RESEARCH ARTICLE

Characterization of a novel variant in the HR1 domain of *MFN2* in a patient with ataxia, optic atrophy and sensorineural hearing loss [version 1; peer review: 2 approved with reservations]

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Abstract

Background: Pathogenic variants in *MFN2* cause Charcot-Marie-Tooth disease (CMT) type 2A (CMT2A) and are the leading cause of the axonal subtypes of CMT. CMT2A is characterized by predominantly distal motor weakness and muscle atrophy, with highly variable severity and onset age. Notably, some *MFN2* variants can also lead to other phenotypes such as optic atrophy, hearing loss and lipodystrophy. Despite the clear link between *MFN2* and CMT2A, our mechanistic understanding of how dysfunction of the MFN2 protein causes human disease pathologies remains incomplete. This lack of understanding is due in part to the multiple cellular roles of MFN2. Though initially characterized for its role in mediating mitochondrial fusion, MFN2 also plays important roles in mediating interactions between mitochondria and other organelles, such as the endoplasmic reticulum and lipid droplets. Additionally, MFN2 is also important for mitochondrial transport, mitochondrial autophagy, and has even been implicated in lipid transfer. Though over 100 pathogenic *MFN2* variants have been described to date, only a few have been characterized functionally, and even then, often only for one or two functions.

Method: Several MFN2-mediated functions were characterized in fibroblast cells from a patient presenting with cerebellar ataxia,

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deafness, blindness, and diffuse cerebral and cerebellar atrophy, who harbours a novel homozygous MFN2 variant, D414V, which is found in a region of the HR1 domain of MFN2 where few pathogenic variants occur.

Results: We found evidence for impairment of several MFN2-mediated functions. Consistent with reduced mitochondrial fusion, patient fibroblasts exhibited more fragmented mitochondrial networks and had reduced mtDNA copy number. Additionally, patient fibroblasts had reduced oxygen consumption, fewer mitochondrial-ER contacts, and altered lipid droplets that displayed an unusual perinuclear distribution.

Conclusion: Overall, this work characterizes D414V as a novel variant in MFN2 and expands the phenotypic presentation of *MFN2* variants to include cerebellar ataxia.

Keywords

Mitochondria, MFN2, Mitochondrial Fusion, Ataxia

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Abbreviations

BDLP: bacterial dynamin like protein
 CADD: combined annotation dependent depletion
 CAPOS: cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss
 CMT2A: Charcot-Marie-Tooth type 2A
 CO₂: carbon dioxide
 DAPI: 4',6-diamidino-2-phenylindole
 DSHB: Developmental Studies Hybridoma Bank
 EDTA: ethylenediaminetetraacetic acid
 ER: endoplasmic reticulum
 FBS: fetal bovine serum
 GAD: glutamic acid decarboxylase
 HR1: heptad repeat 1
 HR2: heptad repeat 2
 IMS: inter membrane space
 MEM: minimum essential media
 MERCs: mitochondria–endoplasmic reticulum contact sites
 MFN: mitofusin
 MRI: magnetic resonance imaging
 mtDNA: mitochondrial deoxyribonucleic acid
 NGS: next generation sequencing
 OCR: oxygen consumption rate
 OMIM: Online Mendelian Inheritance in Man
 OPA1: optic atrophy 1
 PBS: phosphate buffered saline
 PFA: paraformaldehyde
 PLA: proximity ligation assay
 qPCR: quantitative polymerase chain reaction
 SD: standard deviation
 VCFs: Velocardiofacial syndrome
 VEP: Variant Effect Predictor

Introduction

Mitochondria are highly dynamic double membrane-bound organelles that undergo continuous remodelling via fusion and fission events. These dynamic processes determine mitochondrial structure and regulate mitochondrial function.¹ Mitochondrial fusion is a multistep process mediated by several essential proteins and regulators.² Tethering of adjacent mitochondria and fusion of outer mitochondrial membrane (OMM) is performed by mitofusin1 and mitofusin2 (MFN1/2), two homologous proteins with partially redundant functions that are integral to the OMM. Meanwhile, fusion of the inner mitochondrial membrane (IMM) is carried out by optic atrophy 1 (OPA1).^{3,4} Highlighting the importance of mitochondrial fusion is the fact that knockout of *MFN1*, *MFN2* or *OPA1* genes is embryonic lethal.^{5,6} In addition, pathogenic variants in these genes cause human disease, with *OPA1* linked to optic atrophy, and *MFN2* linked to the peripheral neuropathy Charcot Marie Tooth type 2A (CMT2A).^{7,8}

MFNs have an N-terminal GTPase domain that is exposed to the cytosol, and two heptad repeat domains (HR1 and HR2, also referred to as coiled-coil domains), separated by a transmembrane domain. While several structural models have been generated for MFNs,^{9–12} these are from artificial constructs that are based on the notion that N-terminal and C-terminal domains of the protein both face the cytosol and can interact. However, a recent reappraisal of the topology of MFNs showed that the C-terminus of the protein, including the HR2 domain, is exposed to the inner membrane space (IMS), not the cytosol.¹³ This revision raises questions about the validity of the structural models. Thus, there remain many questions as to the exact structure of MFNs and how they mediate mitochondrial fusion.

