

## REPORT

# MCM3AP in recessive Charcot-Marie-Tooth neuropathy and mild intellectual disability

Emil Ylikallio,<sup>1,2,\*</sup> Rosa Woldegebriel,<sup>1,\*</sup> Manuela Tumiaty,<sup>3</sup> Pirjo Isohanni,<sup>1,4</sup> Monique M. Ryan,<sup>5,6,7</sup> Zornitza Stark,<sup>5</sup> Maie Walsh,<sup>5</sup> Sarah L. Sawyer,<sup>8</sup> Katrina M. Bell,<sup>5</sup> Alicia Oshlack,<sup>5</sup> Paul J. Lockhart,<sup>5,7,9</sup> Mariia Shcherbii,<sup>1</sup> Alejandro Estrada-Cuzcano,<sup>10</sup> Derek Atkinson,<sup>10</sup> Taila Hartley,<sup>8</sup> Martine Tetreault,<sup>11,12</sup> Inge Cuppen,<sup>13</sup> W. Ludo van der Pol,<sup>14</sup> Ayse Candayan,<sup>15</sup> Esra Battaloglu,<sup>15</sup> Yesim Parman,<sup>16</sup> Koen L. I. van Gassen,<sup>17</sup> Marie-José H. van den Boogaard,<sup>17</sup> Kym M. Boycott,<sup>8</sup> Liisa Kauppi,<sup>3</sup> Albena Jordanova,<sup>10</sup> Tuula Lönnqvist<sup>4</sup> and Henna Tynjismaa<sup>1,18</sup>

\*These authors contributed equally to this work.

Defects in mRNA export from the nucleus have been linked to various neurodegenerative disorders. We report mutations in the gene *MCM3AP*, encoding the germinal center associated nuclear protein (GANP), in nine affected individuals from five unrelated families. The variants were associated with severe childhood onset primarily axonal (four families) or demyelinating (one family) Charcot-Marie-Tooth neuropathy. Mild to moderate intellectual disability was present in seven of nine affected individuals. The affected individuals were either compound heterozygous or homozygous for different *MCM3AP* variants, which were predicted to cause depletion of GANP or affect conserved amino acids with likely importance for its function. Accordingly, fibroblasts of affected individuals from one family demonstrated severe depletion of GANP. GANP has been described to function as an mRNA export factor, and to suppress TDP-43-mediated motor neuron degeneration in flies. Thus our results suggest defective mRNA export from nucleus as a potential pathogenic mechanism of axonal degeneration in these patients. The identification of *MCM3AP* variants in affected individuals from multiple centres establishes it as a disease gene for childhood-onset recessively inherited Charcot-Marie-Tooth neuropathy with intellectual disability.

- 1 Research Programs Unit, Molecular Neurology, University of Helsinki, 00290 Helsinki, Finland
- 2 Clinical Neurosciences, Neurology, University of Helsinki and Helsinki University Hospital, 00290 Helsinki, Finland
- 3 Research Programs Unit, Genome-Scale Biology, University of Helsinki, 00290 Helsinki, Finland
- 4 Department of Child Neurology, Children's Hospital and Pediatric Research Center, University of Helsinki and Helsinki University Hospital, 00290 Helsinki, Finland
- 5 Murdoch Children's Research Institute, Melbourne, Victoria, 3052, Australia
- 6 Royal Children's Hospital, Melbourne, Victoria, 3052, Australia
- 7 Department of Paediatrics, The University of Melbourne, Melbourne, Victoria, 3052, Australia
- 8 Department of Genetics and Children's Hospital of Eastern Ontario Research Institute, University of Ottawa, Ottawa, K1H 8L1, Canada
- 9 Bruce Lefroy Centre, Murdoch Children's Research Institute, Melbourne, Victoria, 3052, Australia
- 10 Molecular Neurogenomics Group, VIB Department of Molecular Genetics, University of Antwerp, 2610 Antwerpen, Belgium
- 11 Department of Human Genetics, McGill University, Montreal, QC H3A 1B1, Canada
- 12 McGill University and Genome Quebec Innovation Center, Montreal, QC H3A 1A4, Canada
- 13 Department of Paediatric Neurology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands

Received February 8, 2017. Revised April 15, 2017. Accepted May 8, 2017. Advance Access publication June 19, 2017

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- 14 Brain Centre Rudolf Magnus, Department of Neurology and Neurosurgery, University Medical Centre Utrecht, 3508 Utrecht, The Netherlands
- 15 Bogazici University, Department of Molecular Biology and Genetics, Istanbul, Turkey
- 16 Istanbul University, Istanbul Medical School, Department of Neurology, Istanbul, Turkey
- 17 Department of Medical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands
- 18 Department of Medical and Clinical Genetics, University of Helsinki, 00290 Helsinki, Finland

Correspondence to: Henna Tynismaa,  
Biomedicum Helsinki, r.C520b, Haartmaninkatu 8, 00290 Helsinki, Finland  
E-mail: henna.tynismaa@helsinki.fi

**Keywords:** Charcot-Marie-Tooth neuropathy; intellectual disability; *MCM3AP*; GANP; mRNA export

**Abbreviations:** CMT = Charcot-Marie-Tooth; GANP = germinal center associated nuclear protein; NMD = nonsense-mediated decay

## Introduction

Hereditary sensorimotor neuropathies are collectively known as Charcot-Marie-Tooth disease (CMT). Based on nerve conduction velocities, the disease can be divided into demyelinating CMT (CMT1), axonal CMT (CMT2) and intermediate CMT (Reilly *et al.*, 2011). All forms of Mendelian inheritance are possible. Autosomal recessive CMT is typically more severe than the autosomal dominant forms, often with onset in early childhood and severe symptoms such as skeletal deformities and loss of ambulation before adulthood (Tazir *et al.*, 2013). Although more than 70 disease genes for CMT are known, a large number of affected individuals remain without a genetic diagnosis, particularly in CMT2, suggesting that undiscovered disease genes remain (Rossor *et al.*, 2013).

