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Hufnagel		X	X	X	X	X		X
Slone		X	X					X
Wang		X	X					X
Homan		X	X					X
Gutzwiller		X	X					X
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Leslie, N.	X		X					X
Xiao, J.		X	X	X	X	X		X
Hedera		X	X	X	X	X		X
LeDoux		X	X	X	X	X		X
Gebelein		X	X					X
Wilbert		X	X					X
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## A Novel Variant of *ATP5MC3* associated with both dystonia and spastic paraplegia

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## Abstract

**Background:** In a large pedigree with an unusual phenotype of spastic paraplegia or dystonia and autosomal dominant inheritance, linkage analysis previously mapped the disease to chromosome 2q24-2q31.

**Objective:** To identify the genetic etiology and molecular basis of an unusual autosomal dominant spastic paraplegia and dystonia.

**Methods:** Whole exome sequencing following linkage analysis was used to identify the genetic etiology in a large family. Cosegregation analysis was also performed. An additional 384 individuals with spastic paraplegia or dystonia were screened for pathogenic sequence variants in the adenosine triphosphate (ATP) synthase membrane subunit C locus 3 gene (*ATP5MC3*). The identified variant was submitted to the “GeneMatcher” program for recruitment of additional subjects. Mitochondrial functions were analyzed in patient-derived fibroblast cell lines. Transgenic *Drosophila* carrying mutants were studied for movement behavior and mitochondrial function.

**Results:** Exome analysis revealed a variant (c.318C>G (p.Asn106Lys) (NM\_001689.4) in *ATP5MC3* in a large family with autosomal dominant spastic paraplegia and dystonia that cosegregated with affected individuals. No variants were identified in an additional 384 individuals with spastic paraplegia or dystonia. GeneMatcher identified an individual with the same genetic change, acquired *de novo*, who manifested upper limb dystonia. Patient fibroblast studies showed impaired complex V activity, ATP generation, and oxygen consumption. *Drosophila* carrying orthologous mutations also exhibited impaired mitochondrial function and displayed reduced mobility.

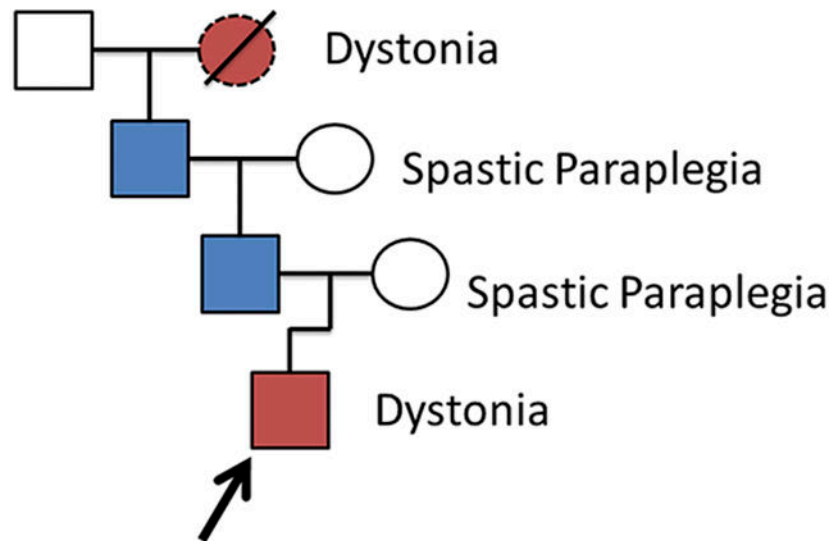
**Conclusions:** A unique form of familial spastic paraplegia and dystonia is associated with a heterozygous *ATP5MC3* variant that also reduces mitochondrial complex V activity.

## TOC Graphic summary

Family-1 (partial) pedigree: symptomatic proband, father, and grandfather presented for neurological consultation. Subsequent investigations identified, in two families, a variant in *ATP5MC3*, which encodes a component of complex V in the mitochondrial electron transport chain. Functional studies in patient-derived fibroblasts and *Drosophila* support a loss of function.

## Graphical Abstract

## Family History



### Introduction

*Dystonia* and *spastic paraplegia* denote neurological conditions with distinct signs and neuroanatomic localization.<sup>1–5</sup> Here, we report evidence from two unrelated families that a novel missense variant in the adenosine triphosphate (ATP) synthase membrane subunit C locus 3 gene (*ATP5MC3*; formerly *ATP5G3*), may cause either condition. After initial linkage analysis in a large, multigenerational family (Family-1) with autosomal dominant inheritance, where affected members manifested with generalized dystonia and/or spastic paraplegia,<sup>6</sup> genetic studies in this and a second family (Family-2) with a proband with generalized dystonia identified an identical *de novo* variant in *ATP5MC3*. We also report results of studies of function of the resulting protein. The structure of subunit c of the mitochondrial  $F_0$  portion of ATP synthase, in complex V of the electron transport chain, is highly conserved. A spontaneous mutation in the *E. coli* ortholog *atpE* at the same position uncouples ATP hydrolysis from proton translocation.<sup>7</sup> Thus, we performed experiments to evaluate whether this human variant would similarly affect mitochondrial function and found supportive evidence in both patient cell lines and *Drosophila*.

### Patients and Methods

#### Protocol Approval and Consents

Written informed consent, as approved by the Institutional Review Board of Cincinnati Children's Hospital Medical Center, was obtained from all the participating members of Family-1. Additional dystonia and spastic paraplegia subjects were obtained under IRB approval by the University of Tennessee Health Science Center. For Family-2, written

informed consent was obtained from the proband and parents under ethics review board-approved protocols from Technical University of Munich, Germany.