Mitochondrial fusion is also important for the maintenance of the mitochondrial genome (mtDNA), which is present in hundreds to thousands of copies per cell. The mtDNA is packaged into nucleoid structures, each of which is approximately 100 nm in size and contains a single copy of the mitochondrial genome.^{14–16} Impairments to mitochondrial fusion can lead to reduced copy number and increased nucleoid size.^{17,18} While the reduced copy number is thought to be a result of decreased distribution of the replication machinery,¹⁹ it is unclear exactly why nucleoid size might change. Meanwhile, mitochondrial respiration can also be compromised upon loss of fusion.^{20,21}

Notably, MFN2 has several functions in addition to its role in mitochondrial fusion. For example, MFN2 mediates mitochondrial autophagy^{22,23} and transport of mitochondria.^{24,25} Meanwhile, MFN2 also localizes to the endoplasmic reticulum, where it mediates interactions between the ER and mitochondria.^{26–28} These mitochondria-ER contact sites (MERCs) are specialized sites for both lipid biogenesis and exchange, are important for regulating calcium signalling, and can also mark sites of mitochondrial fission,²⁹ as well as mtDNA replication.³⁰ Notably, MFN2 has been implicated in directly binding and transferring phosphatidylserine from ER to mitochondria.³¹ While there is some debate whether MFN2 promotes or inhibits MERCs,^{27,28,32,33} it is clear that it plays an important role in these organelle contacts. Finally, in addition to MERCs, MFN2 also mediates interactions between mitochondria and lipid droplets.³⁴

To date, more than 100 pathogenic variants in *MFN2* have been associated with peripheral neuropathy.³⁵ However, despite the general assumption that impaired mitochondrial fusion causes the peripheral neuropathy phenotype, only a few pathogenic *MFN2* variants have been investigated functionally for their effects on *MFN2* functions. While some pathogenic *MFN2* variants do impair fusion,^{36,37} unexpectedly, other pathogenic variants seem to increase fusion,^{38,39} while several pathogenic variants do not appear to affect fusion at all.^{37,40,41} These findings raise the possibility that impaired fusion does not lead to peripheral neuropathy *per se*.

In this context, it is notable that other *MFN2*-mediated mitochondrial functions can also be impacted by pathogenic *MFN2* variants. For example, disruptions to MERCs,^{26,37} lipid metabolism,⁴² mitochondrial respiration,⁴³ mtDNA copy number,^{44–46} and mitochondrial transport,²⁵ have all been observed in association with various pathogenic *MFN2* variants. However, it should be noted these phenotypes have not all been widely investigated across a variety of *MFN2* variants.

Further complicating our mechanistic understanding of how *MFN2* dysfunction causes disease is the fact that additional pathogenic phenotypes can also be linked to *MFN2* variants. Although not common, some *MFN2* variants are linked to other disease phenotypes such as optic atrophy.^{47,48} Central nervous system involvement is also rarely described,⁴⁹ with periventricular and subcortical white matter lesion pathogenic for age, associated with transient neurological deficits in a small minority of cases. Other complex phenotypes have been observed in the presence of homozygous or compound heterozygous mutations. For example, the R707W *MFN2* variant, which causes CMT2A when heterozygous, is also associated with lipodystrophic syndromes when homozygous. Specifically, R707W causes proliferation of adipocytes leading to adipose hyperplasia and lipomatosis.^{42,50,51} Additional atypical features including severe neuropathy with hearing loss has been described with biallelic mutations.⁴⁷

In summary, *MFN2* performs a number of functions, many of which can be impaired by pathogenic variants in *MFN2*. Meanwhile, *MFN2* variants can lead to a number of different patient phenotypes. However, there is no clear understanding of the molecular mechanisms causing disease and whether impairment of specific *MFN2* functions leads to specific phenotypes. Here, in a patient presenting with ataxia, sensorineural hearing loss, and optic atrophy leading to vision loss, we report the presence of a homozygous novel candidate pathogenic variant in *MFN2*, p.(D414V), located in the middle of the HR1 domain. While ataxia has not been associated previously with *MFN2* specifically, it is common in mitochondrial disease. Our characterization of mitochondrial functions in patient fibroblasts is consistent with impairment of *MFN2* functions, expanding the clinical spectrum of phenotypes associated with pathogenic variants in *MFN2*.

Methods

Ethics statement

This study was approved by the University of Calgary Conjoint Health Research Ethics Board with the following approval numbers: REB15-2763 (for exome sequencing), REB17-0850 (for the skin biopsy/fibroblast lines). Written informed consent was obtained from the participant for both of the above projects. Because of the participant's below-mentioned visual impairments, it was necessary to read and verbally explain the consent form to him. The participant's sister, who attends his medical appointments and aids with his care, assisted to ensure he understood the nature of the research and could participate in the informed consent process.

Case report

The case was identified from the clinical practise of one of the authors (GP). The chart and clinical investigations were reviewed retrospectively to produce the summary provided in this report. The patient provided written informed consent for participation in research, for exome sequencing, and for a skin biopsy in order to isolate primary fibroblast cells which were used in this study. All research was part of studies approved by the University of Calgary Conjoint Health Research Ethics Board. The patient was provided with a copy of the finished manuscript and gave consent for the publication of this report.

Exome sequencing and bioinformatics

DNA was extracted from blood collected into EDTA tubes using standard protocols. Library preparation proceeded using the Ion Ampliseq Exome RDY Panel (Thermo Fisher, A38264) according to manufacturer's protocol. Automated chip loading and templating used the Ion Chef system and 540 chip/chef kit (Thermo Fisher, A30011), and sequencing was performed on an Ion S5 system (Thermo Fisher, A27212), according to manufacturer's protocols. Base calling, read alignment to hg19, coverage analysis, and variant calling were performed with Torrent Suite (v. 5.10.1; Thermo Fisher).⁵² Similar analysis can be performed using an alternative bioinformatic pipeline as described previously.⁵³ Patient VCFs were annotated for predicted variant consequence, gnomAD allele frequency,⁵⁴ CADD score,⁵⁵ and OMIM phenotypes,⁵⁶ in addition to default parameters with Ensembl's command line Variant Effect Predictor (VEP).⁵⁷

Cell maintenance

Control and patient fibroblast cultures were generated from skin biopsies and cultured in MEM media (Gibco, 11095080) containing l-Glutamine and supplemented with 10% fetal bovine serum (FBS) and 1 mM sodium pyruvate. Cells were maintained at 37°C and 5% CO₂.