Germinal center associated nuclear protein (GANP) is a multi-domain 208 kDa protein that localizes predominantly to the nucleus and is encoded by 28 exons of *MCM3AP* at chromosome 21q22.3 (Abe *et al.*, 2000). *MCM3AP* is ubiquitously expressed (Abe *et al.*, 2000), but the exact functions of GANP in various tissues remain incompletely understood. GANP has been described to serve as a scaffold for transcription export 2 (TREX-2) complex involved in the export of mRNAs from the nucleus to the cytoplasm (Wickramasinghe *et al.*, 2010; Umlauf *et al.*, 2013). In addition, GANP may associate with NXF1 (nuclear RNA export factor 1) to facilitate the transport of mRNAs from the nuclear interior to the nuclear pores (Wickramasinghe *et al.*, 2010; Jani *et al.*, 2012). In B cells, GANP has been shown to favour transcription complex recruitment and chromatin remodelling at rearranged immunoglobulin variable loci (Singh *et al.*, 2013).

An exome sequencing study by Schuurs-Hoeijmakers *et al.* (2013) investigated families with intellectually disabled siblings, and among their potentially pathogenic findings reported a homozygous *MCM3AP* missense change p.Glu915Lys in an affected sibling pair with progressive polyneuropathy. Another recent study proposed that a *de novo* p.Pro443Ser variant in *MCM3AP* in combination with a *de novo* variant in *POMP*, encoding a

proteasome maturation protein, led to a DNA repair deficit in a child who suffered from immune deficiency (Gatz *et al.*, 2016). The pathogenic role of the *MCM3AP* variants in the former studies is unclear, and thus this gene has not yet been conclusively implicated in human disease.

We present here evidence in multiple families, establishing *MCM3AP* as a disease gene for autosomal recessive CMT and intellectual disability.

