

# PMPCA mutations cause abnormal mitochondrial protein processing in patients with non-progressive cerebellar ataxia

Rebekah K. Jobling,<sup>1,†</sup> Mirna Assoum,<sup>2,3,†</sup> Oleksandr Gakh,<sup>4</sup> Susan Blaser,<sup>5</sup> Julian A. Raiman,<sup>1</sup> Cyril Mignot,<sup>6</sup> Emmanuel Roze,<sup>7,8,9,10,11</sup> Alexandra Dürr,<sup>7,8,9,10,12</sup> Alexis Brice,<sup>7,8,9,10,12</sup> Nicolas Lévy,<sup>2,3,13</sup> Chitra Prasad,<sup>14</sup> Tara Paton,<sup>15</sup> Andrew D. Paterson,<sup>15</sup> Nicole M. Roslin,<sup>15</sup> Christian R. Marshall,<sup>15</sup> Jean-Pierre Desvignes,<sup>2,3</sup> Nathalie Roëckel-Trevisiol,<sup>2,3</sup> Stephen W. Scherer,<sup>15,16</sup> Guy A. Rouleau,<sup>17</sup> André Mégarbané,<sup>18,19,§</sup> Grazia Isaya,<sup>4,§</sup> Valérie Delague<sup>2,3,§</sup> and Grace Yoon<sup>1,20,§</sup>

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<sup>†,§</sup>These authors contributed equally to this work.

Non-progressive cerebellar ataxias are a rare group of disorders that comprise approximately 10% of static infantile encephalopathies. We report the identification of mutations in *PMPCA* in 17 patients from four families affected with cerebellar ataxia, including the large Lebanese family previously described with autosomal recessive cerebellar ataxia and short stature of Norman type and localized to chromosome 9q34 (OMIM #213200). All patients present with non-progressive cerebellar ataxia, and the majority have intellectual disability of variable severity. *PMPCA* encodes  $\alpha$ -MPP, the alpha subunit of mitochondrial processing peptidase, the primary enzyme responsible for the maturation of the vast majority of nuclear-encoded mitochondrial proteins, which is necessary for life at the cellular level. Analysis of lymphoblastoid cells and fibroblasts from patients homozygous for the *PMPCA* p.Ala377Thr mutation and carriers demonstrate that the mutation impacts both the level of the alpha subunit encoded by *PMPCA* and the function of mitochondrial processing peptidase. In particular, this mutation impacts the maturation process of frataxin, the protein which is depleted in Friedreich ataxia. This study represents the first time that defects in *PMPCA* and mitochondrial processing peptidase have been described in association with a disease phenotype in humans.

- 1 Division of Clinical and Metabolic Genetics, The Hospital for Sick Children, University of Toronto, Toronto, ON, Canada
- 2 Inserm, UMR\_S 910, 13385, Marseille, France
- 3 Aix Marseille Université, GMGF, 13385, Marseille, France
- 4 Department of Paediatric and Adolescent Medicine and Mayo Clinic Children's Centre, Mayo Clinic, Rochester, MN, USA
- 5 Division of Neuroradiology, The Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada
- 6 Département de Génétique, Unité de Génétique Clinique, APHP, Groupe Hospitalier Pitié-Salpêtrière; Centre de Référence Maladies Rares 'Déficiences Intellectuelles de Causes Rares'; Groupe de Recherche Clinique UPMC Univ Paris 06; Paris, France
- 7 Sorbonne Université, UPMC Univ Paris 06, UM 75, ICM, F-75013 Paris, France
- 8 Inserm, U 1127, ICM, F-75013 Paris, France
- 9 Cnrs, UMR 7225, ICM, F-75013 Paris, France
- 10 ICM, Paris, F-75013 Paris, France
- 11 AP-HP, Hôpital de la Salpêtrière, Département de Neurologie, F-75013, Paris, France
- 12 AP-HP, Hôpital de la Salpêtrière, Département de Génétique et Cytogénétique, F-75013, Paris, France
- 13 Département de Génétique Médicale, Hôpital d'Enfants de la Timone, AP-HM, Marseille, France
- 14 Medical Genetics Program, Department of Pediatrics, London Health Sciences Centre, London, Ontario, Canada

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- 15 The Centre for Applied Genomics and Program in Genetics and Genome Biology, The Hospital for Sick Children, Toronto, ON, Canada
- 16 McLaughlin Centre and Department of Molecular Genetics, University of Toronto
- 17 Montreal Neurological Institute and Hospital and Department of Neurology and Neurosurgery, McGill University, Montreal, QC, Canada
- 18 Unité de Génétique Médicale and Laboratoire Associé Inserm UMR S\_910, Faculté de Médecine, Université Saint Joseph, Beirut, Lebanon
- 19 Institut Jérôme Lejeune, Paris, France
- 20 Division of Neurology, The Hospital for Sick Children, University of Toronto, Toronto, ON, Canada

Correspondence to: Grace Yoon,  
Division of Clinical and Metabolic Genetics,  
The Hospital for Sick Children  
555 University Avenue, Toronto,  
Ontario, Canada M5G 1X8  
E-mail: grace.yoon@utoronto.ca

Correspondence may also be addressed to: Valérie Delague, UMR\_S 910, Faculté de Médecine de la Timone, 13385 Marseille cedex 05, France. E-mail: valerie.delague@univ-amu.fr

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**Abbreviations:** MPP = mitochondrial processing peptidase; NPCA = non-progressive cerebellar ataxia

## Introduction

Non-progressive cerebellar ataxias (NPCAs) manifest in infancy with abnormal gross motor development and hypotonia, followed by the appearance of ataxia (Steinlin, 1998). Dysarthria, intellectual disability and spasticity are often present (Harding, 1992). Only seven genes/loci have been described for autosomal recessive NPCA with cerebellar atrophy to date including: Cayman type cerebellar ataxia due to mutations in *ATCAY* (OMIM #608179) (Bomar *et al.*, 2003), spinocerebellar ataxia 6 (*SCAR6*), localized to chromosome 20q11–q13 (OMIM #608029) (Tranebjaerg *et al.*, 2003), cerebellar hypoplasia and mental retardation with or without quadrupedal locomotion 1 (previously Disequilibrium Syndrome, OMIM #224050) due to mutations in *VLDLR* (Boycott *et al.*, 2005), spinocerebellar ataxia 14 (*SCAR14*) due to mutations in *SPTBN2* (Lise *et al.*, 2012), a recently described family with mutations in *CWF19L1* (Burns *et al.*, 2014), cerebellar ataxia with mental retardation, optic atrophy and skin abnormalities (*SCAR5*) at chromosome 15q24–q26 (Delague *et al.*, 2002), due to mutations in *ZNF592* (Nicolas *et al.*, 2010), and ‘Norman-type’ ataxia (*SCAR2*) (Mégarbané *et al.*, 1999), at chromosome 9q34–9qter (Delague *et al.*, 2001) (OMIM #213200), for which we report the identification of *PMPCA* as the causative gene. We describe 17 patients with an NPCA phenotype, 16 with a homozygous missense mutation, p.Ala377Thr, and one with compound heterozygous mutations p.Ser96Leu and p.Gly515Arg.