Immunofluorescence staining and microscopy

Fibroblasts were seeded on glass coverslips (Fisherbrand, 12-545-81) placed in 24-well plate, at a density of 2×10^4 cells per well, and incubated for 1–2 days. As described previously,⁵⁸ cells were washed with 1xPBS (37°C) and fixed with 4% paraformaldehyde (37°C), permeabilized with 0.1% TritonX-100, blocked with 5% FBS and target proteins were probed with primary and secondary antibodies indicated below. Phosphate buffered saline (PBS) was used to wash cells between the steps, and to prepare all reagent solutions. Mitochondrial networks were labeled with a rabbit polyclonal anti-TOMM20 antibody (Santa Cruz Biotechnology, F-10 (RRID:AB_628381)) and visualized with a polyclonal goat anti-rabbit secondary conjugated with Alexa fluor 488 (Thermo fisher Catalog # A-11034 (RRID:AB_2576217)). Mitochondrial nucleoids were labelled with a mouse monoclonal autoanti-dsDNA antibody (Developmental Studies Hybridoma Bank, AB_10805293, (RRID:AB_10805293)), and visualized with a polyclonal goat anti-rabbit secondary conjugated with Alexa fluor 568 (Thermo fisher Catalog # A-11004 (RRID:AB_2534072)). Immunostained cells mounted on glass slide, and imaged on an Olympus spinning disc confocal system (Olympus SD OSR) (UAPON 100XOTIRF/1.49 oil objective) operated by Metamorph software. Z-stacks of cells were acquired and their z-projection images were used for data analysis.

Mitochondria-ER contact sites analysis by proximity ligation assay (PLA)

Number and size of MERCs was analyzed using the Duolink[®] In Situ Proximity Ligation Assay (Millipore Sigma), as described previously.⁵⁹ Briefly, fibroblasts cultured on glass coverslips were washed, fixed and permeabilized. Blocking of unspecific binding sites was performed (1 h at 37°C in a humidified chamber). The primary antibodies, TOMM20 (rabbit polyclonal, Sigma-Aldrich: HPA011562-100U (RRID:AB_1080326)) to visualize mitochondria and Calnexin (mouse monoclonal, EMD Millipore: MAB3126 (RRID:AB_2069152)) to visualize ER, were applied at 1:1000 dilution. Cells were then incubated with oligonucleotide conjugated secondary antibodies (donkey anti-rabbit polyclonal PLUS #DUO92002 (RRID:AB_2810940), and donkey anti-mouse polyclonal MINUS #DUO92004 (RRID:AB_2713942)) diluted 1:5 for 1 h at 37°C in a humidified chamber, followed by ligation and amplification steps using the Duolink[®] In Situ Detection Reagents Red kit (DUO92008). Thereafter, the primary antibodies were further labelled with Alexa fluor conjugated secondary antibodies, such that mitochondria were visualized with polyclonal goat-anti-rabbit 647 (Thermo Fisher Catalog # A-21245 (RRID:AB_2535813)), and ER was visualized with polyclonal goat anti-mouse 488 (Thermo Fisher Catalog # A-11029 (RRID:AB_138404)). Coverslips with immuno-stained cells were mounted on a glass slide with ProLong[™] Glass Antifade Mountant with NucBlue[™] Stain (Thermo Fisher Scientific, P36983). Z-stack were acquired by confocal microscopy as described above for signals from PLA, DAPI as well as labelled TOMM20 and calnexin. Maximum intensity projections of the Z-stacks containing the PLA signals were analyzed with ImageJ FIJI using its 'Analyze particle' tool to retrieve the number and sizes of the MERCs.

Lipid droplet staining

Fibroblasts grown on glass coverslips were fixed with 4% PFA for 20 min at 37°C. After washing off the PFA, cells were permeabilized using 0.1% Saponin (Sigma Aldrich, SAE0073-10G) for 15 min at 37°C. Then the neutral lipid dye, HCS LipidTox green (Thermo Fisher Scientific, # H34350), was applied at a dilution of 1:1000 and incubated overnight at 4°C. The dye solution was washed off and coverslips were then blocked 10% FBS in PBS. Stained cells were washed with PBS

and mounted on glass slides with ProLong™ Glass Antifade Mountant with NucBlue™ Stain (Thermo Fisher Scientific, P36983), and microscopy was performed as aforementioned.

Image analysis

Mitochondrial network morphology was quantitatively analyzed by measuring the mitochondrial lengths using ImageJ FIJI. Briefly, background was subtracted, then images of mitochondrial networks were skeletonized and the analyze skeleton function was used to obtain the mitochondrial length. The sum of the lengths of all branches of a mitochondrion was evaluated as the total length of that mitochondrion. For each of control or patient fibroblast, at least 20 cells were evaluated. The results shown are one of three independent biological replicates with the same trends showing mean $\pm$ SD, and P values based on unpaired, 2-tailed Student's t-tests.

Size and number of mitochondrial nucleoids as well as MERCs were analyzed using the 'Analyze particle' tool in ImageJ FIJI.⁶⁰ Mitochondrial nucleoids or PLA signals (indicating MERCs) in the fibroblasts were evaluated from the maximum intensity projection of the z-stacks by using 'Analyze particle' function of ImageJ FIJI (nuclear signal was excluded from images of fibroblasts immunostained with anti-DNA antibody). The analyses were performed on at least 20 fibroblasts for each patient and control lines. The results represent mean $\pm$ SD, and P values were based on unpaired, 2-tailed Student's t-tests. Each data point is presented as the number of nucleoids per cell or the average size of all nucleoids per cell. In most cases there was one cell per image, but in some cases there were multiple cells per image. In the latter case the numbers were averaged for the number of cells in the image. Bar graphs indicate the average nucleoid sizes or counts in all the fibroblasts analyzed $\pm$ SD. P values were based on unpaired, 2-tailed Student's t-tests.