### Whole Exome and Sanger Sequencing

*Family-1* Genomic DNA was isolated from blood using Puregene reagents (Qiagen, Germantown, MD). Whole Exome Sequencing (WES) was performed in the proband and distant cousin with childhood-onset generalized dystonia, restricted to the previously mapped 2q24-31 locus.<sup>6</sup> In the remaining family members, WES was performed by the Cincinnati Children's DNA Sequencing and Genotyping Core. Exome preparation used NimbleGen EZ Exome (Roche, Indianapolis, IN). Sequences were read on an Illumina HiSeq2000 (San Diego, CA). Sanger sequencing was used to determine the *ATP5MC3* mutation for segregation analysis. PCR was performed using primers (5'-TCAGTTTACAAAATCCCCACA-3') and (5'-CAGTTTTTCGGAGTCACAGTAAGA-3') and FastStart TAQ (Roche, Indianapolis, IN). PCR products were treated with ExoSAP-IT (Affymetrix, Santa Clara, CA) and sequenced using BigDye v1.1 chemistry on a 3730XL DNA Analyzer (Life Technologies, Grand Island, NY).

*Family-2* parent-offspring trio WES was pursued using previously described methods.<sup>8,9</sup> Genomic DNA was isolated from whole blood and exonic sequences from DNA were enriched with the SureSelect Human All Exon 60Mb Kit (Agilent Technologies). Paired-end (100-bp) sequencing was done on a HiSeq4000 machine (Illumina) following manufacturer guidelines (sequencing parameters for the trio: generated raw data: 9.7Gb – 10.7Gb; mean coverage: 103x – 112x; >20x-coverage: 97.1% - 97.7%). For variant calling, annotation, and prioritization, we used an established, custom-developed bioinformatics pipeline (Helmholtz Center Munich and Technical University of Munich, Germany). Raw reads were mapped to human reference gene sequences based on genome build hg19/GRCh37 with the Burrows-Wheeler Aligner tool and variant calls were generated with the help of SAMtools, PINDEL, and ExomeDepth. Only calls that met high-confidence quality thresholds (read depth >10x, Phred quality score >30) were retained.

### Bioinformatic Analysis

The Family-1 proband's WES data was searched for rare (minor allele frequency < 0.005 in gnomAD v2.1.1 and ~20,000 in-house controls) protein-altering variants in previously described dystonia-related genes.<sup>8</sup> Next, candidate disease-associated variants were prioritized under both dominant and recessive inheritance models. A single novel, heterozygous variant was identified: chr2:176,043,103C>G (GRCh37) resulting in a missense variant in *ATP5MC3*: NM\_001689.5 c.318C>G (p.Asn106Lys) and was confirmed by bidirectional Sanger sequencing in the proband (Figure 1).

To identify additional individuals with similar clinical phenotype and heterozygous variants in *ATP5MC3*, the Family-1 variant was submitted to GeneMatcher,<sup>10</sup> where a match to Family-2 was identified.

Allele frequency was analyzed using the gnomAD v2.1.1 and v3.1.1 databases.<sup>11</sup> Predictions of the pathogenicity were evaluated with SIFT/PROVEAN (<http://sift.jcvi.org/>), Mutation Taster (<http://www.mutationtaster.org/>), Polyphen2 ([\*Mov Disord.\* Author manuscript; available in PMC 2023 February 01.](http://</a></p></div><div data-bbox=)

genetics.bwh.harvard.edu/pph2/), and CADD (<http://cadd.gs.washington.edu/score>) software.<sup>12–16</sup> Conservation alignments were performed using UCSC Genome Browser (<http://genome.ucsc.edu/>), Wormbase ([wormbase.org](http://wormbase.org)), and Flybase ([flybase.org](http://flybase.org)).<sup>17–19</sup>

### Screening for variants in additional individuals with undiagnosed dystonia and spastic paraplegia

Samples from patients with dystonia<sup>20</sup> (n=362) and HSP (n=22) (see clinical data in Supplemental Table 1), who had been pre-screened and tested for prior known causes of dystonia and HSP, were evaluated by high resolution melting point (HRM) analysis to search for variants in the three *ATP5MC* genes (see Supplementary Table 2). The coding region of *ATP5MC3* was Sanger sequenced in subjects with HSP.

HRM analyses were performed using the LightCycler® 480 Real-Time PCR system and High Resolution Master Mix from Roche Applied Science.<sup>20</sup> Optimized HRM reactions were performed in 384-well plates using 20 ng of template DNA, 1X HRM Master Mix, 2.5 mM MgCl<sub>2</sub> and 200 nM of each primer in a 10- $\mu$ l reaction volume. Using LightCycler 480 Gene Scanning Software, melting curves and difference plots were analyzed. All samples were unambiguously assigned to genotypes by Gene Scanning software.

For sequencing, fourteen pairs of PCR primers were designed with Primer3 ([frodo.wi.mit.edu](http://frodo.wi.mit.edu)) to encompass the coding regions of the three *ATP5MC* genes (Supplementary Table 3). Five  $\mu$ l of the PCR products were cleaned using ExoSAP-IT® (United States Biochemical, Cleveland, Ohio). Then, 1-2  $\mu$ l of the purified PCR products were sequenced in the forward and reverse directions using the Applied Biosystems 3130XL Genetic Analyzer.

