralysis and can be used to evaluate if there is a dysfunction of either gamma aminobutyric acid (GABA) or acetylcholine signalling.\textsuperscript{53,54} To confirm proper aldicarb effect we included the unc-47(e307) and unc-64 mutants. Unc-47(e307) is hypersensitive to aldicarb due to the lack of a vesicular GABA transporter gene (orthologous to SLC32A1 in humans) required for GABA transmission.\textsuperscript{55} Unc-64 (orthologous to STX1A in humans) mutants have reduced cholinergic neurotransmission, making them resistant to aldicarb-induced paralysis.\textsuperscript{56,57} Mutant worms showed greater paralysis in presence of aldicarb, compared to wild type N2 worms (p < 0.0001, log-rank (Mantel–Cox) test, Figure 10D).

**DISCUSSION**

In this study, we report the identification of heterozygous \textit{ATP6V0C} variants in 26 patients with neurodevelopmental phenotypes. Overall, this cohort of patients presented with early-onset epilepsy (mean age of onset: 21.6 ± 8.5 months) and varying severities of intellectual disability and/or developmental delay. Five patients with MRIs show hypoplasia or agenesis of the corpus callosum. Congenital cardiac abnormalities were also observed in four patients. Several patients with cutis laxa caused by biallelic variants in \textit{ATP6V1A} or \textit{ATP6V1E1} are also reported to have congenital heart defects including septal defects, cardiac valve defects and hypoplastic heart syndrome.\textsuperscript{3}
Interestingly, three patients (Patients 4, 15 and 19) were found to be mosaic for their identified \( ATP6V0C \) variant. Based on the available clinical information, the seizure phenotype of these patients may be less severe than for those with a germline variant (Table 1). Patient 4 has seizure-like episodes that started at 14 months, but are not supported electrographically, Patient 15 had seizure onset at 12 years of age, and Patient 19 has not reported any seizure or seizure-like episodes. It is well established that the timing of the post-zygotic mutation event, and the affected tissues, can have a large influence on phenotypic presentation and severity in patients with somatic mosaicism, underlying differences in clinical presentation between patients with germline and mosaic variants.\(^{58}\) Previous work has demonstrated the utility of identifying somatic mosaicism for clinical and genetic counseling outcomes in patients.\(^{59}\)

Nine copies of \( ATP6V0C \) assemble to form the intramembrane c-ring of the V-ATPase which uses a rotary mechanism to translocate protons across the membrane.\(^{21}\) Normally, frameshifting variants are predicted to cause NMD of the mutant mRNA, which would result in reduced protein levels. However, three of the four frameshift variants are located in last exon of \( ATP6V0C \) and are expected to escape NMD, suggesting a possible dominant negative effect. Additionally, modeling showed that the majority of missense variants are likely to act as a ‘stone in the gear’ between \( ATP6V0C \) and \( ATP6V0A \) inhibiting the rotatory mechanism, consistent with a dominant negative mechanism.

\( ATP6V0C \) is an evolutionary constrained gene as reflected by the high degree of amino acid homology between the human and yeast orthologs (72\%), human and worm orthologs (63-66.7\%), and human and Drospohila orthologs (78\%) and the low number of missense variants seen in gnomAD (n=21 compared to an expected 108.5, Figure 2C).\(^{31}\) Of the 18 unique missense variants, 16 are located in TM domains, with nine in TM4 which encompasses the
p.E139 residue that is required for proton transport by the V-ATPase. Consistent with evolutionary constraints on TM4, only one variant in gnomAD is located in this region of the protein (Figure 2B).

Twelve disease-associated missense variants were examined in yeast. When the uptake of LysoSensor was measured, we saw that nine variants were associated with little to no fluorescence, indicating significant reduction or loss of V-ATPase activity. Intermediate levels of fluorescence were seen when the p.G63A, p.F137L, and p.L150F patient variants were expressed (Figure 6). Growth curves generated by yeast expressing the 12 patient variants mirrored observations from the LysoSensor assay, with most variants resulting in little or no growth, and intermediate levels of growth observed with the p.G63A, p.V74F, p.F137L, and p.L150F variants (Figure 7E). To further examine the effect of these intermediate variants on developmental and neurological function, we modeled p.G63A, p.F137L, and p.L150F in worms. Expression of all three variants resulted in morphological delay as indicated by reduced body size and length at day 1 of adulthood (Figure 9A-B). Mutant worms also exhibited greater levels of paralysis and decreased lifespan when compared to N2 wild type worms, and these phenotypes were exacerbated under osmotic stress (Figure 9E-J). Mutants also exhibited increased activity and wave initiation rates but speed was unaltered, suggestive of hyperactive and uncoordinated movement (Figure 10A-C). The p.A95T variant which showed almost no V-ATPase function when tested in yeast (Figures 6-7), resulted in homozygous sterility in worms, suggestive of a greater impact on V-ATPase function.