Lipid droplet numbers were calculated using the same procedure for analyzing the numbers of mitochondrial nucleoids and PLA signals as described above. The distance of individual lipid droplets from nucleus was calculated using ImageJ FIJI. Briefly, the z-projection (maximum intensity) images of cells stained with the LipidTox Green dye and DAPI were processed with the Analyze Particle function to get coordinates for the centre of the individual lipid droplets, as well as the nucleus. These co-ordinates were used to calculate the distance between the centre of each lipid droplet and the center of the nucleus. The distance of each lipid droplet from the nucleus was calculated for at least 20 cells, and used to determine either the distance distribution for all lipid droplets, or the average lipid droplet distance per cell.

mtDNA copy number analysis

Total DNA (nuclear and mitochondrial DNA) was purified from control and patient fibroblasts (seeded at 5×10^5 cells) using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, K182001) according to manufacturer's instructions. The relative mtDNA copy number was assessed using QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific). The mtDNA and the nuclear-encoded housekeeping gene 18S were amplified using primer sequences, and thermocycling conditions exactly as described in.⁶¹ Briefly, the 20 μ L quantitative PCR (qPCR) reaction contained 10 μ L PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, A25742), 100 ng total DNA as template and 500 nM forward and 500 nM reverse primers (final concentrations). MtDNA copy number relative to 18S was analyzed using the delta delta Ct method and represented as percent control.⁶² Reactions were performed in triplicate technical replicates. Data is presented as mean $\pm$ SD and unpaired, 2-tailed Student's t-tests were used to determine statistical significance.

Long range PCR

Long range PCR reactions were performed to examine mtDNA deletions as reported previously.⁶³ The following primers were used to amplify nearly full length mtDNA (16.3 kb),

(1482–1516 F: ACCGCCCCGTCACCCTCCTCAAGTATACTTCAAAGG;

1180–1146 R: ACCGCCAGGTCCTTTGAGTTTAAAGCTGTGGCTCG).

The Takara LA Taq polymerase (Takara Bio, RR002M) was used with 250 ng genomic DNA and 200 nM forward and reverse primers. Cycling conditions for PCR were as follows: 94°C for 1 min; 98°C for 10 s and 68°C for 11 min (30 cycles); and a final extension cycle at 72°C for 10 min, using an Eppendorf® 5331 MasterCycler Gradient Thermal Cycler. PCR products were visualized by electrophoresis on a 0.6% agarose gel, run for approximately 12 h at 20 V.

Mitochondrial respiration

Mitochondrial oxygen consumption rates (OCR) in control and patient fibroblasts were measured using a Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies, Inc) as described previously.²¹ Briefly, cells were seeded in an XF24 microplate (3.75×10^4 /well) and incubated at 37°C, 5% CO₂ for 24 h. Prior to measurement, the growth media was replaced and cells equilibrated in assay media supplemented with d-Glucose (25 mM), sodium pyruvate (2 mM) and l-Glutamine (4 mM). Oxygen consumption rates were calculated following injection of the following compounds: oligomycin (1 µg/mL) (Enzo Life Sciences, BML-CM111), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 1 µM) (Enzo life Sciences, BML-CM120) and Antimycin A (1 µM) (Sigma Aldrich, A8674). Data were normalized to protein content data for each well measured by BCA assay (Thermo Fisher Scientific, 23225).

BioRxiv

An earlier version of this article can be found on bioRxiv (<https://doi.org/10.1101/2021.01.11.426268>).

Results

Case description and clinical diagnosis

The patient initially came to medical attention at eight years of age, having previously had a normal birth history and development. He began to experience visual blurring affecting both eyes, and bilateral optic atrophy was identified on ophthalmologic evaluation. His vision continued to slowly deteriorate over time. In his 20s he developed gait disturbance which was diagnosed as ataxia (though in retrospect teachers had informed him of changes to his gait as early as 12 years of age). The ataxia progressed to affect limb coordination functions in his 30s, in association with dysarthria. Sensorineural hearing loss was diagnosed in his 20s and progressed gradually to deafness. In his 40s he developed hypertension as well as type 2 diabetes mellitus. He was coincidentally diagnosed with a painful small-fibre sensory neuropathy, which was thought to be attributable to his diabetes.

His family history did not include any other individuals with neurologic disease. His parents originated from India and no consanguinity was reported. His two siblings are healthy and do not have a similar condition. The patient does not have any children.

By the time of his assessment by one of the authors (GP) at age 54 years, his family described severe visual and hearing loss. He required use of a wheelchair and assistance for feeding and all daily living tasks due to severe balance and coordination deficits. On neurologic examination, he was alert and attentive. His cognition was difficult to assess due to the above-mentioned visual/hearing loss and communication difficulties, but his overall cognition seemed grossly appropriate. Cranial nerve examination demonstrated bilateral optic atrophy, light perception only for visual acuity, full extraocular movements, no ptosis, and symmetric facial movements. His hearing was poor, but he was able to comprehend speech when spoken in a loud voice directly into his ear. He also had cerebellar dysarthria, but speech was still intelligible. He otherwise had normal lower cranial nerve functions. He had diffuse and symmetric loss of muscle bulk which was attributed to deconditioning, with normal motor tone. Deep tendon reflexes were unobtainable in all extremities. Plantar responses were flexor. Power examination revealed that muscle strength was well preserved, with some minor hip girdle weakness which was again attributed to disuse. Additionally, he exhibited a postural tremor in his upper limbs and bilateral dysmetria and intention tremor on finger-to-nose testing. He required two-person assistance to stand. Sensory examination was fairly unremarkable, with vibration sensory loss below the ankles bilaterally, but was otherwise normal.

