BRAIN REPORT

Variants in *ATP5F1B* are associated with dominantly inherited dystonia

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ATP5F1B is a subunit of the mitochondrial ATP synthase or complex V of the mitochondrial respiratory chain. Pathogenic variants in nuclear genes encoding assembly factors or structural subunits are associated with complex V deficiency, typically characterized by autosomal recessive inheritance and multisystem phenotypes. Movement disorders have been described in a subset of cases carrying autosomal dominant variants in structural subunits genes *ATP5F1A* and *ATP5MC3*.

Here, we report the identification of two different *ATP5F1B* missense variants (c.1000A>C; p.Thr334Pro and c.1445T>C; p.Val482Ala) segregating with early-onset isolated dystonia in two families, both with autosomal dominant mode of inheritance and incomplete penetrance. Functional studies in mutant fibroblasts revealed no decrease of ATP5F1B protein amount but severe reduction of complex V activity and impaired mitochondrial membrane potential, suggesting a dominant-negative effect.

In conclusion, our study describes a new candidate gene associated with isolated dystonia and confirms that heterozygous variants in genes encoding subunits of the mitochondrial ATP synthase may cause autosomal dominant isolated dystonia with incomplete penetrance, likely through a dominant-negative mechanism.

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Introduction

Mitochondrial disorders are a group of heterogeneous multisystem diseases with or without neurological involvement, caused by mitochondrial dysfunction. Dystonia is the most commonly reported movement disorder in children. It is also frequently observed in adults, often in association with basal ganglia structural lesions. Nevertheless, isolated dystonia is very rarely the only manifestation of mitochondrial disorders, as it typically presents in combination with several other neurological (including seizures, visual loss, and neuropathy) or extra-neurological (mainly myopathy) features.[1]

Dystonia is predominantly associated with genetic defects in mitochondrial DNA (mtDNA) but can also be caused by pathogenic variants in nuclear DNA genes. Pathogenic variants have been identified in genes encoding proteins involved in a variety of mitochondrial biological pathways, such as mtDNA maintenance, mitochondrial protein synthesis, cholesterol trafficking, assembly of the mitochondrial respiratory chain (MRC) complexes, etc.[2] Recently, variants in subunits of mitochondrial ATP synthase, i.e. MRC complex V, have been reported to cause variable neurologic phenotypes, often including dystonia.[3]

MRC complex V consists of 16 different polypeptides, two of which are encoded by mtDNA genes. Most isolated cases of complex V deficiency are caused by pathogenic variants in the mitochondrial genes (*MT-ATP6*, *MT-ATP8*) and are associated with different clinical phenotypes. Disease-causing variants in patients with isolated complex V deficiency have been identified in a few nuclear genes, encoding assembly factors (e.g. *TMEM70* and *ATPAF2*) or structural subunits (e.g. *ATP5F1A*, *ATP5F1D*, *ATP5F1E*).[4] Most patients are characterized by autosomal recessive inheritance and complex multisystem phenotypes. Neurological symptoms including seizures, ataxia and peripheral neuropathy are frequently present, often associated with heart, muscle, eyes or kidney involvement.[4] More recently, rare movement disorder cases harbouring autosomal dominant variants in *ATP5MC3*[5] and *ATP5F1A*[3] have been published.

We report here the identification in five members of an Italian family of a monoallelic variant in *ATP5F1B*, encoding for the β subunit of complex V; three members had early-onset generalized dystonia while the remaining two carriers were asymptomatic. By screening a cohort of patients with dystonia, an additional *ATP5F1B* variant was subsequently identified in two subjects from a second unrelated family, with only one of them presenting with early-onset generalized dystonia. Experimental data from mutant fibroblasts supported their deleterious impact on complex V functionality, likely through a dominant-negative effect.

Materials and methods

Genetic analysis

The affected subjects from Family A (Subjects II-2, II-3 and III-2) and an additional 139 patients (including the proband of Family B) presenting with childhood- or adult-onset dystonia were recruited at the Neurological Institute 'Besta', Milan. All members of Families A and B were clinically assessed by clinicians with extensive expertise in the diagnosis of movement disorders. Written informed consents were obtained from the investigated subjects.

Whole-exome sequencing and whole-genome sequencing were performed as previously described.[6-8] Phenolyzer (https://phenolyzer.wglab.org) and Toppgene (https://toppgene.cchmc.org) tools were used for gene prioritization. Presence of short tandem repeats expansions was investigated[9]; copy number and structural variants were assessed with the Parliament framework.[10]

Functional studies

Skin biopsies were taken from family members harbouring the *ATP5F1B* variants and four healthy controls following standard clinical procedure. Methodological details for functional studies in patients' fibroblasts have been described elsewhere: transcript analysis,[11] immunoblotting and blue native polyacrylamide gel electrophoresis (BN-PAGE),[12] measurement of MRC complex activities,[13] oxygraphy,[14] mitochondrial network and membrane potential.[15] For complex V activity, we used the standard protocol based on a coupled reaction measuring ATP consumption,[16] using 2.5 mM ATP as substrate; for estimating Vmax and Km, the same reaction was performed at various concentrations of ATP. The antibodies used were: polyclonal anti-ATP5B (HPA001528, Atlas Antibodies), monoclonal anti-ATP5A1 (459240, Invitrogen), monoclonal anti-GAPDH (MAB374, Millipore), monoclonal anti-UQCRC1 (ab110252, Abcam), monoclonal anti-SDHA (459200, Invitrogen). Immunoblot analysis was performed with the ECL-chemiluminescence kit (Amersham). Mitotracker CMX-Red (ThermoFisher) was used for staining of mitochondria and 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining kit (Sigma, CS0390) was used for mitochondrial membrane potential. Images were acquired with a confocal microscope (Leica TSC-SP8) in live cells.