## Materials and methods

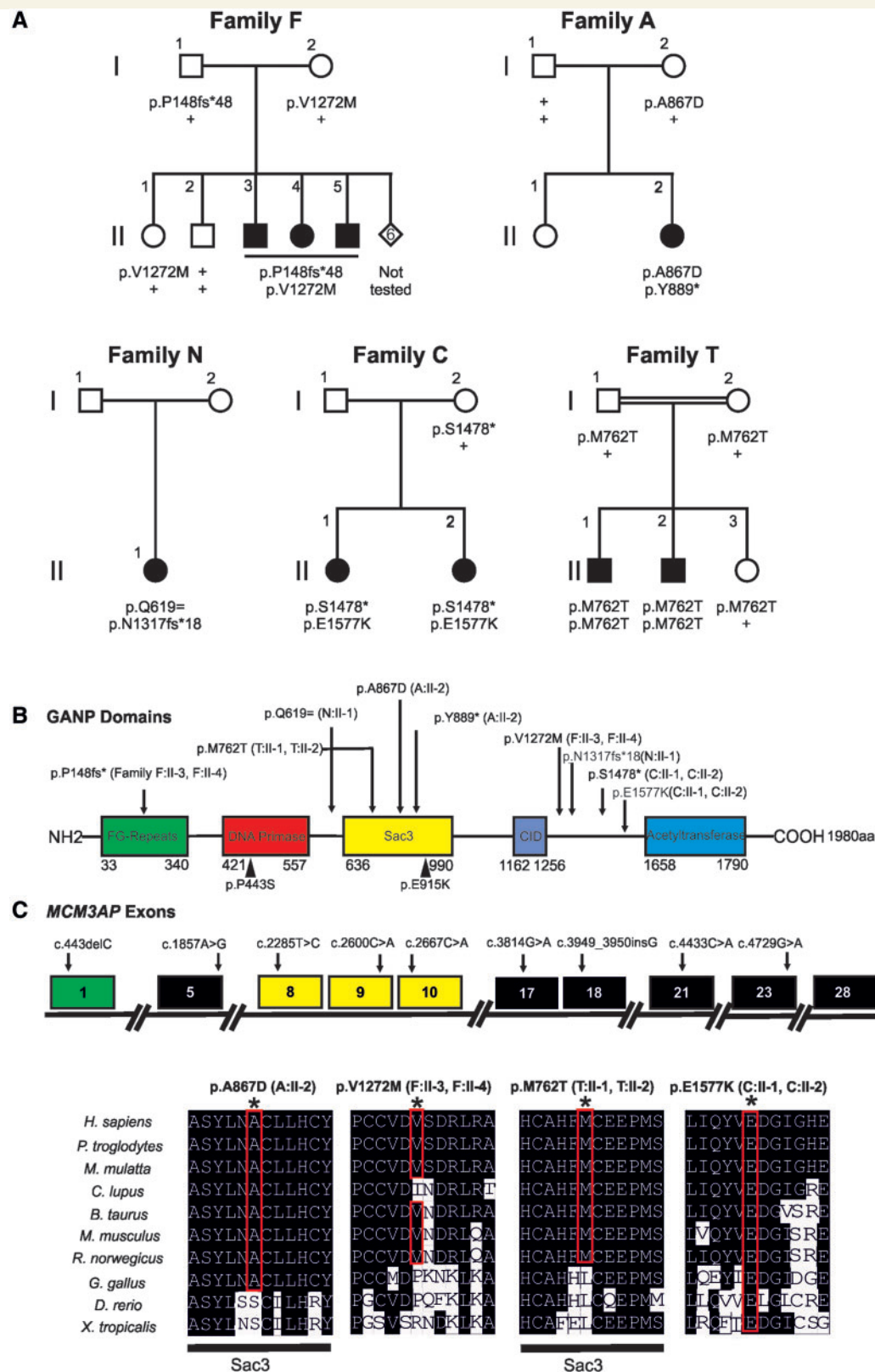
### Patients and sequencing

We studied patients from five families of Finnish (Family F), Australian (Family A), Canadian (Family C), Dutch (Family N) and Turkish (Family T) origin (Fig. 1A). Our study was conducted in accordance with the Declaration of Helsinki, and all participating individuals or their guardians gave written informed consent to participate. The study was approved by the institutional ethics boards. The collaboration was partly established through investigators sharing their genes of interest through the GeneMatcher (Sobreira *et al.*, 2015).

Whole exome sequencing (WES) was performed for Patients F:II-3, F:II-4, F:II-5, A:II-2, N:II-1, C:II-1, T:II-1, and T:II-2, for each family independently. Details of the sequencing methods are in the Supplementary material. *MCM3AP* variants were confirmed by Sanger sequencing; primer sequences are supplied in the Supplementary material.

### Skin fibroblast culture and analysis of RNA and protein

Fibroblast cultures were established from skin punch biopsies by standard methods. Following RNA isolation and cDNA synthesis, cDNA was sequenced with primers specific for exons 1–2 and 16–18 for Family F fibroblasts, and exons 9–10 for Family A fibroblasts. Quantitative PCR (qPCR) was done using DyNAmo™ Flash (Thermo Scientific). Detailed methods and primer sequences are in the Supplementary material. Western blotting was performed with sheep anti-GANP primary antibody provided by Dr Viandha Wickramasinghe, University of Cambridge. To inhibit nonsense-mediated decay



**Figure 1 Pedigrees of affected families and map of variants in MCM3AP/GANP.** (A) Family pedigrees. The genotypes are given for individuals whose samples were available for study, a plus sign (+) indicates a normal allele. (B) The human GANP protein belongs to the Sac3/GANP protein family and consists of 1980 amino acids. The protein contains domains for FG (phenylalanine-glycine) repeat, DNA primase, Sac3 (suppressor of actin 3), CID (Cdc31 interaction domain) and an acetyltransferase domain. The localization of the patient variants in GANP domains and MCM3AP exons are shown with arrows. Arrowheads indicate the positions of two previously reported MCM3AP variants, *de novo* p.Pro443Ser in a child with immunodeficiency, and a homozygous p.Glu915Lys in a sibling pair with intellectual disability. (C) Evolutionary conservation of the amino acids affected by the detected missense variants.

(NMD), cells were treated with cycloheximide (CHX) 20 mg/ml (Sigma) for 24 h. Proteasome inhibition with MG132 was performed as previously described (Ylikallio *et al.*, 2013).

## Minigene mRNA splicing assay

MCM3AP exon 5 and flanking intronic sequences were inserted into pSPL3 minigene vector (Hansen *et al.*, 2008; Wang *et al.*, 2015), a kind gift from Dr Thomas v. O. Hansen. Mutagenesis was used to introduce the splice donor variant c.1857A>G, p.Q619= into exon 5. Two micrograms of plasmids containing the mutated or wild-type exon 5 were transfected into 143B cells with jetPRIME<sup>®</sup> kit (Polyplus). RNA was isolated 24 h later with NucleoSpin<sup>®</sup> kit (Macherey-Nagel) and RT-PCR performed to detect the effects on splicing. Details of the experiment and vector map are supplied in the Supplementary material.

## Mixed neuronal cultures from human induced pluripotent stem cells

To establish a neuronal culture, a previously characterized human induced pluripotent stem cell (iPSC) line HEL11.4 (Mikkola *et al.*, 2013) was differentiated towards neuronal lineage according to Hamalainen *et al.* (2013). Full methods are included in the Supplementary material. Neuronal cells were identified using immunocytochemistry with antibody against neuron-specific class III  $\beta$ -tubulin (TUBJ1, #801201, BioSite), and GANP was detected using an N-terminal antibody (#ab198173, Abcam).

## DNA damage repair

To study DNA damage repair, we used fibroblasts from Patients F:II-3 and F:II-4. Three unrelated normal fibroblast lines served as negative controls, and fibroblasts derived from a xeroderma pigmentosum group C (XPC) patient (Coriell Institute Cell repository, GM16684) and from a DNA ligase 4 syndrome (LIG4) patient (Coriell Institute Cell repository, GM16088) as positive controls for sensitivity to UV and ionizing radiation, respectively. Experimental details are provided in the Supplementary material.