The differential diagnosis at this time was considered to most likely include genetic or metabolic disorders, such as mitochondrial disorders, CAPOS syndrome (cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss, due to mutations in *ATPIA3*), or other multisystem genetic disorders such as Wolfram syndrome (due to *WFS1* mutations). Investigations included vitamin B12, folate, and vitamin E levels, which were normal. Very long chain fatty acids and hexosaminidase A and B were also normal. Testing for anti-GAD antibodies was negative. A muscle biopsy demonstrated some denervation atrophy but did not reveal any changes suggesting a mitochondrial cytopathy. MRI of the brain revealed diffuse cerebral volume loss affecting the cerebrum, cerebellum and brainstem structures, and volume loss was also observable in the optic nerves bilaterally and optic chiasm (Figure 1A). Clinical genetic testing included a first-line genetic screening for spinocerebellar ataxia types 1, 2, 3, 6, 7, 8, and Friedreich ataxia, which was negative. He also had an NGS-based sequencing panel for ataxic syndromes including 277 genes. This found a homozygous variant of unknown significance in *MFN2*, c.1241A>T p.(D414V).

Figure 1. Pathogenic variants in MFN2. Representative images of MRI head scans of the patient at 55 years of age. Top-left: T1-weighted sagittal image through the midline demonstrates volume loss in the frontal lobe, brainstem, and midline cerebellar structures. Top-right: T1-weighted transverse axial image at the level of the lateral ventricles and basal ganglia demonstrate volume loss predominantly affecting the bilateral frontal lobes. Bottom panels: T2-weighted transverse axial images at the levels of the medulla and pons again demonstrate brainstem atrophy as well as accentuated cerebellar foliae indicative of diffuse volume loss in the posterior fossa. (B) Sequencing chromatograms confirms the 1241A>T variant, resulting in a missense mutation of Aspartic acid (D) to Valine (V) at position 414 in the MFN2 protein. Sequencing data are shown from patient (upper panel) or normal control (lower panel) derived fibroblasts. (C) Alignment of the region of the HR1 domain of MFN2, showing D414V is conserved throughout vertebrate species and with MFN1. Residues highlighted in yellow are conserved residues determined by Clustal Omega analysis. (D) Diagram showing the topology and domains of the 757 amino acid MFN2 protein, which contains a GTPase domain, two HR domains and a transmembrane (TM) domain. The number of reported pathogenic missense mutations in the indicated regions of the protein are indicated on the right.

Exome sequencing was performed and variants were filtered as follows: maximum allele frequency in population databases of <0.0001 , predicted to cause protein-coding changes (simple substitutions, frameshifts, splicing alterations, or early termination), and present in genes associated with neurological phenotypes. We considered variants to be reasonable candidates if they met these criteria and if they fit the known mode of inheritance for these conditions (e.g.: recessive disease genes would require two heterozygous variants or a homozygous variant), and were not classified as “benign” or “likely benign” in ClinVar. Using these criteria, we again identified the above-mentioned homozygous variant in *MFN2* (c.1241A>T, p.(D414V)). Using these criteria, we did not identify any other monogenic disease candidates.

Characterization of patient fibroblasts

We obtained skin fibroblast cells from the patient in order to examine whether the D414V variant affects the various functions of *MFN2* and is thus likely to be pathogenic. We first confirmed the presence of *MFN2*-D414V variant in the patient derived fibroblasts by Sanger sequencing (Figure 1B). The aspartate residue at 414 position of *MFN2* is conserved throughout vertebrate orthologs, as well as in the human *MFN1* paralog (Figure 1C). This variant falls in the HR1 domain of the *MFN2* protein (Figure 1D). Notably, a previous bioinformatic study predicted that this amino acid change would be damaging.⁶⁴

Impact of the *MFN2*-D414V variant on mitochondrial dynamics

We compared mitochondrial morphology in patient and control fibroblasts in order to examine the well-defined role for *MFN2* in mediating mitochondrial fusion. While the mitochondria were long and often reticular in the control fibroblasts, those in the patient fibroblasts were noticeably shorter (Figure 2A). We quantified this observation by measuring the mitochondrial length and found shorter mitochondria in patient fibroblasts, indicative of more fragmented mitochondrial networks compared to those of control fibroblasts (Figure 2B). These results are consistent with a reduction in mitochondrial fusion.

Mitochondrial oxygen consumption rate in patient fibroblasts

Given the links between mitochondrial form and function, we checked whether the D414V *MFN2* variant might impact mitochondrial bioenergetics by measuring the oxygen consumption rate. Notably, both basal and maximal oxygen consumption rates were significantly lower in patient fibroblasts when compared to control (Figure 2C, D).