Additionally, we functionally examined, in yeast, three variants from gnomAD (p.R48W, p.G103S, p.M131I) that had high CADD scores and were predicted to be damaging by SIFT or Poly-Phen2 which contrasts the assumption that population variants would be benign (Table 2).
Expression of p.R48W and p.M131I in yeast resulted in growth that was more similar to the wild type rescue compared to patient variants at 5mM CaCl$_2$, and did not significantly differ from wild type rescue as higher concentrations of CaCl$_2$ were tested (Figure 7E, 8C-D). p.G103S, which had the highest CADD score of the gnomAD variants tested, resulted in significantly decreased growth compared to the wild type rescue across all CaCl$_2$ concentrations tested (Figure 7E, 8C-D). Nevertheless, p.G103S still yielded a significantly larger eAUC when compared to the best growing patient variant (p.F137L, p = 0.0006, two sample t-test), suggesting the possibility of a level of decrease in V-ATPase activity that can be tolerated. However, we cannot exclude the possibility that the individuals with the p.G103S variant could have a mild neurological deficit. Further testing of population variants could reveal the minimum level of V-ATPase activity required to maintain normal function.

The cellular mechanism that explains the epilepsy in patients with pathogenic variants in ATP6V0C is not yet known; however, we hypothesize that disruptions in GABAergic signaling and the autophagic pathway might be contributing factors. The V-ATPase plays a role in the acidification of SVs within the central nervous system that allows transporters, such as VGLUT1 and VGAT, to load their respective cargo into SVs.$^{60,61}$ The V-ATPase is also involved in neurotransmitter release.$^{62}$ Recent work has revealed the ability of VGLUT1 to use the Cl$^-$ gradient present in SVs after endocytosis to load glutamate without a H$^+$ gradient; however, VGAT appears to rely solely on the H$^+$ gradient generated by the V-ATPase to load GABA into SVs.$^{60,61}$ Consistent with this prediction of altered GABAergic signaling, worms expressing the p.G63A, p.F137L, and p.L150F variants displayed greater sensitivity to aldicarb and higher rates of paralysis when compared to control N2 worms (Figure 10D).
In addition to deficits in SV loading and fusion, perturbations of lysosomal and autophagy pathways may also contribute to the epilepsy and neurodevelopmental disorders seen in these patients. The V-ATPase plays an essential role in acidifying endosomes, lysosomes, autophagosomes, etc., which ultimately creates the environment needed for proper trafficking and acid hydrolase function within the autophagy pathway.\textsuperscript{1,2} Previous work by Nakamura et al. demonstrated that the V-ATPase is required for protein degradation from autophagic bodies in yeast vacuoles and Fassio et al. showed impairments in autophagic flux caused by pathogenic variants in $ATP6V1A$.\textsuperscript{14,63} In recent years, the contribution of impaired autophagy to neurodegenerative and neurodevelopmental disorders, including epilepsy, has risen in importance.\textsuperscript{14,64,65} We hypothesize that the epilepsy and other neurodevelopmental disorders seen in patients with pathogenic V-ATPase variants are likely due to a combinatorial effect of impaired SV loading and defects within the lysosomal/autophagy degradation pathway. Further research on $ATP6V0C$ variants will be required to better understand underlying disease mechanisms.

The prevalence of neurodevelopmental disorders, including epilepsy, resulting from variants in $ATP6V0C$ is likely underestimated as this gene, to the best of our knowledge, is not currently included on commercially available epilepsy or intellectual disability gene panels. Of the 23 genes that encode for a subunit of the V-ATPase, ten, including $ATP6V0C$, are associated with disease. Ten additional members of the complex are expressed in the central nervous system, but are currently not associated with disease: $ATP6V0B$, $ATP6V0D1$, $ATP6V0E1$, $ATP6V0E2$, $ATP6V1C1$, $ATP6V1D$, $ATP6V1E2$, $ATP6V1F$, $ATP6V1G1$, and $ATP6V1G2$ (Table S1).\textsuperscript{66} Screening of these genes for mutations in patients with neurodevelopmental disorders, such as epilepsy and intellectual disability should be undertaken. Additionally, longitudinal
studies in patients with \textit{ATP6V0C} mutations, and the identification of additional patients, will play an important role in resolving the full spectrum of co-morbidities associated with altered \textit{ATP6V0C} and \textit{V-ATPase} function.