Mitochondrial dysfunction is a frequent cause of cerebellar disorders (Al-Maawali *et al.*, 2012). This dysfunction may be directly related to defects in the respiratory chain or other metabolic pathways within the mitochondria.

*PMPCA* (9q34.3) encodes  $\alpha$ -MPP, the  $\alpha$ -subunit of mitochondrial processing peptidase (MPP), a heterodimeric enzyme responsible for the cleavage of nuclear-encoded mitochondrial precursor proteins after import in the mitochondria (reviewed in Gakh *et al.*, 2002). Although mitochondria contain a separate genome, the vast majority of proteins operating within the mitochondria are encoded in the nuclear genome and synthesized in the cytosol. Most nuclear-encoded mitochondrial proteins destined for the mitochondrial matrix are synthesized with an N-terminal pre-sequence required for targeting to and import into the mitochondrion. These pre-sequences usually comprise a positively charged amphipathic  $\alpha$ -helix with hydrophobic residues on one side and hydrophilic residues at the other side of the  $\alpha$ -helix, ranging on average from 10–60 amino acids in length (Vögtle *et al.*, 2009; Kulawiak *et al.*, 2013). After protein import is complete, the pre-sequence is proteolytically cleaved in one or more steps; a crucial process for normal folding and activity of the mature protein. The initial cleavage of mitochondrial matrix-targeting pre-sequences, and in some cases subsequent cleavage, is performed by MPP. This heterodimeric peptidase consists of an alpha subunit ( $\alpha$ -MPP) and a beta subunit ( $\beta$ -MPP), both essential for enzyme activity (Hawlitshchek *et al.*, 1988). Deletion of the gene encoding  $\alpha$ -MPP or  $\beta$ -MPP is incompatible with the viability of the unicellular organism *Saccharomyces cerevisiae* even during anaerobic growth (Pollock *et al.*, 1988). In yeast cells where either subunit of MPP is depleted, the mitochondria continue to import proteins, but cleavage ceases, precursor proteins accumulate, and the affected cells eventually stop growing (Geli *et al.*, 1990). Thus each of the two MPP subunits is part of a small group of components of the

mitochondrial protein import machinery that are essential for life at the cellular level (reviewed in Neupert, 1997).

The  $\beta$ -MPP subunit is a zinc-metalloprotease containing a highly conserved zinc-binding motif located within the active site, an internal cavity between the two subunits (Taylor *et al.*, 2001). The  $\alpha$ -MPP subunit can bind precursor proteins in the absence of  $\beta$ -MPP with the same efficiency as the MPP heterodimer but does not cleave the substrate (Luciano *et al.*, 1997). Moreover, the  $\alpha$ -MPP subunit undergoes a conformational change in the presence of the substrate, whereas the  $\beta$ -MPP subunit does not (Gakh *et al.*, 2001; Janata *et al.*, 2004). Together these data indicate that  $\alpha$ -MPP is uniquely involved in substrate recognition and binding. Additional data suggest that  $\alpha$ -MPP may achieve its function through a highly conserved, flexible glycine-rich loop which is situated at the entrance to the  $\beta$ -MPP active site. Alterations of the glycine-rich loop dramatically decrease or destroy the affinity for substrate and processing ability of MPP (Nagao *et al.*, 2000). A recent computational study predicted a complex, multi-step role for the glycine-rich loop in recognition and translocation of the substrate into the active site, as well as an important role in overall stability of the quaternary structure of MPP (Kučera *et al.*, 2013).

Of the many mitochondrial protein precursors cleaved by MPP, one of the most well studied is human frataxin (FXN), depletion of which is known to cause Friedreich ataxia (OMIM #229300). FXN is a nuclear-encoded mitochondrial protein which undergoes cleavage by MPP after import into the mitochondria. Unlike most substrates of MPP described thus far which appear to undergo a single cleavage step from precursor to mature protein, FXN is known to undergo two cleavage reactions which require MPP (Cavadini *et al.*, 2000; Schmucker *et al.*, 2008). FXN enters the mitochondria as a precursor polypeptide (FXN1-210), and is sequentially cleaved by MPP to an intermediate size form (FXN42-210) and a shorter form (FXN81-210), which are both present in cells at steady state and appear to play complementary roles in iron-sulphur cluster synthesis (Gakh *et al.*, 2010; Bridwell-Rabb *et al.*, 2014).

We report the identification of *PMPCA* as the causative gene for a non-progressive autosomal recessive cerebellar ataxia syndrome (SCAR2), previously mapped to chromosome 9q34 (Delague *et al.*, 2001) in 17 patients from four families. Functional analyses confirm a significant impact of mutations in *PMPCA* on MPP function. To our knowledge, this is the first report of MPP dysfunction in humans, and further highlights the importance of mitochondrial function in human cerebellar disorders.

## Materials and methods

### Subjects

Seventeen patients from four families were included in this study (Fig. 1). Families 1–3 are Christian Lebanese Maronite