Data availability

Additional images of JC-1 and Mitotracker staining are available on the repository Zenodo: doi:10.5281/zenodo.6826778.

The other data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

Clinical data

The clinical features of the four patients from the two families are reported in Table 1. The three affected subjects from Family A (Subjects II-2, II-3 and III-2; Fig. 1A) had an early-onset progressive isolated dystonia that subsequently generalized, sparing the cranial muscles. All patients retained independent ambulation (Supplementary Videos 1-4). Brain MRI was unrevealing. There were no additional neurological or extra-neurological features and treatment with oral anticholinergic medication (trihexyphenidyl) provided sustained benefit in two subjects.

Dystonia in the proband (Patient III-1) from Family B (Fig. 1A) started at age 5 with right hand dystonic tremor and subsequently spread to involve the left hand at age 8 and finally the neck and

Table 1 Clinical features of the affected subjects with *ATP5F1B* variants

Pt/sex	Site of onset of dystonia (age)	Spreading of dystonia in the other body districts (age)	Distribution of dystonia at last follow-up (age)	Additional features	Medication (daily dosage)	Brain MRI
A-II-2 F	Left leg (3 y)	Left arm (10 y) Trunk + right leg (12 y) Right arm (16 y)	Generalized (48 y)	Writing dystonic tremor	L-DOPA/carbidopa (100/25 mg) – Trihexyphenidyl (12 mg) +	Normal findings
A-II-3 F	Right arm (15 y)	Legs (19 y)	Generalized (47 y)	Writing dystonic tremor	L-DOPA/carbidopa (100/25 mg) –	Normal findings
A-III-2 M	Left leg (2.5 y)	Left arm (8 y) Trunk (12 y)	Generalized (20 y)	None	L-DOPA/carbidopa (100/25 mg) – Trihexyphenidyl (18 mg) +	Normal findings
B-III-1 F	Right hand (5 y)	Right hand (5 y), Left hand (8 y), neck and trunk (20 y)	Generalized dystonia without leg involvement (27 y)	Tremulous rotational torticollis, upper limbs postural and action dystonic tremor. No speech dystonia	Clonazepam (2.5 mg) –	Slight asymmetry of lateral ventricles, without asymmetry of basal ganglia, thalamus or cerebellum

Pt = patient code according to pedigree; F = female; M = male; y = year(s); (–) = no response to treatment; (+) = positive response to treatment.

trunk at age 20 (Supplementary Videos 1–4). Brain MRI showed a slight asymmetry of lateral ventricles, without asymmetry of basal ganglia, thalamus or cerebellum (Supplementary Fig. 1). Clinical examination in the father did not show any neurological sign. Family history was negative for movement disorders.

Genetic studies

Whole-genome sequencing for Family A was performed in the trio Subjects I-1, I-2 and II-3 plus an additional affected member (Subject III-2). Based on the pedigree structure, we assumed either a dominant trait with reduced penetrance or a germline mosaicism in one parent of Generation I, leading to a fully penetrant dominant trait segregating in affected subjects in Generations II and III. No coding variants were present in the affected subjects only. We identified a list of 29 rare coding variants (minor allele frequency <0.0001 in gnomAD v2.1.1) that were shared by the two affected family members, and which were inherited from either unaffected Subjects I-1 or I-2. Among these, according to prioritization tools that rely on phenotypic data, a heterozygous variant in *ATP5F1B* was identified as the top candidate variant (Supplementary Tables 1–3). Detailed copy number variation, structural variant and repeat expansion analyses did not reveal additional possible genetic defects segregating with dystonia. The *ATP5F1B* variant is a nucleotide substitution c.1000A>C (NM_001686.4), predicted to cause the missense change p.Thr334Pro (NP_001677.2), affecting a highly conserved residue (Fig. 1B and C) and with a negative impact on the protein by *in silico* tools (Supplementary Table 4). Sanger sequencing confirmed that the variant was present in all affected family members (Subjects II-2, II-3 and III-2) but also in the unaffected Subjects I-2 and III-1 (Fig. 1A and B). This variant is absent in gnomAD and is classified as 'Variant of Unknown Significance (VUS)' according to the American College of Medical Genetics (ACMG) criteria.[17] Structural analysis showed that the resultant protein is missing polar contact with surrounding residues as well as changes in steric hindrance in the hydrophilic F1 portion of complex V, which contains a hexamer formed by subunits α and β and a central stalk constituted by the subunit γ; F1 catalyses the ATP synthesis (Fig. 1D and E).