In summary, we identified 26 patients with heterozygous \textit{ATP6V0C} variants who presented with intellectual disability, developmental delay, and early-onset epilepsy. \textit{In silico} modeling suggests that the majority of patient missense variants interfere with the interaction between the \textit{ATP6V0C} and \textit{ATP6V0A} subunits, and functional testing in yeast suggests that these variants decrease \textit{V-ATPase} function, and impair motor function, growth and lifespan in worms. Further work is needed to elucidate the exact mechanism(s) by which altered \textit{ATP6V0C} function leads to the range of observed clinical phenotypes, and whether other \textit{V-ATPase} subunits not currently known to cause disease harbor pathogenic variants in patients with neurodevelopmental disorders without a current genetic diagnosis.

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References:


FIGURE LEGENDS

Figure 1. V-ATPase Structure.
The peripheral domain (V₁, uppercase letters, in grey) is the site of ATP binding and hydrolysis. The integral domain (V₀, lowercase letters, in purple, red, blue, and yellow) transports protons across membranes. The c-ring (red) is composed of 9 c-subunits (ATP6V0C) and 1 cʺ-subunit (ATP6V0B) and rotates after ATP hydrolysis to bring protons to ATP6V0A (blue). ATP6V0A possess two hemi-channels and a buried arginine residue (p.R735) which are required along with p.E139 in ATP6V0C for proton translocation.
Figure 2. Conservation and Location of ATP6V0C Variants Identified in Patients with neurodevelopmental disorders.

A) Protein alignments showing conservation of affected residues (germline in yellow, mosaic in magenta). Glutamate residue (p.E139) required for proton transport is bolded. The following ATP6V0C protein sequences were used in the alignments: H. sapiens, NP_001685.1; M. musculus, NP_001348461.1; D. rerio, NP_991117.7; D. melanogaster, NP_476801.1; C. elegans, NP_499166.1; S. cerevisiae, NP_010887.3.

B) Lollipop plot showing the transmembrane structure (green) and location of variants throughout ATP6V0C. Missense (blue) and synonymous (grey) variants observed in gnomAD are shown. Patient missense variants are indicated with red (germline) or magenta (mosaic). Numbers below the figure refer to amino acid number. Based on UniProt accession P27449. There is a significant enrichment of patient variants in TM4 (p = 0.006, Fisher’s Exact test).

C) Plot showing tolerance of missense variants across ATP6V0C. Missense tolerance ratio (MTR) calculated using 21 codon window sizes. A MTR score of < 1 indicates intolerance to missense variation while a score > 1 indicates tolerance to missense variation. Dashed lines on the plot denote gene-specific MTRs: green = 5th percentile, yellow = 25th percentile, and black = 50th percentile.
Figure 3. MRIs from Patients with *ATP6V0C* Show Structural Anomalies.
Sagittal TI weighted MRI from patient 18 demonstrating hypoplasia of the corpus callosum (arrows).
Figure 4. Knockdown of the Drosophila Ortholog of ATP6V0C Induces Seizure Activity.
A) Pan-neuronal (elaV-GAL4) RNAi-mediated knockdown of Dmel\Vha16-3, CG32090 using RNAi (102067) is sufficient to increase the recovery time (RT) of third instar larvae to electroshock-induced seizure. Controls consisted of GFP RNAi under elaV-GAL4 or the RNAi (102067) without a driver. B) Seizure-induction due to expression of 102067 RNAi is preferentially rescued by pre-tretament of larvae with levetiracetam (LEV) or topimarate (TOP). Lamotrigine (LAM) and valproate (VAL) were also active, but not phenytoin (PHY). Data shown as mean ± SEM. P = ****<0.0001; ***<0.001, **<0.01; *<0.05, One-way ANOVA with post-hoc comparison (Dunnett’s).
Figure 5. Molecular Modeling of Patient and gnomAD Variants.
A) ConSurf amino acid sequence conservation data for patient (consP) and gnomAD (consG) variants (red = most conserved, blue = least conserved). A binary color representation is shown for patient (subsP) and gnomAD (subsG), indicating whether the variant is an amino acid substitution present (blue) or not present (red) in the multiple sequence alignment used in ConSurf. B) Structure of the V₀ region of human V-ATPase (PDB: 6wlw). Sites of patient (red) and gnomAD (green) variants are shown superposed on ribbon backbone for two ATP6V0C subunits, one next to the ATP6V0A subunit (pink) and one opposite it across the c-ring. The back part of the c-ring is filled with grey and the front part has been omitted for clarity. C) Isolated view of the interaction between patient variants in ATP6V0C and ATP6V0A. The functional amino acid p.E139 is also displayed (magenta).
Figure 6. Patient Variants Show Decreased LysoSensor Fluorescence.
Quantification of average fluorescent intensity for each variant. Variants are grouped based on region of protein they occur denoted by nearest transmembrane domain. Data was normalized with mean of wild type as 100% (denoted by dotted line) and mean of empty vector as 0%. Data shown as mean ± SEM (n = 71-132 cells/variant). A one sample t-test to a hypothetical mean of 100 was conducted with a Bonferroni correction (adjusted α level = 0.0003125). Significance is shown as: #p < 0.0001.
Figure 7. Patient Variants Cause Decreased Growth at 5mM CaCl₂.
A-D) Growth curves of ΔVMA3 S. cerevisiae expressing patient variants when grown in YPD, pH 5.5 with 5mM CaCl₂. In all panels, wild type is shown in black and the empty vector in grey. Mean of 9 replicates per construct is shown with error bars omitted for clarity. Variants are grouped based on region of protein they occur denoted by nearest transmembrane domain.