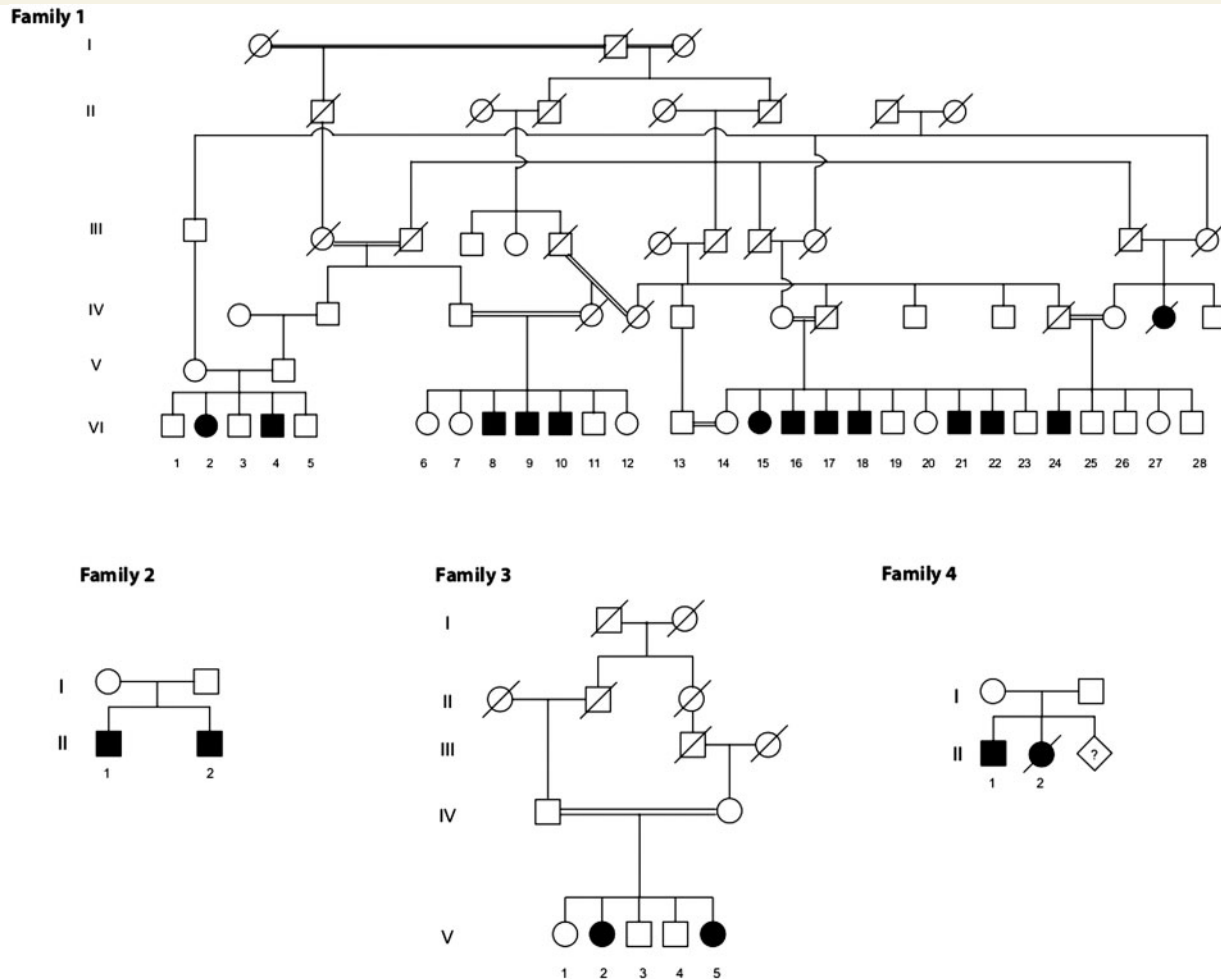
families from North-East Lebanon (Békaa Valley/Mount Lebanon). Clinical characteristics of our patients are summarized in Table 1. After informed consent was obtained from all individuals and parents of children included in this study, EDTA blood samples were collected, and genomic DNA was extracted from lymphocytes with the use of standard methods. All protocols performed in this study complied with the ethics guidelines of the institutions involved.

### Family 1

Family 1 (F1) is a large consanguineous Christian Lebanese Maronite family originating from a village from North-East Lebanon. Thirty-two members from this large family had been previously described as affected with hereditary non-progressive cerebellar ataxia and short stature (Mégarbané *et al.*, 1999). Briefly, the 12 affected members presented with a delay in psychomotor development and ataxic gait which varied from mild to severe. Dysarthria, increased deep tendon reflexes, hypotonia and/or spasticity, slightly diminished muscle strength, flat feet, short stature ranging from <10th to <3rd percentile, moderate to severe intellectual deficiency, and visuospatial defects were also observed. MRI performed on members of this family revealed pronounced cerebellar vermis and bilateral hemisphere atrophy, a dilated fourth ventricle, and a large cisterna magna. Because of similar clinical features, we described this disease as non-progressive cerebellar ataxia of Norman type, also referred to as spinocerebellar ataxia, autosomal recessive 2 (SCAR2) (OMIM 213200).

### Family 2

Patient F2-II.1, a male, was the first child of a G3P0SA2 mother, following an uncomplicated pregnancy. Family history was non-contributory, and the parents, who were both born and raised in Canada, were unaware of any consanguinity, though their ancestors are Christian Lebanese Maronites from the same village in Lebanon. Gross motor milestones were initially normal; the patient stood with support at 10 months of age, and was cruising at 11 months. However, between the ages of 11 and 15 months there was a gradual loss of these skills. His parents noted unsteadiness at 18 months of age, and on examination at 2 years he had truncal and gait ataxia, generalized hypotonia and intention tremor. After this there was stabilization and slow improvement. At the age of 9 years, he could walk short distances unassisted, but required a walker for longer distances. Fine motor skills were also delayed; at 9 years he had difficulty writing and used a computer in class. Initial expressive and receptive language development was normal, but dysarthria was noted between the ages of 2 and 4 years. He was performing at grade level in school. On examination at 9 years of age he had saccadic smooth pursuit, gait ataxia, dysmetria, dysdiadochokinesis and impaired fast finger movements. MRI studies revealed atrophy of the cerebellar vermis and hemispheres (Fig. 2A and B). Sanger sequencing of the coding regions of *FXN*, *POLG*, *APTX* and *PLA2G6*, as well as a microarray did not reveal variants which were bioinformatically predicted to be damaging, and routine metabolic studies were normal. Electromyogram and nerve conduction studies were normal. Muscle biopsy at the age of 4 years revealed mild, non-specific myopathic changes, including rare atrophic fibres and fibres with central nuclei. Electron microscopy studies of the



**Figure 1** Pedigrees from all four families. Affected individuals are shaded in black.

muscle were normal. Respiratory chain analysis on muscle tissue revealed decreased activity of complexes I and III, and II and III. Further studies on fibroblasts revealed mildly decreased activity of complex I and III, and isolated complex III activity in the low-normal range. Coenzyme Q10 analysis of muscle was normal.

Patient F2-II.2, the younger brother of Patient F2-II.1, was born following a normal pregnancy, with delivery at 37 weeks due to foetal bradycardia and a nuchal cord. Delivery was vaginal with forceps. Development was normal until the age of 12 months, at which time he was standing with support, cruising and had seven words. Over the next 5 months he developed limb and gait ataxia. At 15 months he lost his previously normal pincer grasp and used a palmar grasp. Examination at 2 years and 6 months revealed significant truncal and gait ataxia, as well as tremor. Since then there has been stabilization and slow improvement. At the age of 7 years he continued to have very little speech. Neurocognitive assessment revealed intellectual disability. Serial MRI revealed atrophy of the cerebellar vermis and hemispheres identical to that of his brother (Fig. 2C and D).