### Drosophila production

*ATPsynC* cDNA (Annotation symbol: *CG1746*) was isolated from wild type embryonic RNA by RT-PCR using SuperScript III (Life Technologies) and primers (5'-GCCTGTCAACCAATATCTCC-3') and (5'-TGCCAACATGTCAAGCAGTAA-3'). The cDNA was cloned into the TOPO-TA Cloning vector (Life Technologies) and modified by site-directed mutagenesis with QuikChange II (Agilent, Santa Clara, CA). Mutagenesis primers for the human-disease-equivalent p.N102K were (5'-CGGCTACGCCAGGAAGCCATCGCTGAAA-3') and (5'-TTTCAGCGATGGCTTCCTGGCGTAGCCG-3'). For the *E. coli* equivalent mutation p.N102E, the primers were (5'-ATCGGCTACGCCAGGGAGCCATCGCTGAAACAG-3') and (5'-CTGTTTCAGCGATGGCTCCCTGGCGTAGCCGAT-3'). The wild-type, p.N102K and p.N102E mutant constructs were then cloned into the pUAST-attB expression vector.<sup>21</sup> Transgenic fly lines containing a single copy of each UAS construct, specifically integrated at the 51C locus on chromosome 2, were generated using phi3C integrase (performed by Rainbow Transgenic Flies, Inc, Camarillo, CA). Each UAS line was subsequently crossed to either the *armadillo* (*arm*)-*Gal4*, *elav-Gal4*, *tub-Gal4*, or *24B2-Gal4* driver lines available from the Bloomington Stock Center. All *Drosophila* lines were cultured and experiments performed at 25°C.

### Human cell lines and culture conditions

Human fibroblast cells from the Family-1 proband and his father as well as from unrelated, age- and cell-passage matched, de-identified controls were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS) at 37 °C and with 5% CO<sub>2</sub>.

### Drosophila mitochondria isolation

Mitochondria were isolated.<sup>22, 23</sup> In brief, approximately 300 flies were immobilized by chilling on ice. 4 ml of ice-cold isolation medium (250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA, 1% (w/v) bovine serum albumin, pH 7.4 at 4°C) was added. The flies were gently crushed in cold isolation medium with a homogenizer and the homogenate centrifuged at 1000 g for 5 min at 4°C. The supernatant was recentrifuged at 10000 g for 10 min at 4°C and discarded and the mitochondrial pellet was saved.

### Mitochondrial respiratory chain complex I-V activity assay, ATP measurement, and oxygen consumption rate

Methods were modified from previously published protocols.<sup>23</sup> In brief, Complex activities were determined as follows: Complex I (NADH dehydrogenase) as the rotenone-sensitive NADH oxidation at 340 nm, using the coenzyme Q analogue 2,3-dimethyl-5-methyl 6-n-decyl-1,4- benzomethyluinone (DB) as an electron acceptor; Complex II (succinate dehydrogenase) by tracking the secondary reduction of 2, 6-dichlorophenolindophenol by ubiquinone-2 at 600 nm; Complex III (cytochrome bc1 complex) by measuring the reduction of cytochrome c at 550 nm with reduced decylubiquinone; Complex IV (cytochrome c oxidase) by monitoring the oxidation of reduced cytochrome c as a decrease of absorbance at 550 nm; Citrate synthase by the reduction of 5,5'- dithiobis-2-nitrobenzoic acid at 412 nm in the presence of acetyl- CoA and oxaloacetate. For analysis, Complex I-IV activities were normalized to citrate synthase activity. Complex V activity was measured by using the Mitochondrial Complex V Activity Assay kit (EMD Milipore, Billerica, MA) with modifications.<sup>24</sup> The assay relies on linking the ATPase activity to NADH oxidation via the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase (PK) and then pyruvate to ATP production measurement.

The ATP level in cultured cells was determined using the ATP Bioluminescence Assay Kit CLS II (Roche, Indianapolis, IN). ATP was measured by luminescence signal and the ATP production was determined as luciferase activity<sup>22</sup> and lactate by lactate dehydrogenase. The change in absorbance was recorded at 340 nm.

The oxygen consumption rate (OCR) measurements were performed by using a Seahorse Bioscience XF-24 instrument (Seahorse Biosciences, North Billerica, MA, USA). Cells were seeded in XF24-well micro plates in growth medium, which was replaced the next day with 675 µl of assay medium.<sup>23</sup>

### Testing of effects of ATP5MC3 mutations on Drosophila mobility

Drosophila mobility was evaluated using geotaxis in a ten chamber countercurrent device.<sup>25</sup> Flies were phenotypically sorted with humidified carbon dioxide anesthesia and then

returned to a 25°C incubator for a 24 hour recovery period prior to testing. Thirty to fifty flies were introduced into the first lower chamber, tapped to the bottom, and observed for 15 seconds as they climbed the 8 cm distance into the top chamber. At each 15 second interval, the upper chambers were shifted along a sliding mechanism, the flies tapped to the bottom, and the upper chambers returned to their initial position (Supplementary video). Thus, fast climbers enter the upper chamber and are progressively transferred into adjoining chambers, whereas slower flies remain in the same tube. Results are presented as “tube distance,” which represents the average lateral distance traveled, but can be understood as the average number of cycles that a population of flies will travel to the upper chamber.

## Results

### Clinical information for Family-1

Dystonic and spastic paraplegic phenotypes of the affected individuals were previously reported.<sup>6</sup> In brief, the proband had unilateral upper limb onset at age 2 years that generalized to trunk and all limbs, resulting in loss of ambulation. Bilateral pallidal deep brain stimulation, implanted at age 9 years, restored ambulation and limb use. At age 25 years, his response remains excellent. The proband’s father and grandfather manifest the HSP phenotype with onset of progressive leg weakness and spasticity after age 20 years. The autosomal dominant pedigree included 40 individuals, of which 18 were affected with either childhood-onset, progressive generalized dystonia or adult-onset, gradually progressive spastic paraplegia. Less severe, later-onset, focal limb and neck dystonia and spasmodic dysphonia also occurred, possibly related to trauma or overuse. Brain MRIs and peripheral nerve physiology, when performed, were normal. Metabolic studies, including whole blood lactate and pyruvate, serum amino acids, urine organic acids, and acylcarnitine profiles from blood and urine were normal in both the proband and his father.