E) Empirical area under the curve was calculated using Growthcurver. Data was normalized within each plate with wild type as 100% (denoted by dotted line) and empty vector as 0% and is shown as SD ± SEM. A one sample t-test to a hypothetical mean of 100 was conducted with a Bonferroni correction (adjusted α level = 0.0003125). Significance is shown as: ***p = 0.0001, #p < 0.0001.
Figure 8. Growth of Selected Patient Variants at 100mM and 200mM CaCl₂.
Growth curves of ΔVMA3 S. cerevisiae expressing patient variants at A) 100mM CaCl₂ or B) 200mM CaCl₂. In all panels, wild type is shown in black and the empty vector in grey. Mean of 9 replicates per construct is shown with error bars omitted for clarity.
C-D) Empirical area under the curve was calculated using Growthcurver. Data was normalized within each plate with wild type as 100% (denoted by dotted line) and empty vector as 0% and is shown as SD ± SEM. A one sample t-test to a hypothetical mean of 100 was conducted with a Bonferroni correction (adjusted α level = 0.00714 for 100mM and 0.00833 for 200mM). Significance is shown as: ***p = 0.0001, #p < 0.0001.
Figure 9. In presence of osmotic stress, patient variants exacerbate motor dysfunction and reduce lifespan.
A-B) WormLAB analysis of body length and size of worms at day 1 of adulthood. All mutants are shorter and smaller than N2 controls. C-D) Automated analysis of worm movement in liquid culture by WormTracker software. C) In physiological M9 solution, the p.G63A, p.F137L and p.L150F mutants do not show motor deficits. D) In presence of 350mM NaCl concentration the p.G63A (p < 0.0001) and p.F137L (p < 0.0062) mutants show reduced movement scores in liquid culture over 270 minutes. The p.L150F shows a tendency to reduced movement phenotype, which does not result in significance (p = 0.0869). E) All mutants show increased paralysis over 14 days compared to wild type N2 worms (n = 313-317 / strain, p < 0.0001). F-G) In presence of osmotic stress (200 or 300mM NaCl) the paralysis phenotype is exacerbated, leading to almost 100%
paralysis after 8 days for the p.G63A strain (n = 246 – 260 / strain, p< 0.0001). H) The p.G63A, p.F137L and p.L150F mutants have a reduced lifespan compared to wild type worms ( n = 219 – 233 / strain, p< 0.0001). I-J) The p.F137L, p.L150F and particularly p.G63A mutants have reduced lifespans in presence of osmotic stress compared to wild type worms. (200mM NaCl : n = 182 – 228 / strain. 300mM, p< 0.0001) (300mM NaCl: n = 200 – 244 / strain, p< 0.0001). Significance is shown as: *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001 compared to N2 controls.