# Results

## Clinical findings

The affected individuals (Fig. 1A) presented with multiple neurological and variable degrees of non-neurological signs and symptoms. The clinical features are summarized in Table 1. Results from nerve conduction studies are given in Table 2, and detailed case descriptions in the Supplementary material. Sensorimotor neuropathy was a prominent feature in all affected individuals. Nerve conduction studies showed axonal neuropathy in Families F, A, N and C. In Family T, nerve conduction studies of Subject T:II-1 was done at an advanced stage of the disease and thus nerve conduction velocities were unobtainable. In Patient T:II-2, median motor nerve conduction velocity

was decreased to 23 m/s, consistent with a demyelinating component. Patient N:II-1 also had decreased median nerve conduction velocities suggesting demyelination in addition to the axonal degeneration. The affected individuals had severe and progressive distal muscle weakness and atrophy in hands and feet. Motor development was delayed, and independent ambulation was generally lost between ages 10–24 years. Besides CMT, the second most consistent finding was intellectual disability, which was found in seven of nine affected individuals (Supplementary Table 1). The intellectual disability was generally mild to moderate, although more severe intellectual disability was seen in two older patients in Family C. In addition, two individuals (Patients F:II-3 and A:II-2) manifested with delayed speech development and learning difficulties.

## Genetic findings

Sequencing revealed MCM3AP variants segregating with the phenotype in all families. The variants in Family F were a single nucleotide deletion c.443delC p.Pro148Leufs\*48 predicting a frameshift and premature stop in the FG-repeat domain of GANP (Fig. 1B), and a missense variant c.3814G>A p.Val1272Met. The frameshift variant was not found in the ExAC database, whereas p.Val1272Met is a European variant with a further enrichment in Finland with population frequencies of 0.0002 and 0.0057, respectively. No homozygous individuals for the missense variant have been listed in any database. The Combined Annotation Dependent Depletion (CADD) version 1.3 (Kircher *et al.*, 2014) C-score of p.Val1272Met was 10.54. The variants segregated with the disease phenotype: the three affected individuals carried both variants, their father was a heterozygous carrier of p.Pro148Leufs\*48 and their mother was heterozygous for p.Val1272Met. Of the two tested unaffected siblings one was a heterozygous carrier of p.Val1272Met and the other carried neither variant (Fig. 1A).

In the affected Patient A:II-2, we found a nonsense variant c.2667C>A p.Tyr889\* and a missense variant c.2600C>A p.Ala867Asp. Neither variant was present in ExAC. The p.Ala867Asp variant was maternally inherited, and the frameshift variant was found to be *de novo*. This was confirmed using a second independent sample of the patient and father, and family relationships were confirmed using single nucleotide polymorphism (SNP) duo analysis. The missense change p.Ala867Asp, which locates in the Sac3 (suppressor of actin 3) domain of GANP (Fig. 1B), received a CADD C-score of 28.1.

The affected individual from Family N was compound heterozygous for the c.1857A>G p.Gln619= variant, and a single nucleotide insertion c.3949\_3950insG p.Asn1317fs\*18, both of which were absent in ExAC. The latter change, located in exon 18, predicted a frameshift and a premature stop codon. The c.1857A>G variant was positioned in the splice donor position of exon 5. *In silico* prediction with the Human Splicing Finder version



**Table 1** Summary of clinical features of the patients

Patient	Variants	Sex	Origin	Age	Walking	Loss of ambulation (age)	Sensori-motor neuropathy	Intellectual disability	UMN signs	Brain MRI findings	Lower limb reflexes	Other
F:II-3	c.3814G>A p.Val1272Met, c.443delC	M	Finland	19	15 mo	10–11 y	Yes	Difficulties in speech and visuomotor development	+	Mildly increased signal intensity in the right temporal lobe	Accelerated	Ventilator-dependent from age 14, Scoliosis, Short stature
F:II-4	p.Pro148Leufs*48 c.3814G>A p.Val1272Met, c.443delC	F	Finland	13	23 mo	10 y	Yes	No	-	Mildly increased signal intensity in temporal lobes	Normal	Short stature
F:II-5	p.Pro148Leufs*48 c.3814G>A p.Val1272Met, c.443delC p.Pro148Leufs*48	M	Finland	8	2 y	Still ambulant	Yes	Yes	+	None (at 4 y)	Diminished	Bicuspid aortic valve and aortic coarctation, Short stature, left-sided amblyopia and microtia with hearing impairment
A:II-2	c.2600C>A p.Ala867Asp, c.2667C>A p.Tyr889*	F	Australia	8	4 y	Still ambulant for short distances	Yes	Likely	-	Brain normal, cervical myelomalacia	Absent	Sensory ataxia, bulbar dysfunction, speech delay, OSA
N:II-1	c.1857A>G p.Gln619=, c.3949_3950insG p.Asn1317fs*18	F	Netherlands	3		Not ambulant	Yes	Yes	-	None (at 6 mo)	Absent	Decreased pain sensation, hypotonia, congenital ptosis, aestropia, scoliosis
C:II-1	c.4433C>A p.Ser1478*, c.4729G>A p.Glu1577Lys	F	Canada	28	17 mo	15 y	Yes; associated contractures	Can read at a grade 1 level, no addition, Life Skills program	-	None	Absent	Primary ovarian failure, strabismus, obesity
C:II-2	c.4433C>A p.Ser1478*, c.4729G>A p.Glu1577Lys	F	Canada	27	20 mo	24 y	Yes; associated contractures	Can read at a grade 3 level, can do simple addition, no subtraction	-	Mild ventriculomegaly and two small white matter cysts in the left posterotemporal parietal region	Absent	Seizures, primary ovarian failure, strabismus, velopharyngeal insufficiency, corneal hydrops, depression, obesity, dysarthric speech
T:II-1	c.2285T>C, p.Met762Thr	M	Turkey	20	4 y	10 y	Yes	Yes	-	ND	Absent	Ataxia
T:II-2	c.2285T>C, p.Met762Thr	M	Turkey	12	1 y	?	Yes	Yes	-	ND	Absent	-

Age = age at last exam; Walking = age at which patient learned to walk independently or with a walking aid; UMN = upper motor neuron; ND = not determined; OSA = obstructive sleep apnoea; ? = not known; mo = months; y = years.