Mitochondrial nucleoids

In order to understand how the D414V variant might be impacting mitochondrial respiration, we employed several approaches to examine the mtDNA genome, which is essential for respiration, and which can be impacted by impairments to mitochondrial fusion.¹⁹ First, using confocal microscopy, we observed a significant decrease in the average number of nucleoids in patient fibroblasts compared to control (Figure 3A, B). Given previous findings that impaired mitochondrial fusion can lead to larger mitochondrial nucleoids, we also imaged and quantified the size of mtDNA nucleoids. Unexpectedly, we observed a reduction in the average size of nucleoids in patient fibroblasts when compared to those from control (Figure 3C). Consistent with the reduced number of nucleoids evident by confocal microscopy, quantitative PCR analysis also showed a slight reduction in mtDNA copy number in patient fibroblasts (Figure 3D). Meanwhile, D414V patient fibroblasts did not exhibit any evidence for mtDNA deletions (Figure 3E), as has been reported previously for some pathogenic *MFN2* variants.⁴⁸

Mitochondria-ER contacts (MERCs)

Next, we tested whether the D414V variant might impair *MFN2* protein function in mediating MERCs. To estimate the size and number of MERCs in patient and control fibroblasts, we employed a proximity ligation assay (PLA), which indicates when two target proteins (mitochondrial-TOMM20 and ER-calnexin) are within ~40 nm.^{59,65} We observed a significant reduction in both the number and size of MERCs in the patient cells (Figure 4A-D), consistent with the notion that the D414V variant might be causing reduced interactions between mitochondria and ER.

Lipid droplet regulation

Our observations of reduced MERCs, and the probable compromise in tethering function of *MFN2* in the patient fibroblasts, led us to hypothesize that lipid droplet metabolism could also be affected by the *MFN2*-D414V variant. To this end, lipid droplets were imaged by staining with a neutral lipid dye and imaged by confocal microscopy. Notably, the number of lipid droplets was reduced in the patient fibroblasts (Figure 5A, B and C). In addition, the total lipid content, as measured by the total intensity of the lipid dye, was also reduced (Figure 5D). Finally, we also noted an unusual perinuclear arrangement of lipid droplets in patient fibroblasts compared to control fibroblasts, in which lipid droplets were spread throughout the cell (Figure 5A, B). To quantify the differences in lipid droplet distribution, we measured the distance of individual lipid droplets from the center of the nucleus. We found that the average distances of lipid droplets

Figure 2. Mitochondrial dysfunction in *MFN2*-D414V patient-derived fibroblasts. (A) Representative confocal images of healthy control (left panel) and patient derived (right panel) fibroblasts labelled with TOMM20 immunostaining (purple) to visualize mitochondrial networks. Scale bar, 20 μm . (B) Quantification of average mitochondrial length in control and patient cells. The average mitochondrial lengths of 24 control and 27 patient fibroblasts. Error bars indicate mean $\pm$ SD. $p < 0.0001$, unpaired t-test (C) Oxygen consumption rate (OCR) traces in control and *MFN2*-D414V fibroblasts measured using the Seahorse XF24 extracellular flux analyzer. (D) Basal (upper) and maximum (lower) OCR in control and *Mfn2*-D414V fibroblasts calculated from C. Error bars indicate mean $\pm$ SD. $p < 0.0001$, unpaired t-test.

from the nucleus was significantly lower in the patient fibroblasts (Figure 5E, F). Altogether, these observations are consistent with the notion that the D414V variant in *MFN2* affects lipid droplets.

Discussion

We identified a novel homozygous *MFN2* variant, D414V, in a patient with ataxia, deafness and blindness. While hearing and vision loss have been observed in a few cases linked to *MFN2* variants,^{47,48} cerebellar ataxia has not. As pathogenic

Figure 3. Altered mtDNA in MFN2 D414V fibroblasts. (A) Representative confocal images of mitochondria and mitochondrial nucleoids labelled by immunostaining with anti-TOMM20 (purple) and anti-dsDNA (cyan) antibodies in control and *MFN2*-D414V fibroblasts. The area indicated by white box in the upper panel is shown in lower panel at higher magnification. Scale bar, 20 μm . (B) Quantification of the number of mtDNA nucleoids. Each data point indicates the total number of nucleoids in a control or *MFN2* D414V fibroblast. Error bars indicate mean $\pm$ SD. $p = 0.0012$, unpaired t-test. (C) Quantification of the average mtDNA nucleoid size in control (n, 26) or *MFN2*-D414V fibroblast cells (n, 40). Error bars indicate mean $\pm$ SD. $p < 0.0001$, unpaired t-test. (D) Relative mtDNA copy number analyzed using quantitative PCR. (E) Integrity of the mtDNA genome was assessed on 0.6% agarose gel following long-range PCR of DNA isolated from control or *MFN2*-D414V fibroblasts. No mtDNA deletions were detected.

variants in *MFN2* are typically associated with the peripheral neuropathy CMT2A, it was not clear whether the D414V variant is responsible for these patient phenotypes. Notably, though the patient did have a small-fibre sensory neuropathy, this was attributed to diabetes given the relatively late onset compared to the other phenotypes. A second question arising

Figure 4. Mitochondria-ER contacts (MERCs) are reduced in MFN2 D414V fibroblasts. (A and B) Representative images showing MERCs in the control (A) or MFN2 D414V (B) fibroblasts, visualized using a proximity-ligation assay (cyan). Mitochondrial networks and ER are visualized via immunostaining with TOMM20 (pink) and calnexin (purple), respectively. The area indicated by white box in the upper panel is shown in lower panel zoomed in. Scale bar, 20 μm . (C) Quantification of the number of MERCs. Each data point indicates the total number of MERCs from an individual control or MFN2-D414V fibroblast cell. Error bars indicate SD. $p = 0.0002$, unpaired t-test. (D) Quantification of the average size of MERC's in control or MFN2-D414V fibroblasts. Each data point indicates average size of MERCs from an individual cell. Error bars indicate SD. $p < 0.0001$, unpaired t-test.