Figure 10. Patient mutations cause increased uncoordinated movement, neuronal signaling dysfunction in C. elegans.
A-C) Analysis of fine motor movement of worms after 30 minutes in 500mM NaCl liquid culture by WormLAB software after. Mutants show increased activity index and wave initiation (A&B), but no effect on swimming speed (C). D) Synaptic transmission was evaluated by exposing day 1 adult worms to the cholinesterase inhibitor aldicarb. Worms were scored over 2 hours for paralysis. All mutants were hypersensitive to aldicarb treatment compared to N2 worms (n = 236 – 296 / strain, p< 0.0001). Significance is shown as: *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001 compared to N2 controls.
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<td>NA</td>
<td>Cryptogenic focal epilepsy</td>
<td>Infantile spasms, GTCS, At, Myo</td>
<td></td>
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</tr>
<tr>
<td>Intellectual Disabilityd</td>
<td>Too Young for Evaluation</td>
<td>Severe, with regression</td>
<td>NA</td>
<td>Too Young for Evaluation</td>
<td>NA</td>
<td>Severe</td>
<td>Too Young for Evaluation</td>
</tr>
<tr>
<td>Developmental Delay</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes, psychomotor</td>
<td>Yes, non-verbal</td>
<td>No</td>
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</tr>
<tr>
<td>Behavioral</td>
<td>None</td>
<td>ASD</td>
<td>Tantrums, communication related</td>
<td>NA</td>
<td>Head banging and hair pulling</td>
<td>Too young for evaluation</td>
<td></td>
</tr>
<tr>
<td>MRI</td>
<td>20 mo: normal</td>
<td>Normal</td>
<td>30 mo: normal</td>
<td>Cerebellar vermis hypoplasia</td>
<td>21 mo: Widened liquor spaces, thin corpus callosum and delayed myelination</td>
<td>&lt;1yr: mild ventricular dilation</td>
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</tr>
<tr>
<td>Other Features</td>
<td>Bipolar disorder; pulmonary valve stenosis</td>
<td>Hypotonia, seizure-like episodes from 14mo (freezing and eye-rolling) temporary and self-resolved. Not supported as seizures by EEG.</td>
<td>Strong dental enamel defects</td>
<td>Muscle weakness, at 10mo muscle biopsy showed combined complex I and IV defect; feeding difficulties and gastrostomy; slightly thickened left ventricular wall; cryptorchidism</td>
<td>Frequent urinary infections; heart murmur</td>
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</tr>
<tr>
<td>Varianta</td>
<td>Patient 8</td>
<td>Patient 9</td>
<td>Patient 10</td>
<td>Patient 11</td>
<td>Patient 12</td>
<td>Patient 13b</td>
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<td>--------------------</td>
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<tr>
<td></td>
<td>c.220G&gt;T</td>
<td>c.283G&gt;A</td>
<td>c.283G&gt;C</td>
<td>c.284C&gt;T</td>
<td>c.294C&gt;A</td>
<td>c.340_355del16</td>
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</tr>
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<td>Female</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inheritance</td>
<td>de novo</td>
<td>de novo</td>
<td>de novo</td>
<td>de novo</td>
<td>de novo</td>
<td>de novo</td>
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<td>gnomADc</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NA</td>
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<tr>
<td>Poly-Phen2c</td>
<td>Possibly Damaging</td>
<td>Probably Damaging</td>
<td>Probably Damaging</td>
<td>Possibly Damaging</td>
<td>Benign</td>
<td>NA</td>
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<td>SIFTc</td>
<td>Deleterious</td>
<td>Deleterious</td>
<td>Deleterious</td>
<td>Deleterious</td>
<td>Deleterious</td>
<td>NA</td>
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<tr>
<td>Age at Last Visit</td>
<td>8yr</td>
<td>20yr</td>
<td>4yr</td>
<td>25 yr</td>
<td>15yr</td>
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</tr>
<tr>
<td>Growth Parameters at Last Visit</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Height</td>
<td>136cm</td>
<td>68in</td>
<td>115.5cm</td>
<td>171cm</td>
<td>59cm</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-0.65 SD)</td>
<td>(+1.5 SD)</td>
<td>(+3.3 SD)</td>
<td>(-0.78 SD)</td>
<td>(+2.7 SD)</td>
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</tr>
<tr>
<td>Weight</td>
<td>40.6kg</td>
<td>107lbs</td>
<td>19.3kg</td>
<td>72kg</td>
<td>55cm</td>
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</tr>
<tr>
<td></td>
<td>(+1.2 SD)</td>
<td>(-1.3 SD)</td>
<td>(+1.2 SD)</td>
<td>(+0.18 SD)</td>
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<tr>
<td>HC</td>
<td>53cm</td>
<td>53.3cm</td>
<td>12th percentile</td>
<td>59cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-0.9 SD)</td>
<td>(-0.9 SD)</td>
<td>at 13mo</td>
<td>(+2.7 SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seizures (Age at Onset)</td>
<td>Yes (12 mo)</td>
<td>Yes (10 mo)</td>
<td>Yes (10 mo)</td>
<td>Yes (5mo)</td>
<td>Yes (16 mo)</td>
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<td></td>