3.0 suggested that the variant creates an exonic splicing silencer site, suggesting a splicing defect with a likely consequence on mRNA stability.

In Family C, the siblings were compound heterozygous for a nonsense variant c.4433C>A p.Ser1478\* in exon 21, and a missense variant c.4729G>A p.Glu1577Lys. The stop variant was not included in ExAC, whereas the missense variant was present twice (frequency  $1.653 \times 10^{-5}$ ) with a CADD score of 24.4. Sanger sequencing confirmed the variants in the affected siblings, and their unaffected mother was a carrier of the nonsense variant.

Finally, in Family T two siblings were homozygous for the missense variant c.2285T>C p.Met762Thr in exon 8. This variant was not present in ExAC, but a variant in the same codon, p.Met762Leu, had a carrier frequency of  $1.651 \times 10^{-5}$ . The p.Met762Thr variant received a CADD C-score of 25.4.

All *MCM3AP* variants and the evidence of their pathogenicity are summarized in Supplementary Table 2.

## **MCM3AP variants lead to severe depletion of GANP protein**

Skin fibroblasts from affected Patients F:II-3, F:II-4 and A:II-2 in Families F and A were available for testing the effects of the compound heterozygosity on mRNA and protein level. The presence of nonsense variants in these two families suggested the possibility of NMD. Unexpectedly in Family F, the sequencing of *MCM3AP* cDNA showed that mRNAs containing variants c.443delC or c.3814G>A were present in the fibroblasts of the affected individuals (Fig. 2A). Therefore, the nonsense variant c.443delC at least partially escaped NMD, possibly due to its location in exon 1. Variants in the first exon of a gene may escape NMD as the result of inability to recruit essential NMD factors to exon 1 termination sites, enabling translation to start from an alternative downstream start site (Kozak, 1987; Neu-Yilik *et al.*, 2011). Western blots showed no smaller bands below the full length GANP, suggesting that the frameshift mRNA in Family F did not produce a stable truncated protein (Fig. 2C). However, we found the total *MCM3AP* mRNA levels to be reduced to ~50% and GANP protein levels to ~20% of the control levels (Fig. 2B–D). As both alleles could still be detected by cDNA sequencing, it is likely that both mutations partially contributed to the mRNA and protein instability. The level of GANP was not restored by inhibiting the proteasome with MG132, suggesting a mechanism other than proteasomal degradation (Fig. 2D). Hence in Family F, the compound heterozygosity of the mutations results in a severe reduction in the amount of GANP protein.

Sequencing of fibroblast cDNA from Patient A:II-2 showed homozygosity for the c.2600C>A p.(Ala867Asp) variant. Accordingly, the total *MCM3AP* mRNA level was ~50% of control level (Fig. 2B). Treatment of Patient A:II-2 fibroblasts with the NMD inhibitor CHX restored the

stability of the nonsense mutant mRNA (Fig. 2A). This showed that the c.2667C>A p.Tyr889\* mRNA was degraded through NMD, as expected when the stop codon is in the middle of the gene. The level of GANP was also decreased in patient's cells but only to ~85% of the control levels (Fig. 2C and D), potentially as a result of compensatory increase in the stability of the protein produced by the missense allele.

Skin fibroblasts from other affected individuals were not available for investigation of the effects of the identified variants. It is, however, noteworthy that also in Family C, one of the compound heterozygous variants is predicted to directly affect the GANP protein level through mRNA instability, and in Family N, a frameshift variant was found in combination with a probable splicing variant. Using a minigene splicing assay, we confirmed that the donor splice site mutation in exon 5 causes the skipping of that exon (Fig. 2E), resulting in a frameshift and a premature stop codon p.Ser557fs21\*. The minigene assay suggested that the mutant transcript is in part spliced normally, therefore some functional protein is likely to be produced. The missense variants found in Families A, C and T affected conserved residues (Fig. 1C) and received high pathogenicity prediction scores. The variants p.Ala867Asp (Family A) and p.Met762Thr (Family T) localize to the Sac3 domain (Fig. 1B), needed for interaction with TREX-2 components, while p.Glu1577Lys (Family C) is in the C-terminal region of GANP, which is needed for localization to the nuclear envelope (Jani *et al.*, 2012). The missense variants are therefore expected to be deleterious for protein function, but their functional effects need to be addressed in future studies. Particularly, the association of the homozygous p.Met762Thr variant with demyelinating CMT requires confirmation in additional families. In conclusion, our results suggest that the mechanisms of disease variants in our affected individuals are related to partial depletion of the GANP protein and/or its loss of function.

## **GANP localizes to the nuclear envelope in cultured human neurons**

The function of GANP has not been previously investigated in the human nervous system. It is reported as a ubiquitously expressed protein (Abe *et al.*, 2000) and we confirmed by immunocytochemistry in human iPSCs differentiated towards a neuronal lineage that it is also expressed in human neurons. Neurons detected by co-staining with neuron-specific marker TUJ1 showed punctate immunoreactivity of GANP at the nuclear envelope (Fig. 2F), consistent with its described role in nuclear pore complexes.