Next, we queried the whole-exome sequencing data from a cohort of 139 patients presenting with childhood- or adult-onset dystonia as main symptom of their movement disorder. We identified an additional subject with a different heterozygous *ATP5F1B* variant c.1445T>C (NM_001686.4), p.Val482Ala (NP_001677.2) (Fig. 1A). The variant is absent from all control databases and classified as VUS by ACMG criteria.[17] It affects a highly conserved residue and the corresponding amino acid change is predicted to be deleterious by bioinformatics tools (Fig. 1C). Structural analysis revealed missing hydrophobic bonds and high flexibility of the C-terminal α-helix in the mutant protein (Fig. 1D and E). The variant was inherited by the unaffected father Subject II-2 (Fig. 1A and B).

No additional candidate variants in *ATP5F1B* were identified in a larger cohort of dystonic patients (more than 1000 index-case exomes).[18,19]

Functional studies

To experimentally evaluate the effect of the *ATP5F1B* c.1000A>C variant on transcript and protein levels, we exploited cultured skin fibroblasts from every member of Family A harbouring the variant.

Quantitative analysis, amplification and sequencing of the *ATP5F1B* cDNA did not reveal aberrant isoforms and showed biallelic expression and normal or higher levels of the *ATP5F1B* transcript compared to control cells (Fig. 2A and B). Similarly, immunoblot analysis displayed no reduction, but rather a trend towards an increase in ATP5F1B protein levels (Fig. 2C). The same result was obtained for ATP5F1A, the α subunit of complex V. These results confirm that there was not a second genetic hit affecting *ATPF1B*, and suggest that the pathogenic mechanism may be dominant-negative rather than haploinsufficiency.

Assessing the assembly of MRC using BN-PAGE demonstrated normal levels of single MRC complexes. However, by using antibodies against complex V, high molecular weight bands were visualized in patients' samples (Fig. 2D): these correspond to dimeric or oligomeric complex V, or to complex V-containing supercomplexes.

To prove the deleterious role of this *ATP5F1B* variant, we tested the MRC enzyme activities revealing a severe and isolated ATPase deficiency in all *ATP5F1B*-mutant fibroblasts (Fig. 3A), whereas values were in the control range for other MRC complexes, with