### Clinical information for Family-2

A second family with the c.318C>G (p.Asn106Lys) variant was independently identified (Family-2). The proband of this family was a right-handed male, born to healthy unrelated parents of European descent with no family history of dystonia or related movement disorders, who first presented to the Department of pediatric neurology in Freiburg (Germany) at the age of 11 years with a 4-year history of gradually worsening upper limb dystonia. The proband in this family was diagnosed with a childhood-onset dystonic syndrome and enrolled in a research project focusing on the molecular bases of dystonia (Helmholtz Center Munich and Technical University of Munich, Germany). The proband’s progressive condition prompted a comprehensive workup. However, all laboratory studies of blood, urine, and cerebrospinal fluid and testing for dystonia-causing mutations via gene panel analysis were unrevealing. Brain MRI revealed nonspecific mild frontoparietal white-matter changes bilaterally without other associated abnormalities. However, parent-offspring trio WES revealed a NM\_001689.4:c.318C>G (p.Asn106Lys) variant as a heterozygous, *de novo* mutation in the proband (Supplementary Figure 1).



## Candidate gene analyses

Whole exome analysis on the proband and distant cousin with childhood onset dystonia, restricted to novel shared coding variants within the linkage region, identified a single missense variant, (GRCh37/hg19, NC\_000002.11:g.176043127G>C, NM\_001689.4:c.318C>G, NP\_001680.1:p.(Asn106Lys), within *ATP5MC3*. Consistent with the pedigree phenotypes, Sanger sequencing showed only affected individuals carrying the *ATP5MC3* variant.

*ATP5MC3* encodes for the transmembrane-spanning subunit c of the F<sub>0</sub> portion of mitochondrial ATP synthase. The substituted amino acid exists in a highly conserved loop structure that directly interacts with the F<sub>1</sub> subunit (Fig 1).<sup>26</sup> This interaction produces the coupled rotation needed to transduce the potential energy of the proton gradient into the phosphorylation of ADP.<sup>27</sup> This loop structure is conserved in all vertebrates examined, as well as *C. elegans* (*Y82E9BR.3*) and *Drosophila melanogaster* (*CG1746*). The p.Asn106Lys variant has not been documented in gnomAD v2.1.1 or v3.1.1.<sup>11</sup> Computational analyses predict the proband's variant to be damaging by all metrics evaluated, including PolyPhen2 HDIV-score (1.0), MutationTaster score (1), REVEL rankscore (0.75), PROVEAN score (score -5.5), SIFT score (score 0.0), and CADD PHRED score (25.4, GRCh37-v1.6; 24.7, GRCh38-v1.6). Two other extremely rare variants altering Asn106 have been identified, in one individual each, in gnomAD v2.1.1 (p.Asn106Ser) and in v3.1.1 (p.Asn106Tyr). While these are also predicted to be damaging, it is unknown whether these variants were associated with neurological symptoms. Globally, *ATP5MC3* is not highly constrained to missense variation (67% of the expected number missense variants were observed in gnomAD v2.1.1), but such constraint would not be expected for a condition that does not limit reproductive fitness.

*ATP5MC1* and *ATP5MC2* also encode for subunit c. These proteins differ in their N-terminal mitochondrial import sequences while their C-terminal cleavage products, which give rise to the mature subunit c, remain identical.<sup>26</sup> To identify additional subunit c variants in patients with dystonia or spastic paraplegia, 384 patients were screened for variants in *ATP5MC1*, *ATP5MC2*, and *ATP5MC3*. No deleterious variants were identified in the cohort of patients with dystonia or spastic paraplegia (Supplementary Table 1). Identified sequence variants are included in Supplementary Table 2.

## Mitochondrial studies

In fibroblasts from skin biopsies from the Family-1 proband (dystonia) and his affected father (HSP), activities of electron transport chain complexes I through IV were equivalent to controls. However, Complex V activity was reduced by 20% ( $p < 0.01$ ) (Figure 2). Similarly, cellular ATP was reduced 20% to 32% and oxygen consumption 21% to 25% (Figure 2). As loss of one of six genomic copies of *ATP5MC1/2/3* would result in an expected reduction of approximately 17% of complex V activity, these results support the predicted pathogenicity of the p.Asn106Lys mutation. In *E. coli*, subunit c of ATP synthase is encoded by *atpE*. A spontaneous mutation of the equivalent amino acid p.Gln42Glu leads to uncoupling of ATP hydrolysis from proton translocation.<sup>7</sup> Uncoupling of oxidative

phosphorylation, however, was not supported as a mechanism for the human mutation, as both oxygen consumption and ATP were proportionally reduced.

### **Drosophila mutation constructs and geotaxis**

*Drosophila* have one homologous copy of *ATP5MC3* (*CG1746*). Tissue-specific expression of wild type and mutant *ATP5MC3* proteins was performed using the GAL4-UAS transgenic system in which the UAS enhancer sequence activates gene expression in the presence of GAL4.<sup>28</sup> Stable transgenic flies were produced with wild type and mutated forms of *CG1746* (p.N102K and p.N102E) under control of UAS. Multiple GAL4 drivers were tested. The muscle-specific *24B2* and the neuronal-specific *elav* GAL4 drivers produced no discernible phenotypic effects. The constitutive *tubulin*-Gal4 driver was non-viable in all expression lines, including wild-type, precluding further characterization (data not shown). The *armadillo* (*arm*) driver (homologous to human beta-catenin) provided a milder constitutive driver with demonstrable phenotypic effect.

The UAS-response elements were inserted on chromosome 2 and *arm-GAL4* on chromosome 3, allowing for breeding strategies to produce homozygous alleles on both chromosomes. Double homozygous strains of the *arm-GAL4* and *UAS-p.N102K* were non-viable. As such, flies bearing a single copy of *arm-GAL4* and homozygous copies of the UAS-response elements were used.