### Family 3

Family 3 (F3) is a consanguineous Christian Maronite Lebanese family originating from the Békaa valley. Patient

F3-V.2, a female born in Lebanon, was born at term after an uneventful pregnancy. Truncal hypotonia was noted at birth and gradually improved with time. She had global developmental delay; her first words were at 7 years and she walked unaided at 9 years. She attended a special school in Lebanon and was unable to learn to read. Neurological examination at age 33 revealed moderate cerebellar dysfunction; gait ataxia, dysmetric saccades and gaze-evoked nystagmus, dysarthria and dysmetria (Supplementary Video 1). She had no pyramidal, or extrapyramidal manifestations, and no signs suggesting peripheral neuropathy. She was treated with fluoxetine for depression. Routine metabolic testing in blood and CSF, urine purines and pyrimidines, lysosomal enzyme activities in leukocytes, pristanic and phytanic acids and karyotype were normal. Ophthalmologic examination revealed myopia and was otherwise normal. At age 40 years, her height was 156 cm, which was consistent with her family, and her occipitofrontal circumference was normal at 54 cm. Neuropsychological evaluation revealed intellectual deficiency (verbal IQ 47, performance IQ 49). Brain MRI demonstrated cerebellar atrophy with vermian predominance (Fig. 2E). Neurological examination revealed no change from her previous evaluation at 33 years (Supplementary Video 2).

Patient F3-V.5 was the younger sister of Patient F3-V.2. Prenatal and neonatal history was normal. She had always

**Table 1 Clinical characteristics of 17 patients with bi-allelic mutations in PMPCA.**

Patient	Age (years) at most recent assessment	Intellectual disability	Age (years) at language acquisition	Age (years) at independent walking	Dysarthria	Deep tendon reflexes	Gait ataxia	Dysmetria	Gaze-evoked nystagmus
F1-VI.2	25	+	5	10	+	Absent	+	+	+
F1-VI.4	22	+	4–5	9	+	Brisk	+	+	+
F1-VI.8	32	+	3	10	+	Brisk	+	+	+
F1-VI.9	33	+	4	11	+	Brisk	+	+	+
F1-VI.10	23	+	4	10	+	Brisk	+	+	+
F1-VI.15	44	+	5	12	+	Brisk	+	+	+
F1-VI.16	42	+	4	9	+	Brisk	+	+	+
F1-VI.17	40	+	5	12	+	Brisk	+	+	+
F1-VI.18	37	+	3	11	+	Brisk	+	+	+
F1-VI.21	28	+	4	11	+	Brisk	+	+	+
F1-VI.22	27	+	5	9	+	Brisk	+	+	+
F1-VI.24	46	+	4	13	+	Arms absent/legs brisk	+	+	+
F2-II.1	9	–	1	3	+	Normal	+	+	+
F2-II.2	7	+	1 <sup>a</sup>	N/A <sup>b</sup>	+ <sup>c</sup>	Normal	+	+	+
F3-V.2	33	+	7	9	+	Normal	+	+	+
F3-V.5	22	+	4	9	+	Normal	+	+	+
F4-II.1	43	–	U	After 2 years	+	U	+	+	+

N/A = not applicable; U = unknown.

<sup>a</sup>Loss of words after 1 year.

<sup>b</sup>Independent walking not yet achieved.

<sup>c</sup>Minimal language use.

been ataxic and learned to walk unaided at age 9 years. She spoke at 4 years and attended a special school in Lebanon where she learned to read with difficulties. At initial examination at the age of 12 years she had cerebellar dysfunction, and brain MRI revealed cerebellar atrophy. When re-examined at 22 years, she had mild ataxic gait, mild dysarthria, dysmetria of the upper and lower limbs and gaze-evoked nystagmus. Her occipitofrontal circumference was normal. There were no pyramidal or extrapyramidal signs and no peripheral neuropathy. Her intellectual development was that of a 10–12 year-old child although she was not formally tested.

#### Family 4

Patient F4-II.1, a male patient, was born to a non-consanguineous French couple with no familial history of ataxia. His sister was also affected but was never examined. She had depressed mood since the age of 14 and died at 29 years, possibly of suicide. He walked after the age of 2 years and was diagnosed with ataxia because of frequent falls. He is now 58 years of age, and was last examined at the age of 43 years. When examined at 43 years, he had mild gait ataxia, dysmetria of the upper limbs, gaze-evoked nystagmus and dysarthria. He had completed education at the post-secondary level and his neurological status was stable over time. Brain MRI demonstrated cerebellar atrophy with vermian predominance.

## Results

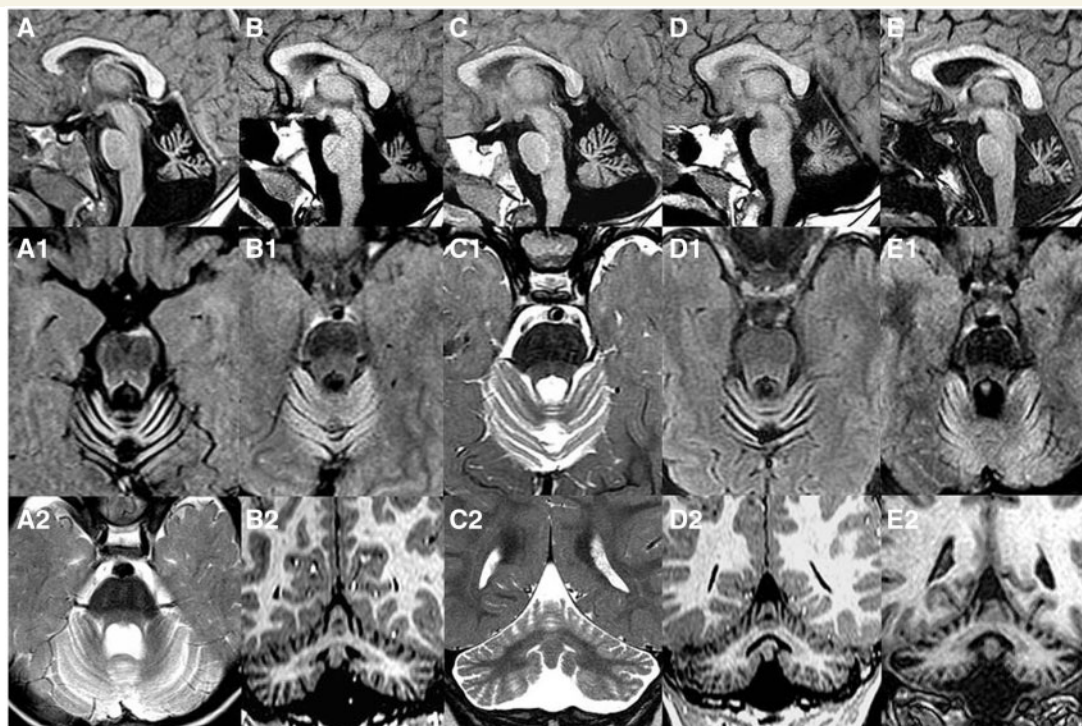
### Mutation identification and modelling

Homozygosity mapping performed in Family 1 previously allowed the assignment of the disease locus to a 12.1-cM

interval on chromosome 9q34-qter between markers D9S67 and D9S312 (Delague *et al.*, 2001). Since the first description of the locus, completion of the genomic sequence at 9q34 led to a reassignment of some microsatellite markers, allowing refining of the linkage region to a 2.85 Mb region between D9S67 and the 9q telomere. In this region, we sequenced 17 candidate genes by standard Sanger sequencing: *BARHL1*, *INPP5E*, *NOTCH1*, *NPDC*, *TRAF2*, *TUBB2C*, *ZYMND19*, *SLC34A3*, *KCNT1*, *LCN1*, *TMEM141*, *PHPT1*, *PAEP*, *QSOX2* and *PMPCA*. A homozygous c.1129G>A transition (p.Ala377Thr) in *PMPCA* (NM\_015160) exon 10 was identified in all patients and found to segregate with the disease phenotype (Fig. 3A and data not shown).