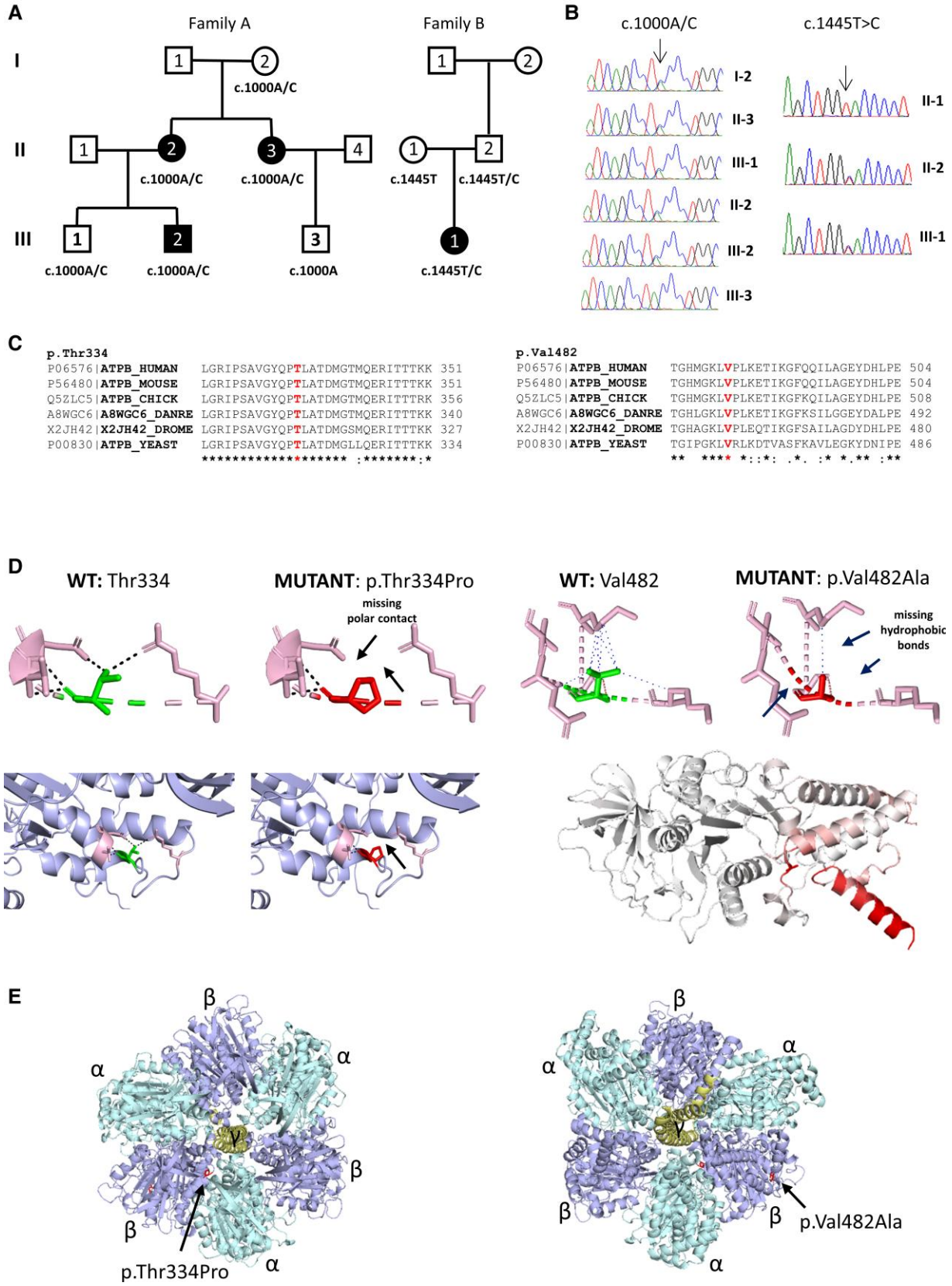

Figure 1 Family pedigrees, genetic and structural analysis. (**A**) Pedigrees of the families, with segregation data on the c.1000A>C and c.1445T>C variants. Black symbols indicate the affected individuals. (**B**) Electropherograms of the *ATP5F1B* regions containing the c.1000A>C and c.1445T>C variants in different members from Families A and B. (**C**) Alignment of ATP5F1B protein homologues shows the conservation of the mutated amino acids (p.Thr334 and p.Val482) in different species, including human, mouse, chicken, zebrafish, fly, yeast (UniProt identifiers are given for the studied species).

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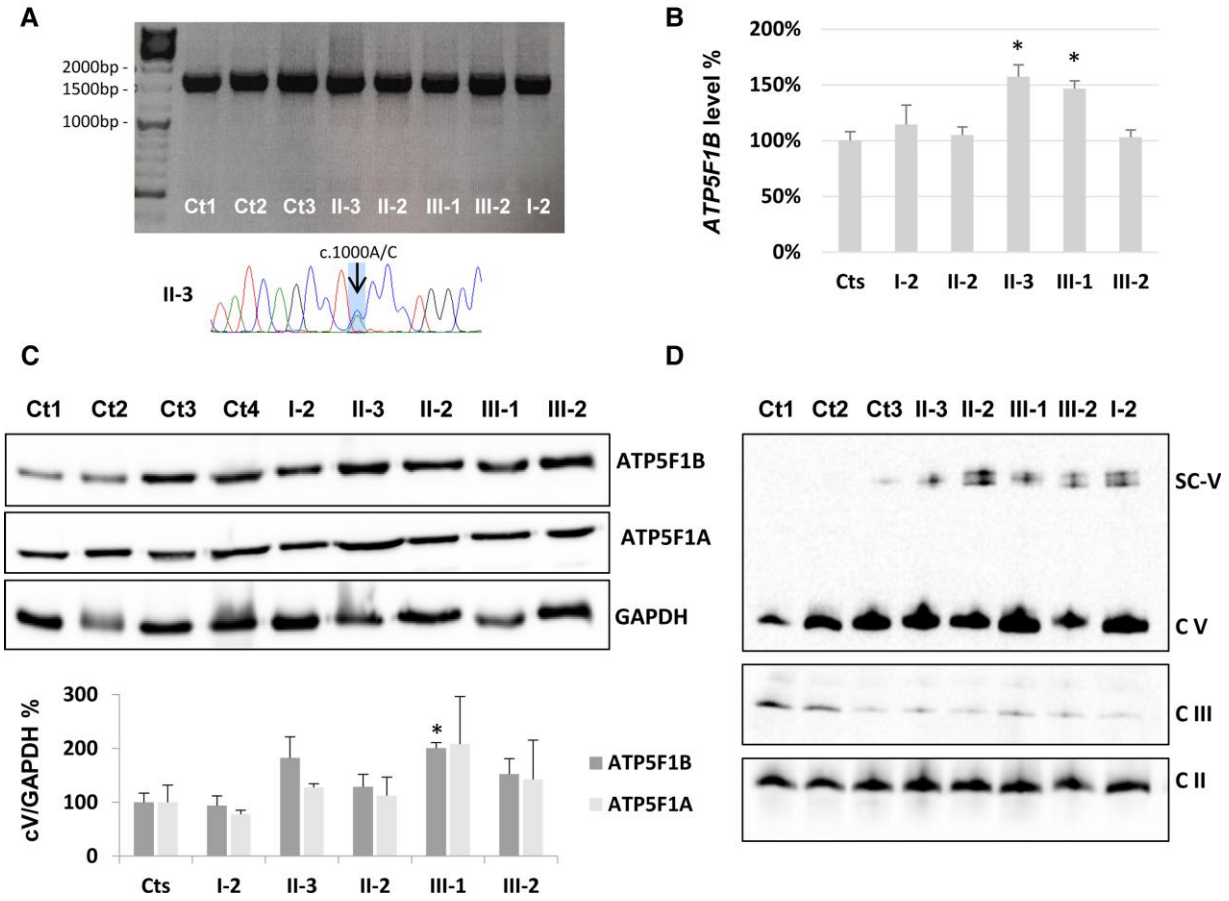