At less than one week of age, the human disease mutant subunit c (p.N102K) and the equivalent bacterial mutation<sup>7</sup> (p.N102E) demonstrated reduced mobility relative to wild type flies and the *arm-GAL4*; *UAS-WT* construct (Fig 3A; Supplementary Video). In serial experiments, Age and Genotype both reduced geotaxis ( $F_{4, 1873} = 292.2$ ;  $p < 0.0001$ ). Genotype had a larger effect than Age; the Age\*Genotype interaction was non-significant (Fig 3B). Both mutants were slower than wildtype ( $p < 0.0001$ ). The human p.N102K mutant was slowest.

In *Drosophila* mitochondria, concordant with the results for geotaxis, the Complex V activity was reduced by 53% for the human p.N102K mutant ( $p = 0.0008$ ) and by 37% for the bacterial p.N102E mutant ( $p = 0.003$ ) (Fig 4). In negative control lines, Complex V activity was similar to wild type for all UAS-response elements without expression drivers, further supporting that the *ATP5MC3* variant (c.318C>G, p. Asn106Lys) is pathogenic.

### **Discussion**

In a large, single-family pedigree manifesting an unusual combination of autosomal dominant dystonia and spastic paraplegia, we report the presence of a novel variant in *ATP5MC3*, a nuclear gene which encodes the c subunit of mitochondrial Complex V. Dystonia in this pedigree can generalize if manifest in childhood, shows incomplete penetrance, and responds to pallidal deep brain stimulation. The spastic paraplegia presents in adulthood, progresses gradually, and is not accompanied by other neurological problems. The pathogenicity of this variant for dystonia has been confirmed by the identification of the same variant in a patient with upper limb dystonia from a second, independent family. *ATP5MC3* remains the primary candidate for the spastic paraplegia in the studied family and

should be considered a target for further study, with evidence for causality based on 2015 American College of Medical Genetics and Genomics/Association for Molecular Pathology Standards and Guidelines<sup>29</sup> including strong functional studies, low frequency in the general population, supportive *in silico* predictions, and cosegregation.<sup>30</sup>

To date, variants in *ATP5MC3* have not been reported in other human diseases, nor could we identify a similar mutation in any additional patients manifesting dystonia or spastic paraplegia. To further address the pathogenicity of this variant, we undertook additional experiments to elucidate function and clarify possible pathophysiological mechanisms.

*ATP5MC3* encodes a component of ATP synthase (electron transport chain complex V), which is critical to oxidative phosphorylation, the aerobic process by which energy substrates are converted to ATP.<sup>26</sup> The electron transport chain uses energy substrates to form an electrochemical gradient of protons. ATP synthase then uses the proton gradient as an energy source to drive conversion of ADP to ATP. To do this, the protons are translocated across the inner mitochondrial membrane by the F<sub>0</sub> subunit of complex V. As the protons traverse, they induce a structural change in F<sub>0</sub> causing its rotor component to engage and rotate the F<sub>1</sub> subunit. This rotation brings ADP and inorganic phosphate into alignment, allowing phosphorylation.<sup>31</sup> *ATP5MC3* encodes the monomer, subunit c, which aggregates to form the F<sub>0</sub> rotor. The variant in these two families substitutes asparagine for lysine at position 106, in a loop of *ATP5MC3* with complete homology from human to *Drosophila*. This conserved loop acts as the point of contact between F<sub>1</sub> and F<sub>0</sub>.<sup>32</sup> As such, we hypothesized that the p.Asn106Lys variant would impair oxidative phosphorylation by disrupting this point of contact.

Understanding pathophysiology of this variant should account for the fact that two paralogs, *ATP5MC1* and *ATP5MC2*, also encode subunit c. These functionally identical proteins have divergent mitochondrial import sequences which are cleaved following import.<sup>33</sup> Assuming equal expression of the three alleles, haploinsufficiency would be unlikely to account for the severe phenotypes, as 83% of the active protein would still remain. Thus, we hypothesized that the presence of the mutant allele interfered with the F<sub>1</sub>-F<sub>0</sub> interaction which could be tested through overexpression.

To this end we conducted several experiments, all of which support the pathogenicity of this *ATP5MC3* mutation. First, *in vitro* functional studies of fibroblasts from the proband of Family-1 and his father demonstrated a reduction of complex V activity. Second, overexpression of the mutant form of the homolog in *Drosophila* resulted in statistically significant impairment of mobility (geotaxis), whereas overexpression of wildtype protein did not. Third, as a naturally occurring mutation, p.N102E, in the homologous *E. coli* gene *atpE* reduces Complex V function and ATP production (while preserving proton translocation),<sup>7</sup> we studied this mutation and found that both *Drosophila* geotaxis and complex V activity were significantly reduced, although to a lesser extent than with the human mutation. Finally, the human mutation reduced complex V activity in *Drosophila* with a severity that matched that observed motor slowing in the geotaxis assays. Taken together, these experiments strongly support the pathogenicity of this *ATP5MC3* variant.

Further study may clarify mechanisms of phenotypic heterogeneity. Ten to fourteen copies of subunit c create the transmembrane spanning domain of F<sub>0</sub>. The relative composition of normal versus mutant subunit c in the overall structure will likely influence the function of complex V. In *Drosophila*, different GAL4 drivers and their relative dosage produced different degrees of severity. Thus, we suspect that relative expression of the mutated gene may mediate phenotypic expression. The specific regulatory control of *ATP5MC* genes remains unstudied, but expression assays suggest that the expression levels of *ATP5MC1*, 2, and 3 respond independently when exposed to the same stressor.<sup>34–40</sup> Differential expression patterns for *ATP5MC* genes within the nervous system (e.g. striatum versus spinal cord) or resulting from environmental factors such as oxidative stress,<sup>39–42</sup> might increase the proportion of mutant *ATP5MC3*, influencing the phenotype.