Figure 5. The intracellular distribution and number of lipid droplets (LD) are reduced in MFN2 D414V fibroblasts. (A and B) Representative confocal images of control (A) and MFN2 D414V (B) fibroblasts, with lipid droplets stained with LipidTox Green (green) and mitochondria visualized following immunofluorescence with TOMM20 (purple). The area indicated by white box in the upper panel is shown in lower panel zoomed in. Scale bar, 20 μ m. (C) Quantification of the number of LDs. Each data point indicates the total number of LDs in a control or MFN2 D414V fibroblasts. Error bars indicate SD. $p = 0.0002$, unpaired t-test. (D) Quantification of total lipid content. Each data point indicates the total intensity given by the LipidTox Green dye from each of the control or MFN2 D414V fibroblasts. Error bar indicates SD. $p = 0.0073$, unpaired t-test. (E) Quantification of the intracellular distribution of LDs. Distance of each LD from centre of the nucleus of a respective cell was calculated for control (n, 8303) and MFN2-D414V (n, 5259). The median and inter-quartile ranges are indicated in the violin plot. $p < 0.0001$, unpaired t-test. (F) The average distance of LDs from nucleus. Each data point indicates the average distance of all the LDs from the center of respective cell's nucleus for control or MFN2 D414V. Error bar indicates SD. $p = 0.0006$, unpaired t-test.

from the D414V variant is whether different mechanisms underlying MFN2 dysfunction might explain the distinct patient phenotypes. Here, we discuss our characterization of the D414V variant, and the novel insight provided into the function of MFN2 and molecular mechanisms underlying pathology associated with dysfunction of this protein.

Despite the limitations of existing structural models, homology modelling of MFN2 with the bacterial homolog BDLP may still be informative.^{66,67} These reports suggest that the HR1 domain, which is predicted to form part of a larger helical bundle, comprises two alpha helical subdomains (~350-383, and ~390-419) that are separated by a flexible hinge domain. Notably, of the 167 reported MFN2 missense/nonsense variants in the Human Gene Mutation Database,⁶⁸ only a handful are found in three positions in the second helical domain of HR1 (aa390-419), with two of the affected residues at the extremities of this subdomain (i.e. aa390 and aa 418). These variants have been published in the context of classic CMT2A phenotypes, including: C390R,⁴⁷ C390F,⁸ R400X,⁶⁹ R400P,⁷⁰ R418Q⁷¹ and R418X.⁷² However, none of these

variants have been characterized functionally in human cells. Moreover, in contrast to the homozygous D414V reported here, the other published variants in the second helical domain of HR1 are all heterozygous. Notably, two variants were compound heterozygous, with the R400X variant found in combination with another pathogenic variant (R250W, also linked to CMT2A), and the C390R variant in combination with N214D. As such, some functional rescue by the other allele is possible for these other variants. These observations suggest that the region comprising the second helical domain within HR1 (aa390-419), where the D414V variant is present, is of critical importance for proper MFN2 function.

It is also worth briefly discussing a study in which two rare human *MFN2* variants were modelled in *Drosophila*.⁶⁴ Tissue-specific overexpression of these variants (M393I and R400Q) led to cardiac and eye phenotypes. Notably, these variants are also present in the second helical region of the HR1 domain, suggesting a distinct role for this region of the HR1 domain in mediating MFN2 function, and hint at additional phenotypes that could be associated with human disease due to pathogenic variants in *MFN2*.

To investigate whether the D414V variant is indeed pathogenic, we performed a comprehensive analysis of mitochondrial functions linked to MFN2, beginning with the well-recognized role of MFN2 in mediating mitochondrial fusion. Patient fibroblasts harboring the D414V variant displayed fragmented mitochondrial networks compared to control, consistent with impaired fusion in these cells. This finding is notable in comparison to the few pathogenic MFN2 variants causing CMT2A that have been investigated for their role in fusion. While a few of the CMT2A *MFN2* variants studied appear to be fusion incompetent,^{5,64,73} there are other CMT2A *MFN2* variants that do not seem to affect mitochondrial morphology.^{40,74} Conversely, some CMT2A *MFN2* variants actually enhance fusion.^{38,39} Thus, impaired mitochondrial fusion does not appear to be necessary to cause CMT2A.

As mitochondrial fusion is important for maintenance of the mitochondrial genome, we also investigated mtDNA in patient fibroblasts. The reduced mtDNA copy number and nucleoid number observed in patient fibroblasts is consistent with impaired mitochondrial fusion in these cells, as reduced copy number is proposed to be due to inefficient fusion-dependent distribution of the mtDNA replication machinery.¹⁹ Surprisingly, we also observed slightly smaller nucleoids in patient cells, an unexpected finding given that impaired mitochondrial fusion has previously been linked to enlarged mitochondrial nucleoids.^{19,21,75} However, it should be noted that it is not clear why nucleoid size increases in response to impaired fusion. One possible explanation for this discrepancy in nucleoid size could be that size does not correlate directly with the degree of fusion impairment. Perhaps, slight impairments to mitochondrial fusion lead to smaller nucleoids, while greater impairments lead to larger nucleoids. The fact that each nucleoid is estimated to contain ~1.4 copies of the mtDNA genome,⁷⁶ is consistent with observations that a significant subset of nucleoids is actively undergoing replication.³⁰ If one logically assumes that replicating nucleoids are larger than non-replicating nucleoids, then the smaller nucleoids we observe could be due to a reduced number of nucleoids being actively replicated.