Figure 2 *ATP5F1B* transcript and protein analysis on mutant fibroblasts from Family A. (**A**) Amplification of the *ATP5F1B* transcript in cDNA, retrotranscribed from available Family A members and control fibroblasts RNA. *Bottom*: An electropherogram of the region containing the *ATP5F1B* c.1000A>C variant, with overlapping peaks confirming biallelic expression. (**B**) Graph reporting *ATP5F1B* transcript level normalized to a housekeeping gene (*ACTN* or *GAPDH*), being 100% the mean value of controls (Cts). Three replicates (with different primer pairs) were assessed and reported as means ± standard deviation. Asterisks indicate statistical significance by *t*-test ($P < 0.001$). (**C**) SDS gel electrophoresis of fibroblasts from available family members and four controls (Ct1–4). For immunoblotting, we used antibodies against ATP5F1B (complex V subunit β), ATP5F1A (complex V subunit α) and GAPDH (as loading control). The graph reports the densitometric analysis (means ± standard deviation) of three independent experiments. All comparisons 'patient versus controls' were not significant, except III-1 versus controls for ATP5F1B (asterisk, $P < 0.01$ by *t*-test). (**D**) One-dimensional blue native gel electrophoresis of mitochondrial-enriched fibroblast samples from available family members and three controls (Ct1–3). We used an antibody against ATP5F1A (complex V subunit α) for complex V (cV), an antibody against SDHA for complex II (cII) and an antibody against subunit UQCRC1 for complex III (cIII). High-molecular weight bands (SC-V) were detected in patients' samples using the ATP5F1A antibody in two biological replicates: they correspond to dimeric/oligomeric complex V or to complex V-containing super-complexes.

partial increased activity of citrate synthase (Fig. 3B). High-resolution respirometry did not show impaired rates of oxygen consumption, in basal conditions or after oligomycin injection, nor maximal respiratory capacity (data not shown).

The activity of complex V depends on the proton gradient created by MRC complexes; hence, JC-1 staining was used to evaluate the mitochondrial membrane potential. Red JC-1 aggregates indicate high membrane potential and were observed in all mitochondrial networks of the control cells. Conversely, *ATP5F1B*-mutant fibroblasts presented with remarkable areas of green staining corresponding to monomeric JC-1, resembling the mitochondrial network. The green signal was not diffused in the cytosol, as expected for completely abolished potential: this indicates the presence of altered, partly reduced mitochondrial membrane potential (Fig. 3C). No gross morphological alterations in the mitochondrial network were observed by visualization using a mitochondrion-specific dye (Fig. 3D).

Similarly, functional investigations for c.1445T>C were carried out on skin fibroblasts from the two Family B members. Immunoblot analysis displayed slightly increased amount of ATP5F1B and ATP5F1A protein levels (Supplementary Fig. 2). We measured the MRC enzyme activities: Patient B-III-1 had normal complex V activity while her father (Subject B-II-2) showed a mild complex V defect (88% of the lower value in the control range). No defects were present in other MRC

Figure 1 Continued
(**D**) Three-dimensional structural modelling (PDB: AF-P06576-F1-model_v3) shows that the mutant Pro334 (*left*) causes the loss of polar contacts (dotted black lines indicated by arrows) with surrounding amino acids of the subunit β as well as changes in steric hindrance while the mutant Ala482 (*right*) causes the loss of hydrophobic bonds (dotted blue lines indicated by arrows) with surrounding amino acids and increased flexibility of the C-terminal α helix (in dark red). (**E**) Structure (PDB: 1NBM) of the F1 module of bovine complex V with the hexamer formed by 3 α (azure) and 3 β (blue) subunits and a central stalk constituted by the subunit γ (yellow): red residues (indicated by arrows) correspond to mutant amino acids Pro334 and Ala482. Pro334 is located close to α and γ subunits, possibly impairing the contact between β and these subunits while Ala482 is located in the outer part of the hexamer.