In parallel, exome sequencing was performed in Patient F1-VI.2. After variant annotation and filtering, two rare candidate variants were located within 2.95 Mb linkage region distal to D9S67 identified previously: the c.1129G>A transition (p.Ala377Thr) already identified in *PMPCA* (NM\_015160) by Sanger sequencing of candidate genes, and a c.2453C>T transition (p.Ala818Val) in *CAMSAP1* (NM\_015447).

Exome sequencing in Patient F2-II.1 was undertaken. Homozygosity mapping performed previously in Family 2 allowed the identification of seven candidate homozygous by descent regions. Analysis of the homozygous variants, within the linked regions, revealed only one variant present at <1% frequency in the public population databases: the same missense variant in *PMPCA*, NM\_015160.1:c.1129G>A p.Ala377Thr identified in Family 1, which after Sanger sequencing, was shown to segregate in



**Figure 2 Serial brain images of affected patients.** Sagittal T<sub>1</sub>-weighted image (A) demonstrates moderately severe vermian atrophy in Patient F2-II.1 at 21 months of age. Axial FLAIR (A1) and T<sub>2</sub> (A2) images obtained at the same time demonstrate atrophy and abnormal signal of the cortex of the vermis and cerebellar hemispheres. Similar findings are present on subsequent sagittal T<sub>1</sub> (B), axial FLAIR (B1) and coronal T<sub>1</sub>-weighted (B2) MRI images of the same child at 6 years 5 months of age. Sagittal T<sub>1</sub> weighted image (C) demonstrates moderately severe vermian atrophy in his sibling, Patient F2-II.2 at 15 months of age. Axial (C1) and coronal T<sub>2</sub>-weighted images (C2) obtained at the same time demonstrate atrophy and increased signal of the cortex of the vermis and cerebellar hemispheres. Similar findings are present on subsequent sagittal T<sub>1</sub> (D), axial FLAIR (D1) and coronal T<sub>1</sub> (D2) weighted images of the same child at 4 years 3 months of age. Finally, moderately severe vermian atrophy and abnormal signal of the cortex of vermis and cerebellar hemispheres is shown on sagittal T<sub>1</sub> (E), axial FLAIR (E2) and coronal T<sub>1</sub> (E2) weighted MRI in Patient F3-V.2 at 40 years of age. Supratentorial structures (not shown) were normal in signal and appearance in all at all ages. Diffusion weighted imaging for Patients F2-II.1 and F2-II.2 showed no diffusion restriction and short echo supratentorial magnetic resonance spectroscopy was age appropriate without demonstrable lactate. Magnetic resonance spectroscopy of the cerebellum was not performed.

the pedigree. The variant in *CAMSAP1* was absent in this family. The *PMPCA* p.Ala377Thr variant was absent in 916 Lebanese control chromosomes, and was not present in any of the public single nucleotide polymorphism or variant databases.

Reverse transcriptase PCR on total RNA extracted from lymphoblastoid cell lines from Patients F1-VI.8, F1-VI.15, and F1-VI.16 from Family 1, using primers located in *PMPCA* exons 8 and 12 did not show evidence of any abnormal transcript, thus ruling out a possible splicing defect associated with this specific variant (Fig. 3A and data not shown).

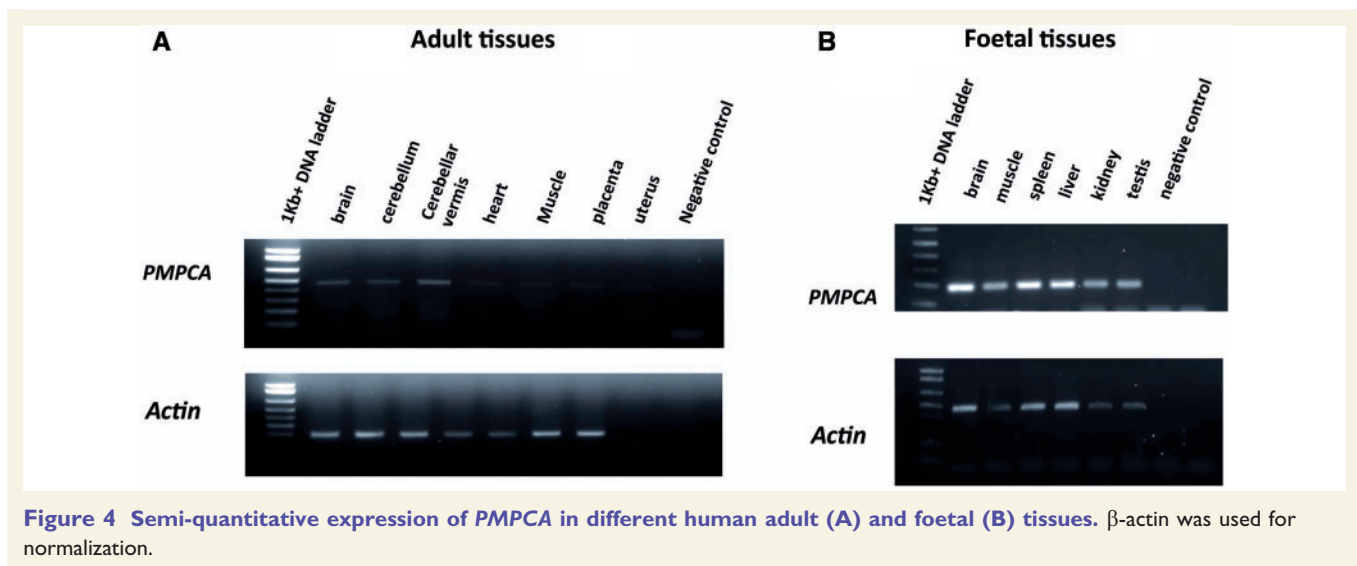
We screened the *PMPCA* coding sequence in a cohort of 39 French patients with NPCA and identified two additional patients with variants in *PMPCA*: Patient F3-V.2, who was homozygous for the same missense p.Ala377Thr variant and Patient F4-II.1, who was compound heterozygous for two missense variants: c.287C>T (p.Ser96Leu) and c.1543G>A (p.Gly515Arg) (Fig. 3A and data not shown). Patient F3-V.2

proved to be of Lebanese origin and her sister, Patient F3-V.5, was also homozygous for the same variant. The c.287C>T (p.Ser96Leu) and c.1543G>A (p.Gly515Arg) variants were absent in 372 French control chromosomes and not present in any public single nucleotide polymorphism or variant databases.