## **Severe depletion of GANP does not compromise DNA repair**

*MCM3AP* has previously been linked to a DNA repair defect (Gatz *et al.*, 2016), and thus we asked if the

**Table 2 Motor nerve conduction studies**

Patient	Age	Median			Ulnar			Lower limb		
		Latency (ms)	Amp (mV)	CV (m/s)	Latency (ms)	Amp (mV)	CV (m/s)	Latency (ms)	Amp (mV)	CV (m/s)
F:II-3	8 y 3 m	-	-	No response	5.7 (distal)	0.3 (distal) 0.5 (prox)	51	5.8 (distal)	0.3 (distal) 0.3 (prox)	38
F:II-4	1 y 10 m	-	-	-	-	-	-	5.6 (distal)	-	38
F:II-5	2 y 1 m	4.1 (distal)	0.7	28.1	-	-	-	4.0	1.4	26.3
A:II-2	8 y	7.0	0.26 (distal)	-	6.6 (distal) 12.9 (prox)	0.47 (distal) 0.25 (prox)	30	7.1 (distal) 17.0 (prox)	0.55 (distal) 0.16 (prox)	28
N:II-1	2 y 8 m	9.44 (distal) 17.6 (prox)	0.10 (distal) 0.11 (prox)	15.3	4.39 (distal) 8.71 (prox)	0.75 (distal) 0.43 (prox)	37	-	-	-
C:II-1	-	-	-	-	-	-	-	-	-	-
C:II-2	-	-	-	-	-	-	-	-	-	-
T:II-1	15 y	-	-	Unobtainable	-	-	-	-	-	Unobtainable
T:II-2	7 y	-	0.5	23	-	-	-	-	-	-

Amp = amplitude; CV = conduction velocity; m = months; prox = proximal; y = years; - = no record available.

For affected individuals in Family C, nerve conduction studies showed axonal sensorimotor neuropathy, but records of the specific measurements are not available. The lower limb recordings were from tibial nerve in Patient A:II-2 and from peroneal nerve in Patients F:II-3, F:II-4 and F:II-5.

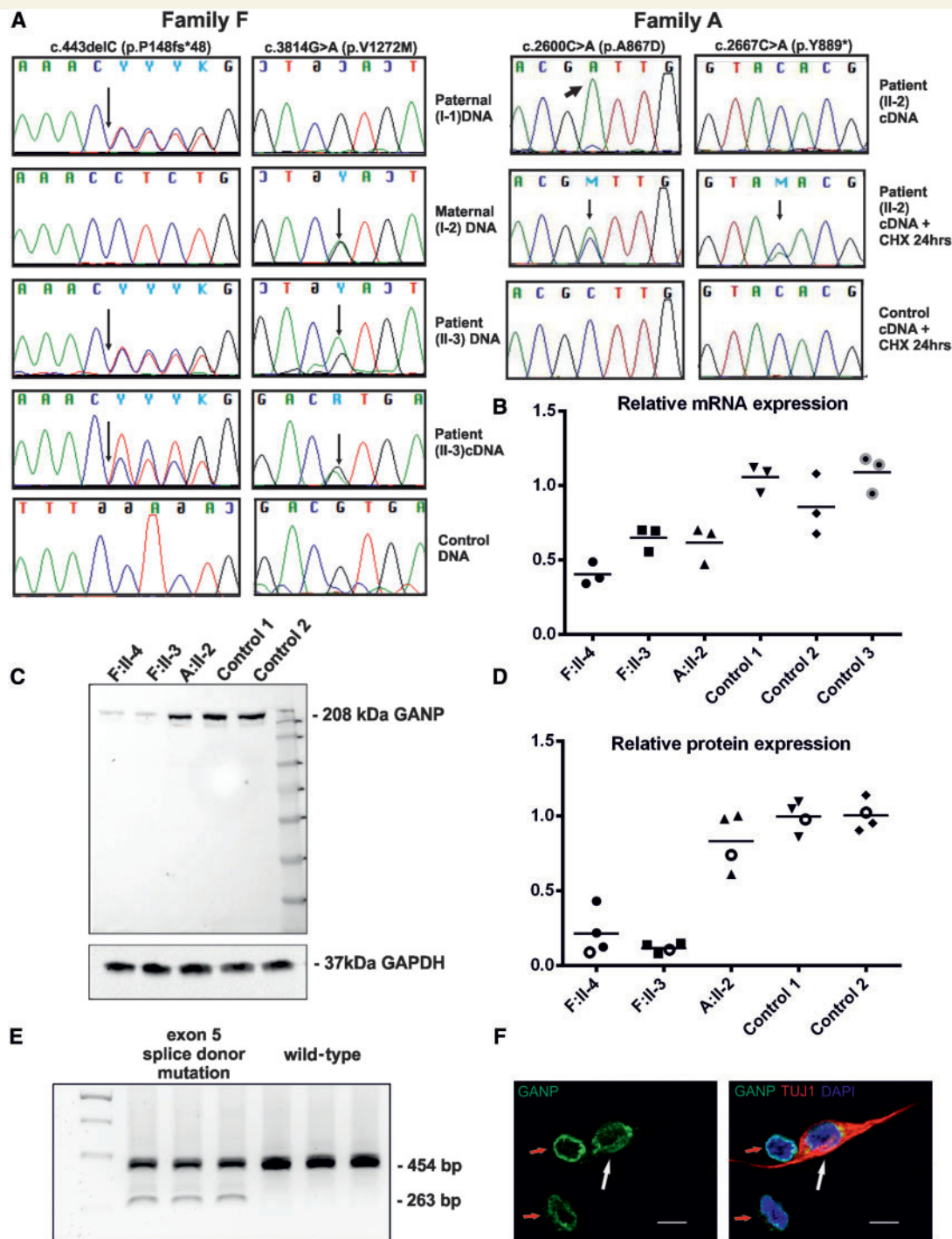
significant depletion of GANP observed in the cells from affected individuals in Family F led to sensitivity to DNA damage. When subjected to UV or ionizing radiation treatment, the cells from Patients F:II-3 and F:II-4 behaved similar to healthy control cells, and no defect in the repair of DNA damage could be identified (Supplementary Fig. 1). This result indicates that severe depletion of GANP does not impair the ability to repair these types of DNA damage.