Although this pedigree has unusual features, other dystonias and spastic paraplegias may involve similar pathophysiological mechanisms.<sup>3</sup> For example, variants in *MTATP6* cause mitochondrially-inherited neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) syndrome,<sup>43</sup> infantile bilateral striatal necrosis,<sup>44</sup> and Leigh Syndrome.<sup>45</sup> Two extremely rare and severe autosomal recessive diseases, resulting from mutations in transmembrane protein *TMEM70*<sup>46</sup> and *ATPAF2*,<sup>47</sup> involve defective F1 complex assembly. Other mitochondrial diseases can also present with secondary dystonia.<sup>48</sup> Spastic paraplegias involving mitochondrial pathology can be pure or complex.<sup>49</sup> These include a late onset HSP-like disorder linked to ATP synthase 6<sup>50</sup> as well as a variable onset, autosomal dominant variant of neurodegeneration with iron accumulation type-4, NBIA/HSP-*C19orf12*, due to deletion the *C19ORF12*.<sup>51, 52</sup>

In conclusion, in a large pedigree with an unusual autosomal dominant form of dystonia and spastic paraplegia, we identified a cosegregating p. Asn106Lys variant of *ATP5MC3*. The association with dystonia has been confirmed in a second family, supporting pathogenicity for the dystonia phenotype. Functional experimental studies show this mutation reduces complex V activity and, in a *Drosophila* model, impairs mobility. Further studies are needed to clarify the mechanisms whereby disorders involving energy metabolism may induce neurological disease with varying phenotypes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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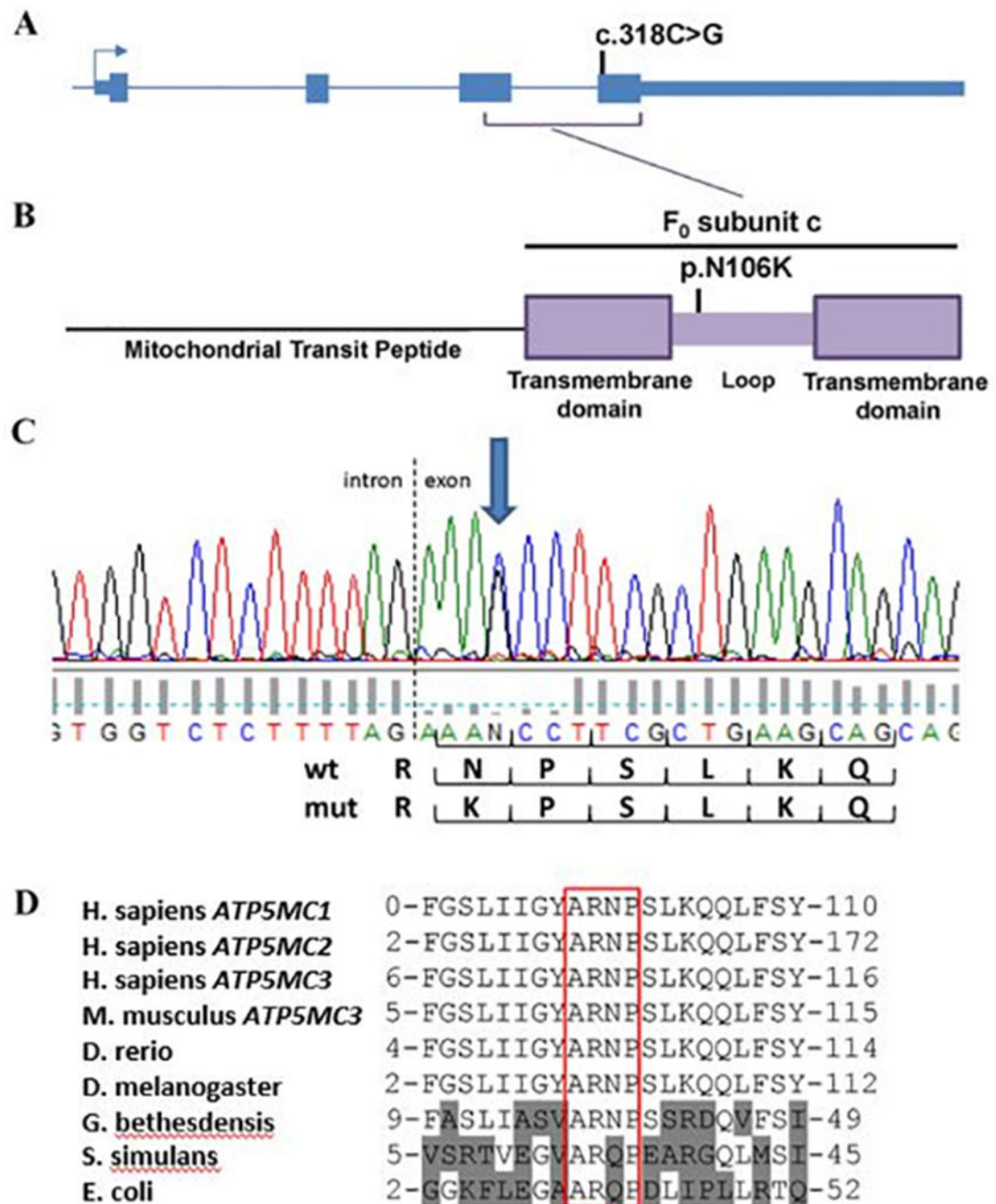
This work was previously presented in part at the 65th Annual Meeting of the American Academy of Neurology, San Diego, CA, March 21, 2013; at the 17<sup>th</sup> International conference of Parkinson's disease and Movement Disorders, Sydney, Australia June 19, 2013; and at the 63<sup>rd</sup> Annual Meeting of the American Society of Human Genetics, Boston, MA, October 22, 2013.