All three variants affect amino acid residues highly conserved in  $\alpha$ -MPP sequences across vertebrates, invertebrates and most metazoans (Fig. 3B). They are predicted to be pathogenic by multiple prediction software programs including SIFT, Provean, Polyphen-2, MutationTaster, Provean, Panther and UMD predictor.

The alanine residue at position 377 (human numbering) lies only 20 residues downstream from the glycine-rich region (Fig. 3B). Homology modelling suggests that Ala377 is in close proximity to the glycine-rich loop (Fig. 3C). The bulkier side chain of threonine at position 377 in our patients'  $\alpha$ -MPP may interfere with the flexibility of the glycine-rich loop, and therefore the ability of MPP to bind





Densitometry measurements revealed that  $\alpha$ -MPP levels were 33% and 34% in Patients F2 II.1 and F2 II.2, and 65% and 57% in the two carrier parents, respectively relative to  $\alpha$ -MPP levels in the controls (Fig. 5A and B). The intermediate  $\alpha$ -MPP level in the carrier parents supported that it was the Ala377Thr mutation which was responsible for the decreased  $\alpha$ -MPP. Levels of  $\alpha$ -MPP were also quantified in lymphoblastoid cells and fibroblasts from patients from Family 1 and controls. The results from two independent experiments showed a mean decrease of 25%, 50% and 44% of  $\alpha$ -MPP levels in lymphoblastoid cells from Patients F1-VI.8, F1-VI.15 and F1-VI.22, respectively, and a 54% decrease in fibroblasts from Patient F1-VI.22 (data not shown).

The levels of  $\beta$ -MPP were examined in Family 2 by western blotting with anti- $\beta$ -MPP polyclonal antibody, and densitometry and found to be similar between the two patients and the carriers or controls (Supplementary Fig. 1).

## MPP function

To assess potential functional effects of the Ala377Thr mutation, we analysed the steady-state levels of the mature forms of four nuclear-encoded mitochondrial matrix proteins: dihydrolipoamide dehydrogenase (DLD), cysteine desulphurase (NFS1), peroxiredoxin 3 (PRDX3) and FXN (Fig. 5A), in patients and carriers from Family 2 and controls. The levels of DLD, NFS1 and PRDX3 appeared similar in both carrier and patient lymphoblasts compared to controls, suggesting that the mutation does not alter the ability of MPP to process these particular precursor proteins, at least not to an extent appreciable by western blotting. However, the cleavage of FXN appeared altered in carriers and patients. The carrier parents' lymphoblast cell extracts demonstrated a small accumulation of the intermediate isoform FXN42-210, but no noticeable change in

FXN81-210 or other FXN isoforms compared to controls (Figs 5A and 6). Moreover, the affected patients demonstrated several abnormalities of FXN processing including accumulation of FXN42-210 (~300% of normal levels), reduction of FXN81-210 (~50% of normal levels) (Figs 5A and 6), and accumulation of the FXN56-210 isoform, which was not detected in the controls and was detected as a faint band in one of the carrier parents (Fig. 5A). The total levels of all FXN isoforms in the patients were ~150% of control levels (Fig. 6).

As a measure of REDOX balance in the mitochondria, the ratio of oxidized/reduced PRDX3 was assessed via western blotting and densitometry (Fig. 5A) (Kumar *et al.*, 2009). The ratio in the patients was increased over both the control and carrier levels while they were similar between carriers and controls (Supplementary Fig. 2).