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<tr>
<td>Seizure Types</td>
<td>GTCS, Ab, FOA</td>
<td>GTCS, FOA, Febrile</td>
<td>GTCS, staring</td>
<td>Febrile, Ab, Myo, Nocturnal T</td>
<td>Focal with secondary generalization</td>
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<td></td>
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<tr>
<td>Intellectual Disabilityd</td>
<td>Severe</td>
<td>Profound</td>
<td>Too Young for Evaluation</td>
<td>Moderate</td>
<td>Yes</td>
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<tr>
<td>Developmental Delay</td>
<td>Yes, motor and speech</td>
<td>Yes, regression to non-verbal</td>
<td>Yes, motor and non-verbal</td>
<td>No</td>
<td>Yes, speaks only in short sentences</td>
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<td></td>
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<tr>
<td>Behavioral</td>
<td>ASD, ADHD, Food seeking</td>
<td>ASD</td>
<td>Picky eater</td>
<td>ASD, ADHD, Aggression</td>
<td>ASD</td>
<td></td>
<td></td>
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<tr>
<td>MRI</td>
<td>Normal</td>
<td>10 mo: Normal 34 mo: Bilateral hippocampal sclerosis</td>
<td>Normal</td>
<td></td>
<td></td>
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<tr>
<td>Other Features</td>
<td>Unusual breathing patterns with “breath-holding”/hyperneic-like fluctuations in respirations (seems semi-purposeful)</td>
<td>Hypotonia starting at 15 months with gait ataxia and intentional tremors</td>
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<td>Hypertrophic cardiomyopathy with moderate LVOT; mitral valve prolapse; mild-moderate mitral valve regurgitation with moderate mitral stenosis; Previous symptoms for cryptorchidism.</td>
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<tr>
<td>Patient 14</td>
<td>Patient 15</td>
<td>Patient 16</td>
<td>Patient 17</td>
<td>Patient 18</td>
<td>Patient 19</td>
<td></td>
<td></td>
</tr>
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<td>-----------</td>
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<td>Female</td>
<td>Male</td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inheritance</td>
<td>de novo</td>
<td>de novo, mosaic</td>
<td>de novo</td>
<td>de novo</td>
<td>de novo, mosaic</td>
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<td></td>
</tr>
<tr>
<td>gnomAD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CADD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA</td>
<td>25.3</td>
<td>25.2</td>
<td>25.5</td>
<td>25.5</td>
<td>24.7</td>
<td></td>
</tr>
<tr>
<td>Poly-Phen2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA</td>
<td>Possibly Damaging</td>
<td>Probably Damaging</td>
<td>Probably Damaging</td>
<td>Probably Damaging</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIFT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA</td>
<td>Deleterious</td>
<td>Deleterious</td>
<td>Deleterious</td>
<td>Deleterious</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at Last Visit</td>
<td>34yr</td>
<td>13yr</td>
<td>17.5yr</td>
<td>9yr</td>
<td>16mo</td>
<td>26mo</td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td>NA</td>
<td>NA</td>
<td>184.8cm (+1.5 SD)</td>
<td>NA</td>
<td>81cm (+0.4 SD)</td>
<td>84cm (-0.89 SD)</td>
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<tr>
<td>Weight</td>
<td>NA</td>
<td>22.68kg (-3.5 SD)</td>
<td>64.6kg (+0.07 SD)</td>
<td>NA</td>
<td>9.3kg (-1.8 SD)</td>
<td>9.24kg (-2.7 SD)</td>
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</tr>
<tr>
<td>HC</td>
<td>56.5cm (+1.97 SD)</td>
<td>NA</td>
<td>56cm (+0.63 SD)</td>
<td>NA</td>
<td>43.5cm (-3.4 SD)</td>
<td>41.5cm (-4.3 SD)</td>
<td></td>
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<tr>
<td>Seizures (Age at Onset)</td>
<td>Yes (30 mo)</td>
<td>Yes (12 yr)</td>
<td>Yes (13 mo)</td>
<td>NA</td>
<td>Yes (6 mo)</td>
<td>NA</td>
<td></td>
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<tr>
<td>Seizure Types</td>
<td>GTCS, focal to bilateral TCS, Ab, FIA</td>
<td>GTCS, Myo, At, FOA</td>
<td>TCS</td>
<td>Multifocal, GTCS</td>
<td>NA</td>
<td></td>
<td></td>
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<tr>
<td>Intellectual Disability&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Mild</td>
<td>Yes</td>
<td>Profound</td>
<td>NA</td>
<td>Too young for evaluation</td>
<td>Too young for evaluation</td>
<td></td>
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<tr>
<td>Developmental Delay</td>
<td>Yes, motor and speech</td>
<td>Yes</td>
<td>Yes, motor and non-verbal</td>
<td>Yes</td>
<td>Yes, motor and speech</td>
<td>Yes, motor and non-verbal</td>
<td></td>
</tr>
<tr>
<td>Behavioral</td>
<td>ASD</td>
<td>Tantrums</td>
<td>Stereotypies</td>
<td>NA</td>
<td>Screams to indicate wants</td>
<td></td>
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<tr>
<td>MRI</td>
<td>Small white matter changes in left hemisphere</td>
<td>12 yr: normal</td>
<td>NA</td>