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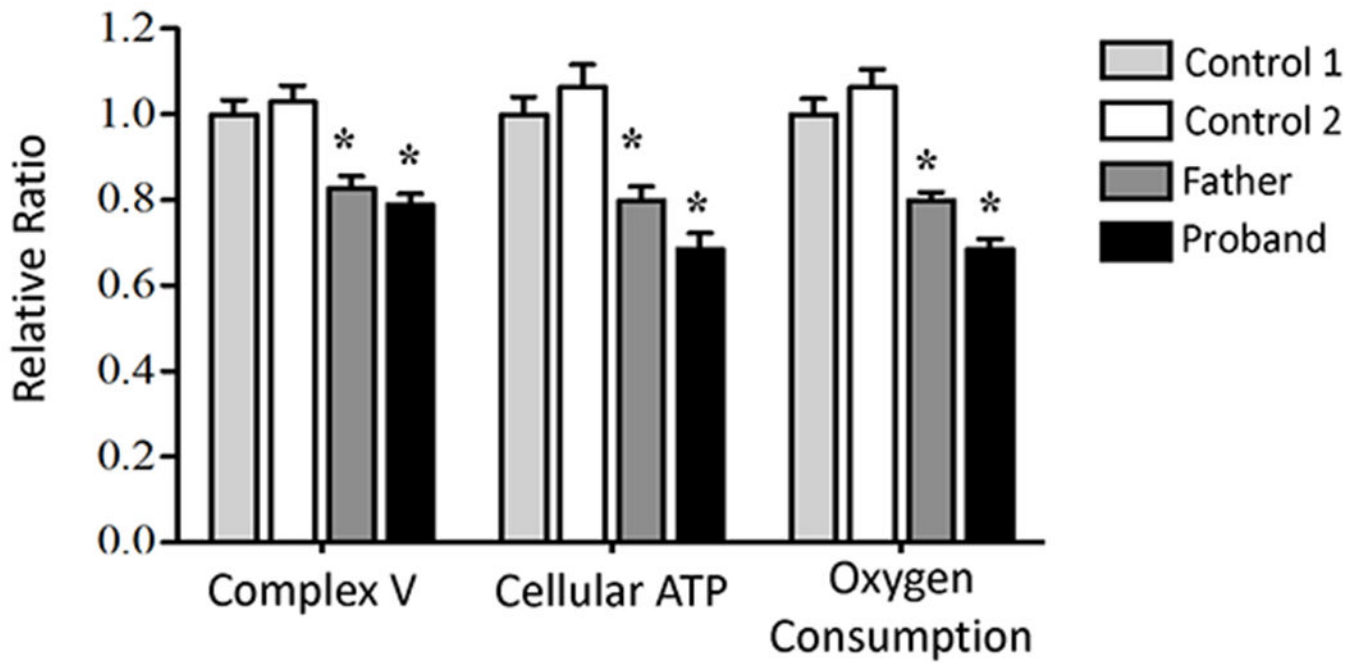
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**Figure 1. ATP5MC3 mutation and conservation analysis.**

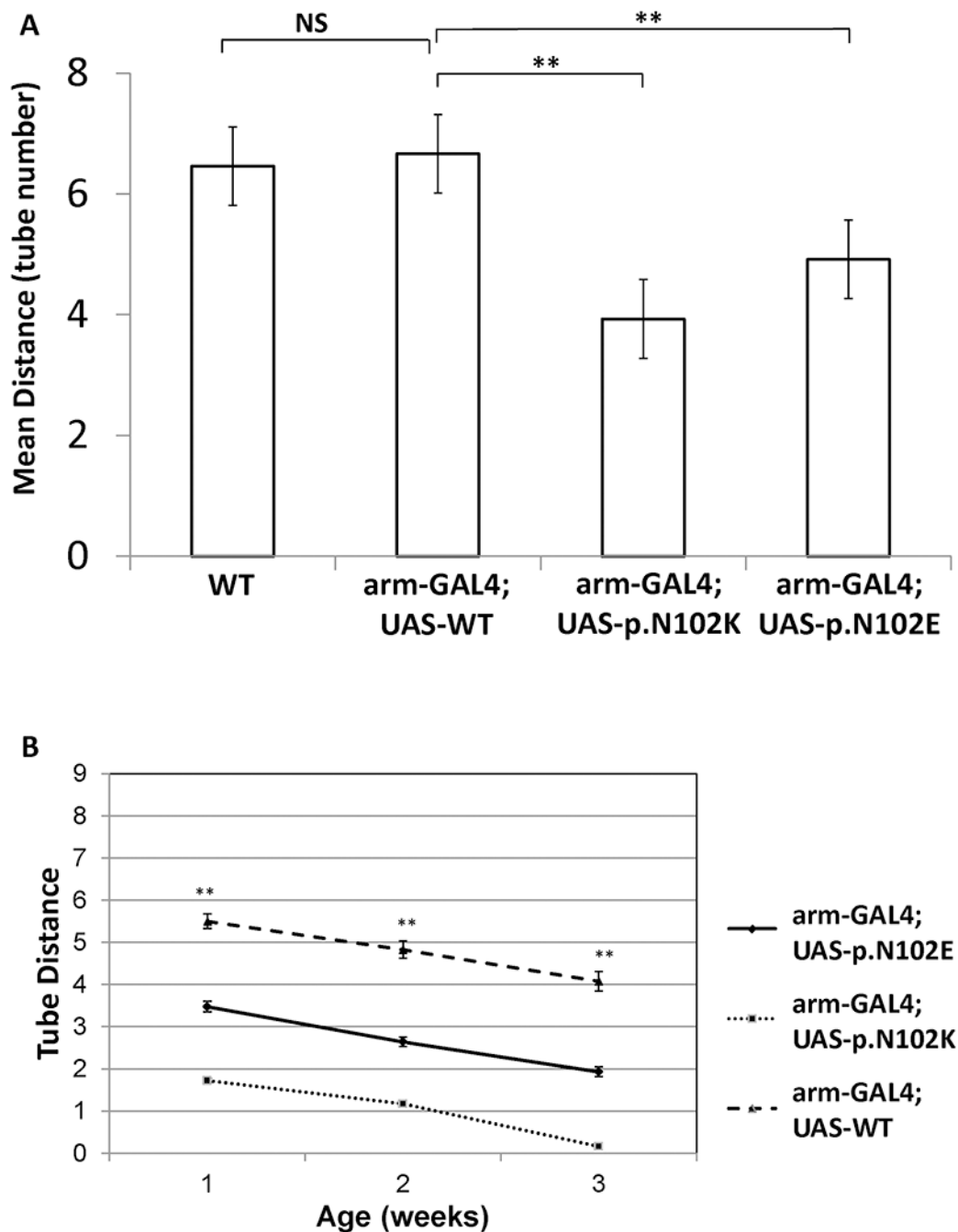
(A) c.318C>G mutation in exon 4 of *ATP5MC3* resulting in (B) p.Asn106Lys substitution in the c subunit. (C) Electropherogram demonstrating heterozygous mutation near the start of exon 4. (D) ARNP motif conserved among c subunit genes *APT5MC1–3*, *Drosophila*, and bacteria including *G. bethesdensis*. Gln is substituted for Asn in *S. simulans* and *E. coli*.