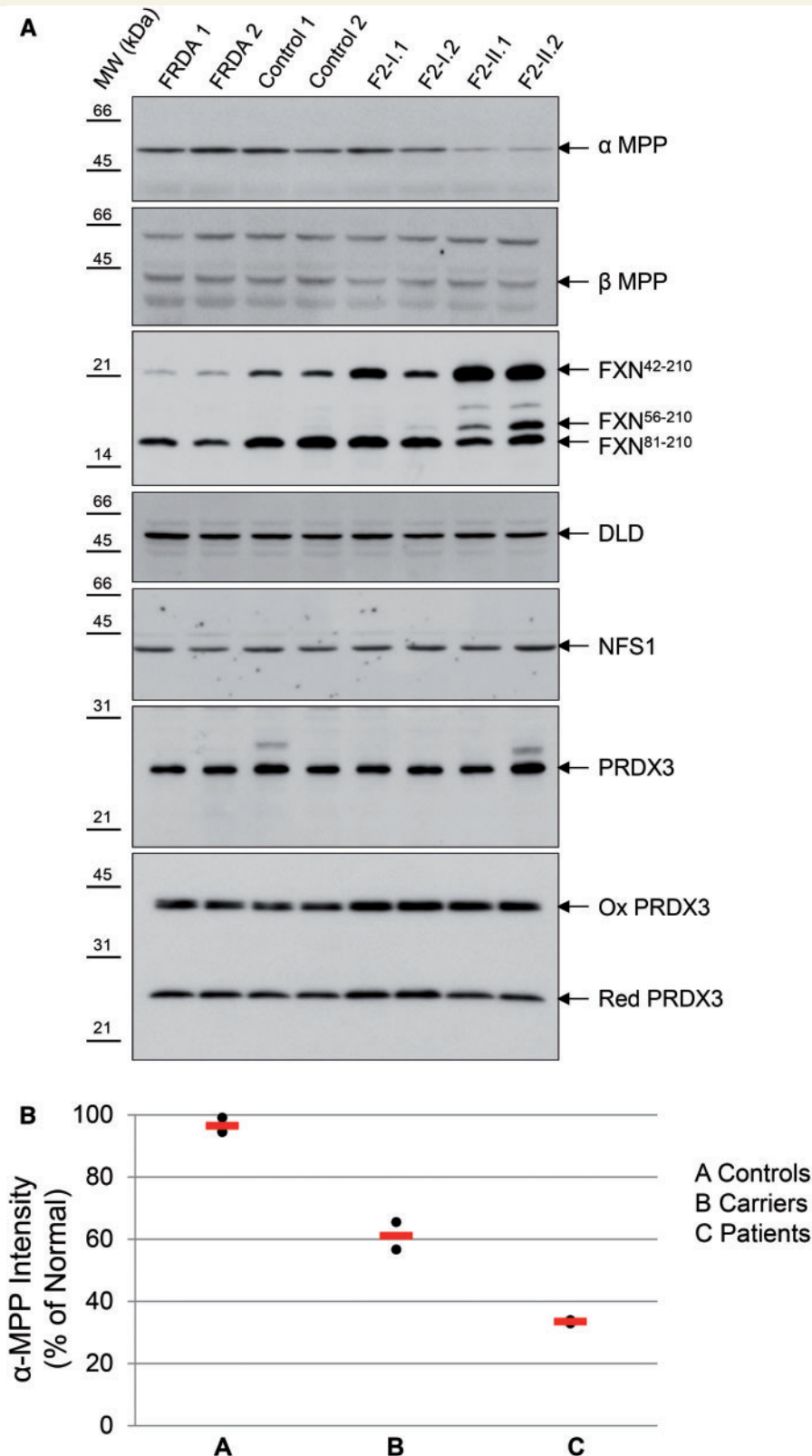
## Subcellular localization

We carried out immunofluorescent labelling of  $\alpha$ -MPP and CoxIV, one of the nuclear-encoded subunits of cytochrome *c* oxidase, the terminal enzyme complex of the mitochondrial electron transport chain located at the inner mitochondrial membrane, in fibroblasts from Patient F1-VI.22 and one control. Co-localization of  $\alpha$ -MPP and CoxIV and the morphology of the mitochondrial reticulum appeared similar between patient and control cells (Fig. 7).

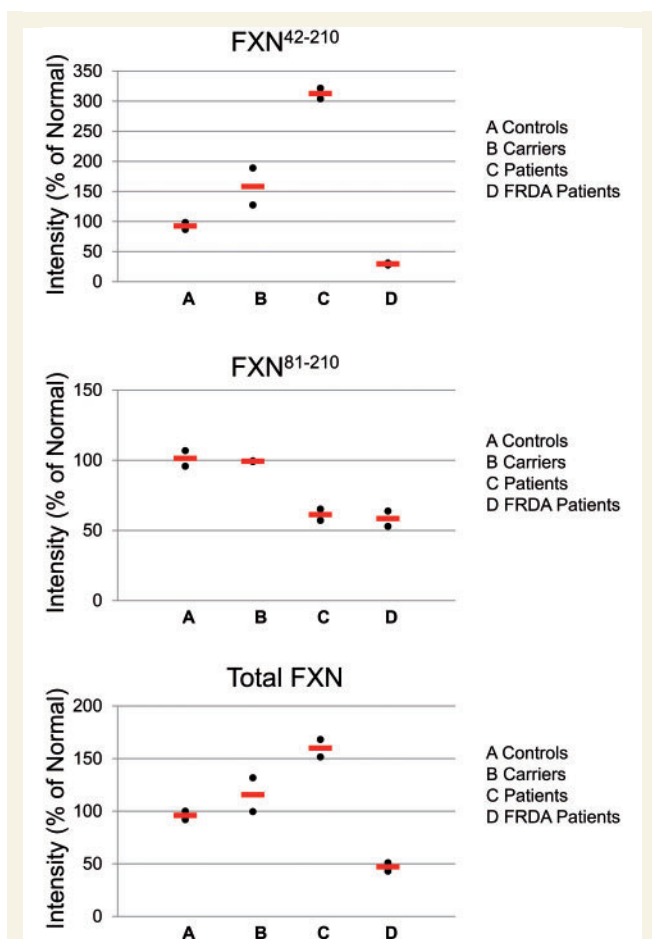
## Discussion

The  $\alpha$ -MPP protein, encoded by *PMPCA*, is necessary for cell survival. Without functional  $\alpha$ -MPP, nuclear-encoded mitochondrial precursor proteins accumulate inside mitochondria and the MPP-deficient cells stop growing (Geli *et al.*, 1990). The requirement of MPP, and therefore





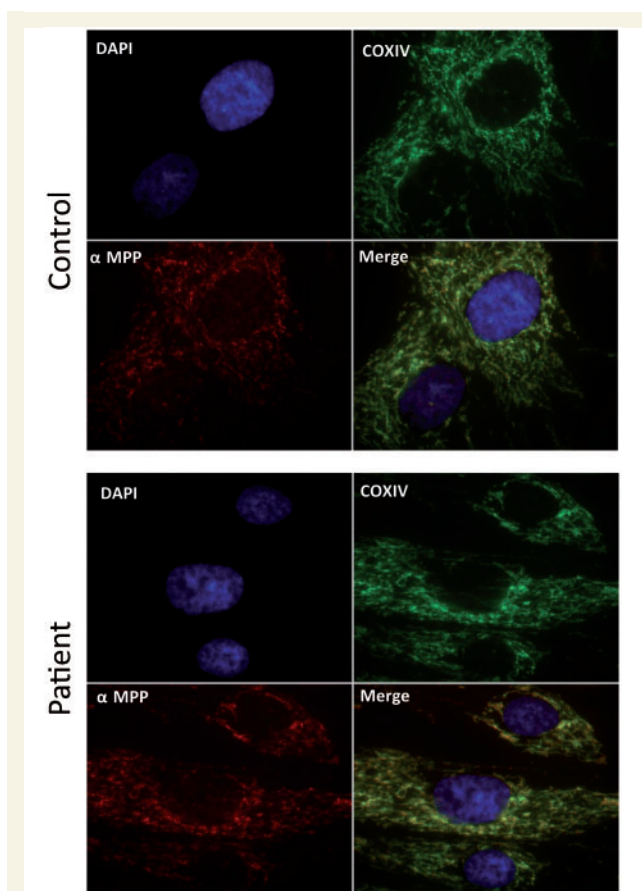
**Figure 5 Functional analysis of MPP in cultured cells.** (A) Cell extracts from the indicated subjects were prepared from exponentially growing lymphoblastoid cell cultures and each sample (30 µg of total protein) was analysed by 15% SDS/PAGE and western blotting with specific antibodies as described in the Supplementary material. Blots were cropped to enable direct comparison of several different mitochondrial proteins in one figure. Full length blots are provided in Supplementary Fig. 4A–C. (B) Densitometry measurements of α-MPP protein bands were collected from the subjects analysed in A, as described in the Supplementary material. The total number of α-MPP protein bands analysed was ≥ 6 for each carrier and each patient, and ≥ 3 for each of the controls. Each data point shows the mean of the technical replicates for each subject in the group.



**Figure 6** Quantification of FXN isoforms in cultured cells.

Densitometry measurements of FXN 42-210 and FXN 81-210 protein bands were collected from the same subjects analysed in Fig. 5 and densitometry was performed as described in the Supplementary material. Two FXN42-210 or FXN81-210 protein bands were analysed for each control, FRDA patient, MPP carrier and MPP patient. Each data point shows the mean of the technical replicates for each subject in the group. FRDA = Friedreich ataxia.