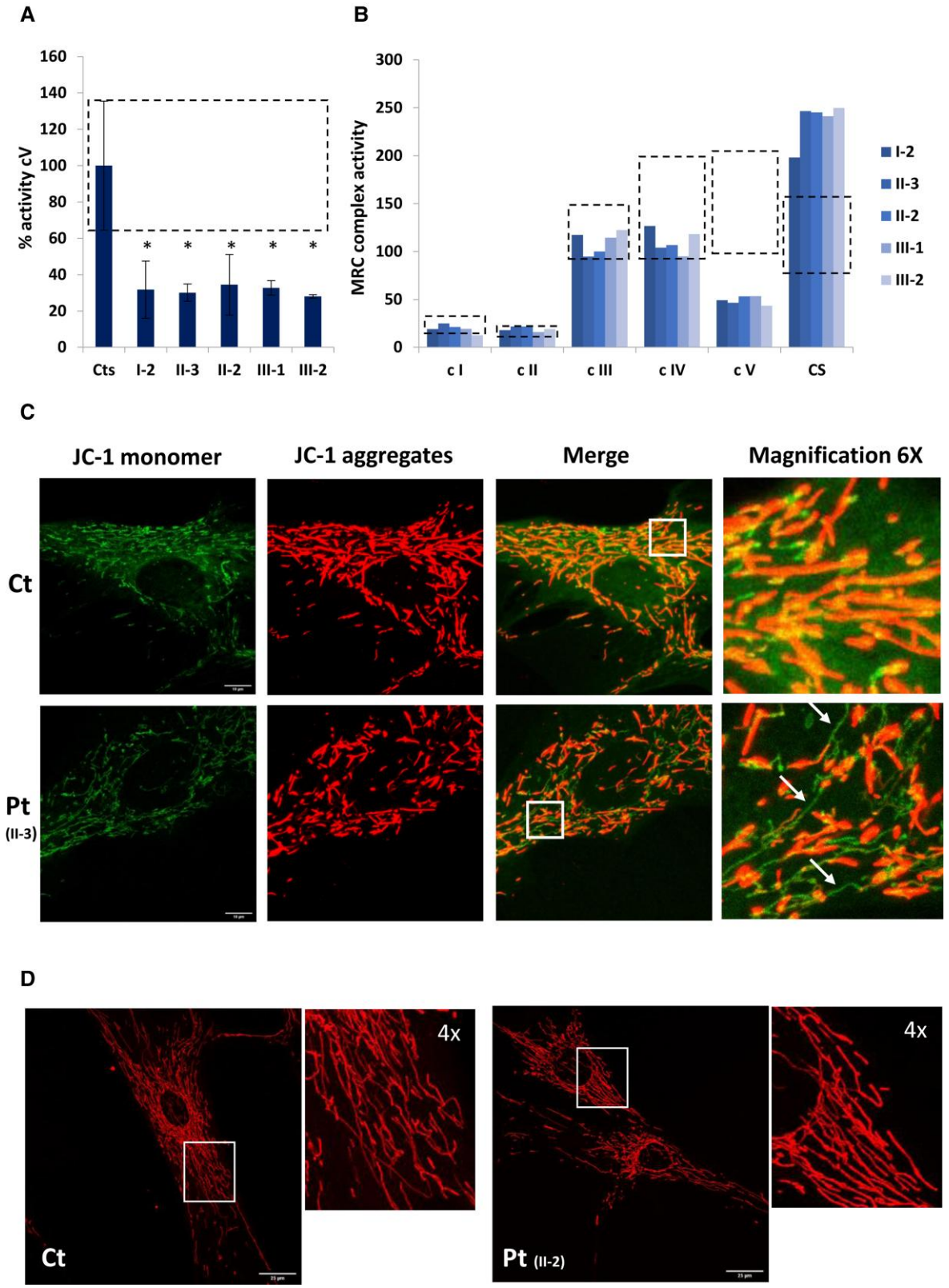

Figure 3 Functional studies on mutant fibroblasts from Family A. (**A**) Assessment of complex V (cV) activity normalized to citrate synthase, in fibroblast cell lines from controls (Cts) and family members. Mean complex V/citrate synthase activity of control fibroblasts is set at 100%, and error bars (and dotted box) represent 1 standard deviation from 290 controls measured in our laboratory. Two biological replicates were quantified for mutant fibroblasts and reported as means ± standard deviation. Asterisks indicate statistical significance by *t*-test ($P < 0.001$). (**B**) Values of respiratory chain enzyme activities normalized to citrate synthase (CS), in fibroblast cell lines from family members. Dotted boxes represent the control range for activities of each complex obtained from 290 laboratory controls. Citrate synthase values are reported as nmol/min mg. (**C**) Representative images of JC-1 staining on fibroblasts from controls

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complexes (Supplementary Fig. 2). In addition, high-resolution respirometry did not reveal any impairment in oxygen consumption (data not shown). However, by testing different concentrations of ATP, the substrate of the complex V/ATPase reaction, we observed a clear defect of complex V activity in Family B members compared with controls (Supplementary Fig. 2). These findings indicate an increased Km, index of reduced affinity of the enzyme for the substrate. Finally, to assess the effects of this variant on mitochondrial membrane potential, we used JC-1 staining as reported above. As observed in Family A, *ATP5F1B*-mutant fibroblasts from Family B presented with areas of green staining, resembling the mitochondrial network. In Patient B-III-1, some intracellular regions displayed a green diffused signal in the cytosol, as expected for severely decreased mitochondrial membrane potential (Supplementary Fig. 3).

By adding these functional data to the ACMG criteria,[17] both variants can be reclassified as 'Likely Pathogenic'.

Discussion

Dystonia is genetically heterogeneous; numerous disease-causing genes have been reported in recent years, but several cases remain without a molecular diagnosis.[18] A model describing the interaction between proteins linked to hereditary dystonia has been proposed.[20] The cellular pathways involved in genetic dystonias include a chaperone function for proper protein folding and prevention of abnormal aggregation; dopamine signalling; secretory pathways and impaired synaptic recycling or plasticity; DNA binding factors with roles in proliferation, apoptosis, cell cycle and transcriptional regulation; cellular stress response and signalling pathways.

We describe here, for the first time, two likely pathogenic monoallelic missense variants (p.Thr334Pro and p.Val482Ala) in *ATP5F1B* associated with dystonia with incomplete penetrance in two independent families. Recently, a *de novo* heterozygous *ATP5F1B* variant has been reported in identical twin boys who presented with congenital hypermetabolism[21]; the authors suggested a mitochondrial uncoupling syndrome. Notably, the identified variant c.1004T>C, p.Leu335Pro hits the amino acid adjacent to the residue mutated in Family A. The phenotype of these cases included infantile onset, developmental delay and episodic hyperthermia in these two brothers. Conversely, all affected individuals from our study were characterized by early-onset isolated and slowly progressive dystonia sparing the cranial muscles, resembling the *DYT1* phenotype, without any additional neurological or systemic feature.