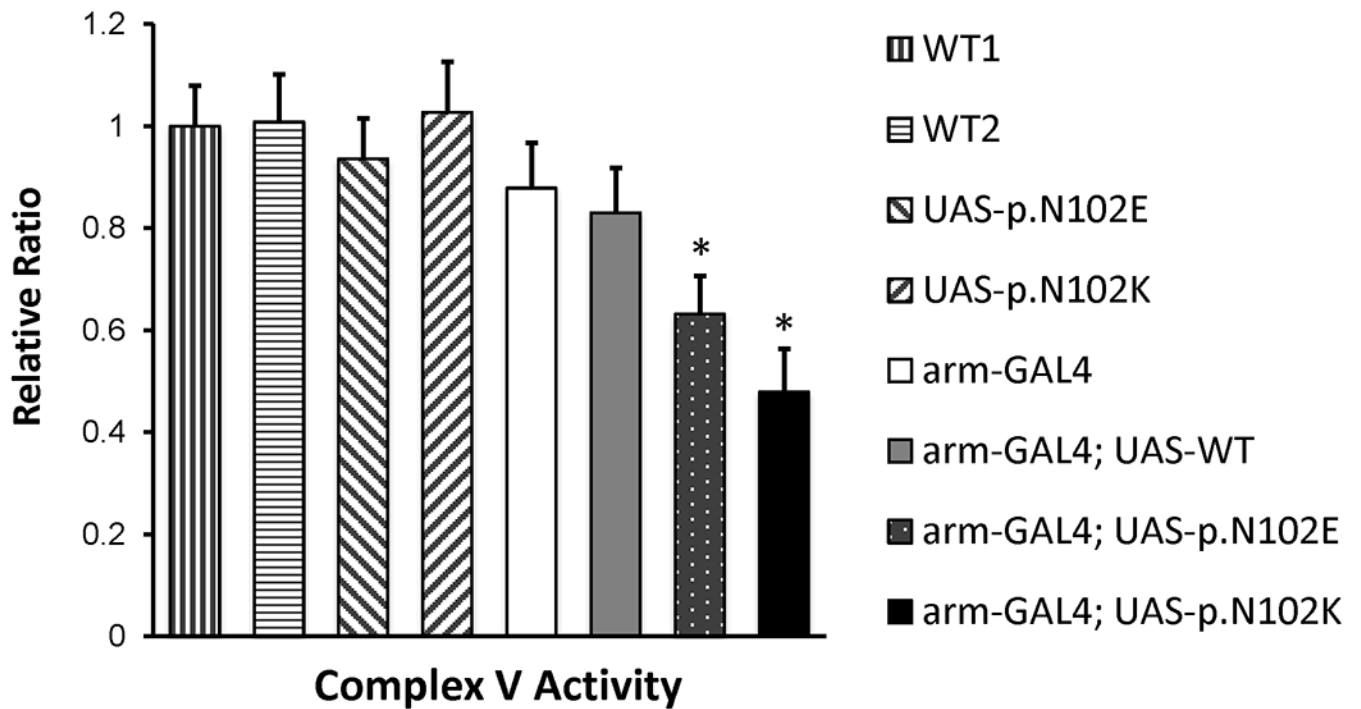
**Figure 2. Decreased complex V function in ATP5MC3-associated disease.**

A significant decrease was observed in relative ATP synthase activity in fibroblast cell lines derived from proband and affected father compared to two unrelated control fibroblast cell lines, with an associated decrease in cellular ATP and oxygen consumption in patient fibroblast cells lines. (\* p-value <0.01).



**Figure 3. *Drosophila* geotaxis assay.**

(A) Mean distance traveled in geotaxis assay for transgenic flies with *arm-GAL4* driver with or without UAS constructs (see also supplementary video). Wild type flies compared to *UAS-WT* overexpression flies showed no significant difference. Flies carrying p.N102K and p.N102E expression constructs traveled significantly shorter distances. (B) Aging flies showed decreasing activity with age but demonstrated consistent differences between transgenic lines. (\*\* p-value <0.001).



**Figure 4. *Drosophila* complex V activity in transgenic flies.**

Relative ATP synthase activity among control and *UAS-GAL4* transgenic flies. Significant reductions were noted in flies containing both *arm-GAL4* driver and UAS constructs with either p.N102E or p.N102K mutations, but not with wild type. WT1 is a *yellow* mutant, which retains normal neuromuscular function. WT2 contains the *CyO* (curly wing) balancer used to create *UAS-GAL4* transgenic lines. (\* p-value <0.01; \*\* p-value <0.001).