$\alpha$ -MPP, for cell survival can be attributed to the large number of mitochondrial proteins that require precursor cleavage to attain their mature and functional form. Accumulation of non-functional or poorly functional mitochondrial proteins disrupts mitochondrial function and causes loss of cell viability even in the facultative anaerobe *S. cerevisiae* (Pollock *et al.*, 1988; Geli *et al.*, 1990). This vital biological function renders it unlikely that mutations that cause complete loss of  $\alpha$ -MPP or widespread disruption of MPP cleavage reactions would be common in humans. However, our patients demonstrate that partial disruption in MPP function can be compatible with life although with severe phenotypic consequences. Indeed, the c.1129G>A (p.Ala377Thr) mutation decreased the level of  $\alpha$ -MPP, yet the residual level of functioning MPP was sufficient to maintain mitochondrial biogenesis and overall viability.



**Figure 7** Immunolabeling of  $\alpha$ -MPP and CoxIV in fibroblasts from Patient VI.22 from Family I and one control.

Mitochondria were labelled using a mouse monoclonal antibody against CoxIV (cytochrome oxidase IV), located at the inner mitochondrial membrane (green signal), and  $\alpha$ -MPP was detected using a rabbit polyclonal antibody (Novus Biologicals-NBP89126) against human  $\alpha$ -MPP (red signal). Nuclei are stained with DAPI (blue). No abnormal localization or accumulation of  $\alpha$ -MPP was found in the 108 patients' fibroblasts as compared to 68 control cells from three independent experiments.

The presence of this mutation does perturb the function of MPP with respect to the cleavage of FXN. The initial cleavage step from precursor FXN1-210 to intermediate FXN42-210 appears to be intact as we did not detect any accumulation of FXN1-210. However, the subsequent cleavage to FXN81-210 is impaired, leading to a 3-fold accumulation of FXN42-210. The generation of excess FXN56-210 is also seen in the patient cells. This isoform is detected at very low levels under normal conditions (Gakh *et al.*, 2010), but has been demonstrated to accumulate in the context of disruption of either the initial or second cleavage steps, or with a 20-fold over-expression of FXN1-210 (Schmucker *et al.*, 2008).

The second cleavage step occurs at a slower rate than the first, and is rate-limiting for the formation of mature protein (Cavadini *et al.*, 2000; Schmucker *et al.*, 2008). We proposed that the structure of the pre-sequence of the

human frataxin precursor is responsible for the low rate at which FXN42-210 is processed to mature form and/or that the first cleavage results in a conformational change that affects the ability of MPP to carry out the second cleavage (Cavadini *et al.*, 2000). If the Ala377Thr mutation impacts the flexibility of the glycine-rich loop of  $\alpha$ -MPP, which is important for recognition and translocation of the substrate into the peptidase active site (Kučera *et al.*, 2013), a cleavage reaction that is particularly dependent on structural determinants would be more likely to be affected. The Ser96Leu and Gly515Arg mutations may affect the folding of other conserved regions of the peptidase, and perhaps the stability of the entire alpha subunit given the longer side chains of Leu and Arg; however this remains to be verified in future studies.

The non-progressive clinical phenotype of our patients differs from the progressive course with death at a young age typically seen in patients with Friedreich ataxia, which results from global depletion of all frataxin isoforms. In this respect, it is notable that while the levels of FXN81-210 in our patients were as low as those in patients with Friedreich ataxia, the levels of FXN42-210 were much higher in our patients compared to patients with Friedreich ataxia and, to a lesser extent, carriers and controls. FXN42-210 and FXN81-210 have been proposed to have complementary functions and were consistently detected in normal lymphoblastoid cells and cerebellum with a FXN81-210:FXN42-210 ratio of approximately 2:1 (Gakh *et al.*, 2010). Therefore, it is possible that abnormal FXN processing contributes to disease pathophysiology in our patients by altering the FXN81-210:FXN42-210 ratio.

Our analysis also suggests the Ala377Thr mutation leads to more general abnormalities of mitochondrial function. The increased ratio of oxidized to reduced PRDX3 is an indication of increased hydrogen peroxide in mitochondria (Kumar *et al.*, 2009), which may be due to reduced electron flux through the respiratory chain, leading to increased reactive oxygen species formation. It is possible that the mild disturbance of oxidative phosphorylation in muscle tissue from Patient F2-II.1 may be a secondary consequence of one or more defects in the processing of nuclear-encoded respiratory chain proteins.

Mitochondrial dysfunction is an established cause of cerebellar atrophy. It is unlikely that the pathogenesis of cerebellar atrophy in these patients is solely related to dysfunction of FXN, since our patients do not display the typical clinical features or MRI findings of Friedreich ataxia (Supplementary Fig. 3). Moreover, the vast majority of precursor cleavage reactions performed by MPP have not been evaluated in our patients and it is possible that other cleavage reactions are also affected. Nevertheless, the finding of both decreased levels of  $\alpha$ -MPP and disruption of FXN processing in association with mutations in *PMPCA* in multiple patients from different families with almost identical phenotypes argues strongly for a role for MPP dysfunction in this disease process.

We present 17 patients from four families with cerebellar atrophy and NPCA. All patients presented with hypotonia and gross motor delay, with or without initial worsening of their symptoms which then stabilized and did not progress even after decades, and serial brain imaging revealed stable cerebellar atrophy. We identified a homozygous missense c.1129G>A (p.Ala377Thr) mutation in *PMPCA* (NM\_015160) in 16 Lebanese patients from three families and two compound heterozygous missense variations in one French patient: c.287C>T (p.Ser96Leu) and c.1543G>A (p.Gly515Arg), in *PMPCA* exons 3 and 13, respectively. All mutations segregated with the disease phenotype, were not found in control chromosomes, nor in any of the public single nucleotide polymorphism or variant databases. We describe a founder effect for the c.1129G>A (p.Ala377Thr) mutation, as patients from three *a priori* non-related Lebanese families share a common 2.3 Mb homozygous region at 9q34.3 surrounding the *PMPCA* gene. Functional studies confirmed the deleterious effect of *PMPCA* mutations on MPP function, which definitely implicates *PMPCA* as the causal gene in this rare form of NPCA. This is the first time that the MPP enzyme, vital to life at the cellular level, has been associated with a clinical phenotype in humans. Disruption of mitochondrial protein precursor cleavage represents a new avenue for investigation of the pathogenesis of non-progressive cerebellar ataxia.

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## Conflict of interest

Mayo Clinic has a financial interest associated with technology used in the author's research, which has been licensed to a commercial entity. Mayo Clinic, but not the authors, has received royalties of less than the federal threshold for significant financial interest.

## Supplementary material

Supplementary material is available at *Brain* online.

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