As a distinctive clinical sign, three of four patients had also a dystonic tremor (writing tremor in two individuals; postural and action dystonic tremor affecting head and upper limbs in the third). Age of onset ranged from infancy to adolescence. Severity was also variable, but all patients were still ambulant at last follow-up visits. Furthermore, penetrance was incomplete with two individuals from Family A, aged 22 and 76 years, respectively, and one in Family B (57 years), who were completely asymptomatic at the time the assessment was performed.

An interesting aspect of this new genetic entity relates to the autosomal dominant mode of inheritance. Most genetic defects in nuclear-encoded structural complex V subunits display a recessive pattern of transmission. Conversely, single heterozygous dominant variants in *ATP5MC3* and *ATP5F1A* typically occur *de novo*.[3] Similarly, the pathogenic *ATP5F1B* variant in the recently published twins with mitochondrial dysfunction[21] was also *de novo*. Additionally, a heterozygous *ATP5MC3* variant has been recently described in a dominantly transmitted movement disorder featuring combined dystonia and spasticity in two independent families. Notably, reduced penetrance was also reported in these pedigrees.[5] In accordance with the dominant negative effect we hypothesized for the *ATP5F1B* variants identified in our study, cellular models expressing the p.Leu335Pro variant (associated with congenital hypermetabolism) showed that heterologous expression of the pathogenic variant is sufficient to produce a defective phenotype.[21] Dominant-negative effects were also hypothesized for *ATP5F1A* and *ATP5MC3* variants, supported by the observation of mild/moderate defects of complex V activity or assembly in several patient lines.[3]

ATP5F1B is a nuclear gene that encodes the β subunit of complex V. Due to the high energy demand of the nervous system and the important role of mitochondria in neuronal homeostasis, neurological impairment is expected in ATP synthase deficiency. However, the exact mechanisms through which these variants are disease-causing remain not fully understood. Our data from *ATP5F1B* mutant fibroblasts suggest an impairment of ATP synthase activity possibly due to altered mitochondrial membrane potential. Alternatively, a less-efficient coupling between proton flow and 'rotary catalysis' by the F1 module is also possible.[22] An increasing number of pathological conditions are characterized by partial or full uncoupling, thus creating an energy-dissipating structure.[23] The same mechanism has also been suggested for the other recently published *ATP5F1B* dominant variant (p.Leu335Pro), with loosened coupling between dissipation of the proton motive force and the generation of ATP.[21] Cells from the twins with congenital hypermetabolism showed increased mitochondrial oxygen consumption as possible compensatory effect to a less-efficient complex V; this could explain the clinical picture of these patients with excessive caloric intake and hyperthermia. Despite the presence of defective complex V and impaired mitochondrial membrane potential, the patients with dystonia did not present alterations in oxygen consumption. The presence of larger complex V-containing structures observed in mutant fibroblasts could be a compensatory mechanism to decreased metabolic efficiency, as formation of dimers and oligomers regulates the efficiency of ATP synthase.[24]

Our structural predictions (Fig. 1E), supported by similar findings by others,[21] indicated that both p.Thr334Pro and p.Leu335Pro are located near the α and γ subunits, possibly impairing the contact between the α–β hexamer and the γ subunit. The p.Val482Ala variant hits a different position, in the external part of the hexamer, and thus it is not possible to predict whether it acts with the same mechanism proposed for the other two variants. Furthermore, p.Val482Ala does not affect hydrophilic/polar interaction with surrounding amino acids but causes increased flexibility of the C-terminal part of the

Figure 3 Continued
(*top row*) and *ATP5F1B*-mutant subjects (*bottom row*). Red fluorescence (**C**), corresponding to JC-1 aggregates and sign of preserved mitochondrial membrane potential, is present in almost all controls, whereas several mutant cells display green fluorescent signals (JC-1 monomer) with distribution resembling the mitochondrial network: this finding indicates partial mitochondrial membrane depolarization. Scale bars = 10 µm. *Right*: Digital magnification at ×6. (**D**) Representative images of mitochondrial morphology obtained with Mitotracker red staining, showing the filamentous mitochondrial network of fibroblasts from both control (Ct) and *ATP5F1B*-mutant (Pt) subjects. Scale bars = 25 µm. The smaller panels show digital magnifications (×4) of the *insets*.

mutant protein. Functional data showed some differences between p.Thr334Pro and p.Val482Ala, in particular the severity of complex V deficiency. However, the impact of the two variants on the membrane potential of cells deriving from the subjects of the two families was quite similar. This, together with the similar clinical presentation, suggests a similar pathophysiological mechanism between the two variants. Overall, we can speculate that they both disrupt the efficiency of ATP synthesis, though with different degrees of severity, not influencing other respiratory chain complex activities and oxygen consumption. This less-efficient ATP production may be insufficient for energetic requirements of selective cell types (e.g. basal ganglia or cerebellar neurons) in specific conditions, thus possibly explaining the incomplete penetrance and the phenotype of our patients.