<td>7 mo: delayed myelination, hypoplastic corpus callosum, frontal atrophy</td>
<td>10 mo: Pineal region cyst</td>
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<tr>
<td>Other Features</td>
<td>Strange odor, especially before or after GTCS; increased amino acids (glycine, alanine and proline) in plasma.</td>
<td>Noisy breathing; mild ataxia</td>
<td>Broad-based gait</td>
<td>Neonatal apneic episodes</td>
<td>Hypotonia, dystonic posturing of upper limbs; poor eye contact</td>
<td>Hypotonia</td>
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<tr>
<td>Variant</td>
<td>Patient 20</td>
<td>Patient 21</td>
<td>Patient 22</td>
<td>Patient 23</td>
<td>Patient 24</td>
<td>Patient 25</td>
<td>Patient 26</td>
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<td>Male</td>
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<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td>Inheritance</td>
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<td>de novo</td>
<td>de novo</td>
<td>de novo</td>
<td>de novo</td>
<td>de novo</td>
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<tr>
<td>gnomAD^b</td>
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<td>0</td>
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<td>CADD^c</td>
<td>23.3</td>
<td>24.3</td>
<td>24.3</td>
<td>25.1</td>
<td>25.1</td>
<td>24.7</td>
<td>NA</td>
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<tr>
<td>Poly-Phen2^d</td>
<td>Possibly Damaging</td>
<td>Probably Damaging</td>
<td>Probably Damaging</td>
<td>Possibly Damaging</td>
<td>Possibly Damaging</td>
<td>Probably Damaging</td>
<td>NA</td>
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<tr>
<td>SIFT^e</td>
<td>Deleterious</td>
<td>Deleterious</td>
<td>Deleterious</td>
<td>Deleterious</td>
<td>Deleterious</td>
<td>Deleterious</td>
<td>NA</td>
</tr>
<tr>
<td>Age at Last Visit</td>
<td>17yr</td>
<td>5yr 8mo</td>
<td>16yr</td>
<td>11yr 6mo</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Growth Parameters at Last Visit</td>
<td>Height: 166cm (-1.2 SD)</td>
<td>109cm (-0.75 SD)</td>
<td>180cm (+1.2 SD)</td>
<td>30.9kg (-1.2 SD)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Weight: 57.2kg (-0.79 SD)</td>
<td>17.2kg (-1.2 SD)</td>
<td>79.6kg (+1.4 SD)</td>
<td>49cm (-3.9 SD)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td></td>
<td>HC: 50.2cm (+0.5 SD)</td>
<td>48cm (-2.4 SD)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Seizures (Age at Onset)</td>
<td>Yes</td>
<td>Yes (3yr 2mo)</td>
<td>Yes (6mo)</td>
<td>Yes (6 mo)</td>
<td>Yes (12 mo)</td>
<td>Yes (2 yr)</td>
<td>NA</td>
</tr>
<tr>
<td>Seizure Types</td>
<td>NA</td>
<td>Febrile seizures; TCS, Myo, Ab</td>
<td>Infantile flexor spasms, T (with asymmetrical limb stiffening)</td>
<td>GTCS, afebrile, TCS, At</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Intellectual Disability^d</td>
<td>Profound</td>
<td>Mild (IQ 71)</td>
<td>Profound</td>
<td>Profound</td>
<td>Mild</td>
<td>Yes, with regression</td>
<td>NA</td>
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<tr>
<td>Developmental Delay</td>
<td>Yes, speech</td>
<td>Yes, motor and speech</td>
<td>Yes, motor and speech</td>
<td>Fine motor delay</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Behavioral</td>
<td>Autistic traits</td>
<td>None</td>
<td>ASD-like sensory, Self-biting and scratching</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>MRI</td>
<td>4.7yr: Diffuse cerebral atrophy with diffuse T2 hyperintense signal involving white matter bilaterally. Bilateral hippocampal sclerosis. Findings consistent with HIE or a metabolic insult</td>
<td>normal</td>
<td>4yr: delayed myelination</td>
<td>Delayed myelination</td>
<td>Corpus callosum agenesis/dysplasia</td>
<td>7, 9, and 16 yrs: Unchanged slightly smaller size of left hippocampus</td>
<td>NA</td>
</tr>
<tr>
<td>Other Features</td>
<td>Frequent falling</td>
<td>viral cerebellitis with transient ataxia at 3y; hypernasal speech</td>
<td>vagus deformities of the feet</td>
<td>Mild scoliosis; no enamel on teeth; soft, brittle nails</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
Table 1. Clinical Presentation of Patients with Variants in *ATP6V0C*.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Based on reference sequence NM_001694.3.</td>
</tr>
<tr>
<td>b</td>
<td>Number of times observed in the gnomAD database (v.2.1.1) out of approximately 200,000 alleles.</td>
</tr>
<tr>
<td>c</td>
<td>CADD, Poly-Phen2 and SIFT score missense variants. Scores obtained using CADD GRCh37-v1.6.</td>
</tr>
<tr>
<td>d</td>
<td>Intellectual Disability can usually be first assessed at 5 years of age</td>
</tr>
<tr>
<td>e</td>
<td>Previously published as Patient T1911 in Carvill <em>et al.</em></td>
</tr>
<tr>
<td>f</td>
<td>ClinVar Variant: VCV000870676</td>
</tr>
<tr>
<td>g</td>
<td>Previously published as DDD4K.04123 in DDD study</td>
</tr>
<tr>
<td>h</td>
<td>Previously published in Ittiwut <em>et al.</em></td>
</tr>
</tbody>
</table>