## Discussion

Our results demonstrate that *MCM3AP* mutations cause early-onset recessive CMT with intellectual disability. Variable additional clinical features included pyramidal signs and brain MRI abnormalities. Affected individuals in Family C had primary ovarian failure and Patient F:II-4 had increased gonadotropin levels; however, gonadotropin levels were not measured in the other affected individuals and thus we cannot conclude that gonadal failure is a consequence of the *MCM3AP* variants. The sensorimotor neuropathy was predominantly axonal except in Family T, where decreased nerve conduction velocities suggested demyelination. Indications of demyelination were also detected in Patients C:II-2 and N:II-1. Thus, recessive loss-of-function variants in *MCM3AP* should be considered as a cause in patients with either axonal or demyelinating features and autosomal recessive inheritance (CMT4). Variants in *GDAP1* can also cause demyelinating CMT4A (Baxter *et al.*, 2002), axonal CMT2K (Cuesta *et al.*, 2002; Claramunt *et al.*, 2005), or intermediate CMT type A (Nelis *et al.*, 2002). The causality of either axonal, demyelinating or intermediate phenotypes is also well documented for variants in *MPZ* (Shy *et al.*, 2004). Similarly, *NEFL* variants cause predominantly axonal neuropathy but nerve conduction velocities can sometimes be decreased to the CMT1 range (Mersiyanova *et al.*, 2000;

Jordanova *et al.*, 2003). *GJB1* is another gene associated with both axonal and demyelinating forms (Ionasescu *et al.*, 1994). The identification of both axonal and demyelinating phenotypes for the same disease gene suggests a defect in the interplay between axons and glia. It is also possible that the observed decrease of nerve conduction velocities in the patients reported here is caused by loss of myelin that develops secondary to severe degeneration of axons.

Neuropathy has been described as a common comorbidity in intellectual disability (Chiurazzi and Pirozzi, 2016). However, it is uncommon for affected individuals diagnosed with CMT to have concomitant intellectual disability. For *MFN2* variants, which are the most common cause of CMT2 (CMT2A2) (Zuchner *et al.*, 2006; Rossor *et al.*, 2013), cases of intellectual disability and cerebral MRI abnormalities together with CMT have been reported (Brockmann *et al.*, 2008; Del Bo *et al.*, 2008; Tufano *et al.*, 2015). Variants in *DYNC1H1* are known to cause a spectrum of diseases that includes spinal muscular atrophy with lower extremity predominance (SMA-LED) (Harms *et al.*, 2012), intellectual disability with malformations of cortical development (MCD) (Poirier *et al.*, 2013), and CMT2O (Weedon *et al.*, 2011). Speech delay and/or learning difficulties were reported in some individuals with SMA-LED or CMT2O (Weedon *et al.*, 2011; Harms *et al.*, 2012), and several individuals with MCD also had neuropathy (Poirier *et al.*, 2013). Furthermore, Cowchock syndrome (CMTX4) consists of CMT in combination with intellectual disability and sensorineural hearing loss (Cowchock *et al.*, 1985). Lastly, giant axonal neuropathy is a disorder caused by recessively inherited variants in *GAN* (Bomont *et al.*, 2000), which can present with a severe CMT2-resembling neuropathy and multiple additional symptoms and signs including curly hair, CNS manifestations and intellectual disability (Vijaykumar *et al.*, 2015). Our findings add *MCM3AP* to the disease genes associated with CMT and intellectual disability.



**Figure 2** Consequences of *MCM3AP* variants on mRNA and protein level. (A) Sequencing traces for the variants in Families F and A. Total blood DNA and fibroblast cDNA sequencing traces are shown for Family F. The presence of both variants c.443delC p.Pro148fs48\* and c.3814G>A p.Val1272Met were detected in the cDNA. Sequencing of Family A patient fibroblast cDNA showed that the variant c.2667C>A p.Tyr889\* was undetectable under normal culture conditions, and the cDNA was homozygous for the c.2600C>T p.Ala867Asp variant. Treatment of fibroblasts with the nonsense mediated decay (NMD) inhibitor cycloheximide (CHX) restored stability of the mRNA containing the c.2667C>A variant. (B) *MCM3AP* mRNA levels in fibroblasts from affected individuals in Families F and A, and from controls. The quantification is shown for three biological triplicates. Mean is indicated by a line. (C) Western blotting results show the relative level of GANP protein (208 kDa) in patient and control fibroblasts. The 37 kDa GAPDH was detected as a loading control. (D) Quantification of GANP protein level relative to GAPDH in patient and control cells. Black symbols show the results from three independent blots. Mean is indicated by a line. White circles are the quantifications from a blot of cells treated with proteasome inhibitor MG132. (E) Minigene splicing assay using the pSPL3-vector containing *MCM3AP* exon 5 and flanking intronic sequences revealed that the exon 5 splice donor variant c.1857A>G, p.Gln619=, resulted in skipping of exon 5. 143B cells transfected with wild-type or mutant plasmids were subjected to RT-PCR using the vector primers. The 454 bp product resulted from correctly spliced transcript, and the 263 bp product from the exon skipping transcript. Biological triplicates are shown. (F) Mixed neuronal culture differentiated from human iPSCs co-immunostained with GANP antibody (green) and neuronal marker TUJ1 (red), and with DAPI (blue). White arrows indicate a neuron, while red arrows indicate other cell types. Scale bars = 10 μm.