In conclusion, our study confirms that pathogenic variants in genes encoding subunits of mitochondrial ATP synthase/complex V cause multifocal or generalized isolated dystonia, also with dominant inheritance and incomplete penetrance, likely through a dominant negative-effect on protein function. Additional cases harbouring *ATP5F1B* variants are needed to better define the complete spectrum of phenotypes associated with variants in this gene. Indeed, variable neurological phenotypes have been reported for genes encoding other complex V subunits. Accordingly, the recent report of a complex multisystem presentation in subjects with a pathogenic *de novo ATP5F1B* variant suggests a probable broad range of clinical presentations.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

References

1. Schreglmann SR, Riederer F, Galovic M, *et al*. Movement disorders in genetically confirmed mitochondrial disease and the putative role of the cerebellum: Mitochondrial movement disorders. *Mov Disord*. 2018;33:146-155.
2. Musumeci O, Oteri R, Toscano A. Spectrum of movement disorders in mitochondrial diseases. *J Transl Genet Genomics*. 2020;4:221-237.
3. Zech M, Kopajtich R, Steinbrücker K, *et al*. Variants in mitochondrial ATP synthase cause variable neurologic phenotypes. *Ann Neurol*. 2022;91:225-237.
4. Fernandez-Vizarra E, Zeviani M. Mitochondrial disorders of the OXPHOS system. *FEBS Lett*. 2021;595:1062-1106.
5. Neilson DE, Zech M, Hufnagel RB, *et al*. A novel variant of *ATP5MC3* associated with both dystonia and spastic paraplegia. *Mov Disord*. 2022;37:375-383.
6. Carecchio M, Invernizzi F, Gonzàlez-Latapi P, *et al*. Frequency and phenotypic spectrum of *KMT2B* dystonia in childhood: A single-center cohort study. *Mov Disord*. 2019;34:1516-1527.
7. Kremer LS, Bader DM, Mertes C, *et al*. Genetic diagnosis of Mendelian disorders via RNA sequencing. *Nat Commun*. 2017;8:15824.
8. Gauthier J, Meijer IA, Lessel D, *et al*. Recessive mutations in *VPS13D* cause childhood onset movement disorders. *Ann Neurol*. 2018;83:1089-1095.
9. Dolzhenko E, Bennett MF, Richmond PA, *et al*. ExpansionHunter DeNovo: A computational method for locating known and novel repeat expansions in short-read sequencing data. *Genome Biol*. 2020;21:102.
10. English AC, Salerno WJ, Hampton OA, *et al*. Assessing structural variation in a personal genome—Towards a human reference diploid genome. *BMC Genomics*. 2015;16:286.
11. Alahmad A, Nasca A, Heidler J, *et al*. Bi-allelic pathogenic variants in *NDUFC2* cause early-onset leigh syndrome and stalled biogenesis of complex I. *EMBO Mol Med*. 2020;12:e12619.
12. Diodato D, Invernizzi F, Lamantea E, *et al*. Common and novel *TMEM70* mutations in a cohort of Italian patients with mitochondrial encephalocardiomyopathy. *JIMD Rep*. 2015;15:71-78.
13. Bugiani M, Invernizzi F, Alberio S, *et al*. Clinical and molecular findings in children with complex I deficiency. *Biochim Biophys Acta*. 2004;1659:136-147.
14. Invernizzi F, D'Amato I, Jensen PB, Ravaglia S, Zeviani M, Tiranti V. Microscale oxygraphy reveals OXPHOS impairment in MRC mutant cells. *Mitochondrion*. 2012;12:328-335.
15. Zanellati MC, Monti V, Barzaghi C, *et al*. Mitochondrial dysfunction in Parkinson disease: Evidence in mutant PARK2 fibroblasts. *Front Genet*. 2015;6:78.
16. Ragan CI, Wilson TM, Darley-Usmar VM, Lowe PN. Sub-fractionation of mitochondria and isolation of the proteins of oxidative phosphorylation. Mitochondria: A Practical Approach. 1987:79-112.
17. Richards S, Aziz N, Bale S, *et al*. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17:405-424.
18. Zech M, Jech R, Boesch S, *et al*. Monogenic variants in dystonia: An exome-wide sequencing study. *Lancet Neurol*. 2020;19:908-918.
19. Dzinovic I, Boesch S, Škorvánek M, *et al*. Genetic overlap between dystonia and other neurologic disorders: A study of 1,100 exomes. *Parkinsonism Relat Disord*. 2022;102:1-6.
20. Gonzalez-Latapi P, Marotta N, Mencacci NE. Emerging and converging molecular mechanisms in dystonia. *J Neural Transm*. 2021;128:483-498.
21. Ganetzky RD, Markhard AL, Yee I, *et al*. Congenital hypermetabolism and uncoupled oxidative phosphorylation. *N Engl J Med*. 2022;387:1395-1403.

22. Devenish RJ, Prescott M, Rodgers AJW. The structure and function of mitochondrial F1F0-ATP synthases. *Int Rev Cell Mol Biol*. 2008;267:1-58.
23. Lippe G, Coluccino G, Zancani M, Baratta W, Crusiz P. Mitochondrial F-ATP synthase and its transition into an energy-dissipating molecular machine. *Oxid Med Cell Longev*. 2019;2019:8743257.
24. Strauss M, Hofhaus G, Schröder RR, Kühlbrandt W. Dimer ribbons of ATP synthase shape the inner mitochondrial membrane. *EMBO J*. 2008;27:1154-1160.