Abbreviations: Ab = absence, At = atonic, ASD = autism spectrum disorder, FDS = focal dyscognitive seizures, FOA = Focal onset aware (Partial), FIA = Focal impaired aware, GSW = Generalized spike-wave, HC = Head Circumference, HIE = hypoxic ischemic encephalopathy, LVOT = left ventricular outflow tract, MFD = Multifocal discharges, Myo = Myoclonic, NA = not available, T = Tonic, TCS = Tonic-clonic seizures
Table 2. gnomAD Variants Functional Assessed in Yeast.

<table>
<thead>
<tr>
<th>gnomAD Variant&lt;sup&gt;a&lt;/sup&gt;</th>
<th>gnomAD count&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CADD Score&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Poly-Phen2&lt;sup&gt;c&lt;/sup&gt;</th>
<th>SIFT&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.142C&gt;T p.R48W</td>
<td>1</td>
<td>21.2</td>
<td>Benign (0.235)</td>
<td>Deleterious (0.04)</td>
<td>CL1</td>
</tr>
<tr>
<td>c.307G&gt;A p.G103S</td>
<td>2</td>
<td>24</td>
<td>Probably Damaging (0.957)</td>
<td>Deleterious (0.05)</td>
<td>TM3</td>
</tr>
<tr>
<td>c.393G&gt;C p.M131I</td>
<td>1</td>
<td>23</td>
<td>Benign (0.168)</td>
<td>Deleterious (0.04)</td>
<td>TM4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Based on reference sequence NM_001694.3.

<sup>b</sup>Number of times observed in gnomAD (v.2.1.1) out of approximately 200,000 alleles.

<sup>c</sup>CADD, Poly-Phen2 and SIFT scores obtained from CADD GRCh37-v1.6

Abbreviations: CL = cytoplasmic loop, TM = Transmembrane domain