The identification of variants in five families of various ethnicities suggests that *MCM3AP* may not be an uncommon disease gene for this clinical presentation.

Our results suggest that GANP has an important role in the development of the nervous system in addition to its previously identified physiologic role in B cell antibody maturation (Singh *et al.*, 2013). GANP expression has been detected in the brain (Abe *et al.*, 2000), but its function in neural cells has not been characterized. We show that GANP localizes to the nuclear envelope in cultured human neurons. Involvement in DNA repair has been suggested for GANP (Eid *et al.*, 2014; Gatz *et al.*, 2016) as well as for another recently identified CMT gene *MORC2* (Li *et al.*, 2012; Albulym *et al.*, 2016; Sevilla *et al.*, 2016), suggesting these genes may share a common pathway as the underlying cause for CMT. However, our results in this study do not support a role for a DNA repair defect in GANP deficiency. Interestingly, a study in *Drosophila* identified the orthologue of *MCM3AP*, *xmas-2*, to suppress the toxic effect of cytoplasmic accumulation of TDP-43 (TAR DNA binding protein 43 kDa, encoded by *TBPH*) in fly motor neurons, fitting with *xmas-2* having a crucial role in the nuclear export of *TBPH* mRNA in neurons (Sreedharan *et al.*, 2015). GANP could thus be involved in the regulation of mRNA export also in human neurons. Defects of RNA metabolism have been previously implicated in motor neuron degeneration (Strong, 2010). In particular, *GLE1*, which is mutated in foetal motor neuron disease (Nousiainen *et al.*, 2008) or amyotrophic lateral sclerosis (ALS) (Kanab *et al.*, 2015), encodes a factor that is essential for mRNA export (Folkman *et al.*, 2013). Moreover, a recent study showed that disordered nucleocytoplasmic transport may contribute to the neurodegeneration caused by protein aggregates in the cytoplasm (Woerner *et al.*, 2016). Thus, it is conceivable that GANP dysfunction leading to nuclear retention of mRNAs that are crucial for neuronal function is the cause of axonal degeneration in our affected individuals. Further studies are needed to elucidate the identity of the particular mRNAs, or other potential roles of GANP in neurons.

In summary, we have identified recessive mutations *MCM3AP* associated with neuropathy and intellectual disability and a functional decrease of GANP protein. Our findings propose a new molecular mechanism for neuropathy involving mRNA export from the nucleus.

## Web resources

GeneMatcher, <http://www.genematcher.org>  
 Exome Aggregation Consortium (ExAC), <http://exac.broad-institute.org/>  
 Combined Annotation Dependent Depletion (CADD), <http://cadd.gs.washington.edu/home>  
 Human Splicing Finder, <http://www.umd.be/HSF3/>  
 HAL Splice Prediction, <http://splicing.cs.washington.edu/SE>  
 OMIM, <http://www.omim.org/>

## Acknowledgements

We thank the affected individuals and their families for participating in this study. Riitta Lehtinen and Sundar Baral are thanked for technical assistance. Drs Jemeen Sreedharan and Matthew White are acknowledged for discussions and advice. Dr Vihandha Wickramasinghe, University of Cambridge, is acknowledged for contributing the GANP antibody, and the Biomedicum Stem Cell Centre, the iPSCs.

## Funding

Patient A's sequencing was completed as part of a study funded and supported by the Melbourne Genomic Health Alliance. This work was supported in part by the Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIISS. Whole exome sequencing for Family C was performed as part of the Care4Rare Canada Consortium, funded by Genome Canada, the Canadian Institutes of Health Research, the Ontario Genomics Institute, Ontario Research Fund, Genome Quebec, and Children's Hospital of Eastern Ontario Foundation. Analysis of the Turkish family is supported partially by Bogazici University Research Fund Grant Number 14B01D5. The following funding sources are also gratefully acknowledged: Academy of Finland (H.T., E.Y., L.K.), Sigrid Juselius Foundation (H.T., L.K.), University of Helsinki (H.T., E.Y., M.T.), Biocentrum Helsinki (L.K.), Finska läkaresällskapet (E.Y.), Helsinki University Hospital (E.Y.), Foundation for Pediatric Research (P.I.), Fund for Scientific Research-Flanders (FWO) (A.J.), University of Antwerp (A.J.), Belgium Association for Neuromuscular Disorders (ABMM) (A.J.), research fellowships from the Fund for Scientific Research-Flanders (FWO) (D.A., A.E.-C), NHMRC Career Development Fellowship (GNT1032364) (P.J.L.).

## Supplementary material

Supplementary material is available at *Brain* online.

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