Meanwhile, the larger nucleoids described in cells completely lacking fusion are due to clustering of multiple individual nucleoids that cannot be resolved by traditional confocal microscopy.¹⁹ Notably, cells where mitochondrial fission is inhibited also exhibit large clustered nucleoids, demonstrating that mitochondrial dynamics are important for the distribution of nucleoids.^{77,78} Perhaps in cells with more severe fusion impairment, individual mtDNA nucleoids also cluster, leading to apparently larger nucleoids. It is also notable that while nucleoid size has not previously been quantified in cells with different pathogenic MFN2 variants, a subset of pathogenic MFN2 variants are linked to mtDNA depletion and deletions.^{46,79}

Given the links between mitochondrial structure and function, as well as the critical role for mtDNA encoded proteins in oxidative phosphorylation, we also examined mitochondrial function in patient fibroblasts. The reduced OCR we observed in *MFN2*-D414V patient fibroblast demonstrated clear mitochondrial dysfunction in these cells, possibly as a result of reduced mitochondrial fusion leading to reduced levels of mtDNA, or impaired MERCs affecting Ca^{++} transfer to mitochondria. While MFN2 is important for maintaining mitochondrial bioenergetics,⁸⁰ the consequences of pathogenic CMT2A *MFN2* variants on mitochondrial function are conflicting, raising questions about the contribution of impaired mitochondrial bioenergetics to CMT2A. For example, some pathogenic CMT2A *MFN2* variants lead to a decrease in the mitochondrial bioenergetic function,^{40,80,81} others cause an increase,^{82,83} while still more variants do not cause any change at all.^{25,37,41}

MFN2 also plays a key role in mediating contact sites between mitochondria and ER, though conflicting reports debate the exact role of MFN2 in maintaining MERCs. Thus, we also investigated MERCs in D414V patient fibroblasts, where we observed a decrease in number as well as size. These results suggest that the D414V variant reduces MERCs. While only a limited number of CMT2A *MFN2* variants have been investigated for their role in MERCs,^{26,37} disruption of contacts between mitochondria and ER seems to be a common theme underlying neuropathies.^{73,84,85} Further, a study in

Drosophila indicated that MFN2 could regulate ER stress,⁸⁶ and peripheral neuropathy in diabetic patients is also thought to be partly due to ER stress.^{87,88} These observations suggest that deregulation of MERCs and their many functions could be a significant contributor to the peripheral neuropathy phenotype associated with CMT2A *MFN2* variants.^{37,73}

Although a peripheral neuropathy noted in the D414V patient was initially attributed to a diabetic neuropathy, it is worth considering the contribution of MFN2 dysfunction to this phenotype. Notably, peripheral neuropathy can appear at later stages in life and with varying severity.^{35,89} Phenotypic variation may occur between individuals with the same mutation suggesting a potential role for other genetic or environmental factors. In this regard, the alteration to MERCs are consistent with the fact that impaired MERCs are a feature of MFN2 CMT2A variants.^{37,90} As such, it may simply be a coincidence that the peripheral neuropathy appeared around the same time as the patient's diabetes, and that MFN2 dysfunction was responsible. Alternatively, it is possible that the development of diabetes exacerbated an already unstable situation due to the *MFN2* variant.

Next, we examined lipid droplets, another organelle whose interactions with mitochondria are mediated by MFN2.³⁴ We found that the abundance of neutral lipid signal and the number of lipid droplets are reduced in patient fibroblasts. In contrast to our finding, a previous report has shown that CMT2A *MFN2* variants increase the lipid droplet signal and apparent abundance.³⁷ Interestingly, we also discovered an unexpected perinuclear accumulation of lipid droplets in fibroblasts with the D414V variant when compared to healthy control. This phenotype has not been described previously in the context of MFN2 dysfunction.

It is also worth discussing the role of MFN2 in cellular lipid homeostasis, though there is clearly more to learn, especially in the context of disease. In addition to regulating lipid droplets and MERCs, MFN2 was recently shown to have a direct role in transferring phosphatidylserine from the ER to mitochondria, a function implicated in non-alcoholic steatohepatitis.³¹ Moreover, in adipose tissue the association between mitochondria and lipid droplets is important for both lipid storage and consumption,^{91,92} while adipocyte specific knockout of *MFN2* leads to obesity in mice.^{34,93,94} Finally, the R707W *MFN2* variant, which causes CMT2A when present heterozygously, also causes lipomatosis when present homozygously.^{50,51} Thus, it is clear that the roles of MFN2 in maintaining lipid homeostasis are important, though the exact molecular mechanisms remain undefined.

Our results indicate that the D414V *MFN2* variant behaves differently than the few other CMT2A variants that have been investigated in the context of lipid droplets. Furthermore, in contrast to complete loss of MFN2 function, which seems to increase lipid accumulation, the reduced lipid droplet abundance we found in D414V fibroblasts could be due to reduced lipid storage or increased lipid consumption. We suggest that this reduced lipid storage is most likely due to impaired lipid droplet tethering, given the reduced oxidative phosphorylation we observed is inconsistent with an increase in lipid consumption. Furthermore, the distinct distribution pattern of lipid droplets in D414V fibroblasts is consistent with a role of MFN2 regulating interactions between mitochondria and lipid droplets via perilipin,³⁴ an important mediator of lipid droplet distribution.⁹⁵ In contrast, the other CMT2A *MFN2* variants investigated previously are proposed to increase lipid droplet signal via alterations to MERCs.³⁷ Though we also see evidence of MERC disruption in D414V cells, the differences in lipid droplet signal between the D414V and other CMT2A variants could also be due to the severity of MERC disruption, which cannot be compared across studies, as different methods of analysis were used.

In comparison to the characteristics of previous published CMT2A variants, our findings describing the cellular characteristics of the D414V variant, begin to provide insight that may explain the different patient phenotypes and the underlying mechanisms of disease. Though mounting evidence clearly shows that dysregulation of MFN2 causes CMT2A, the exact molecular mechanism underlying this pathology is complicated by the fact that MFN2 is a multi-functional protein. As such, impairment of any or all functions performed by MFN2 could be pathogenic. Here, our description of the D414V variant suggests that impairment of different MFN2 functions may be associated with different pathological phenotypes. Notably, the patient phenotypes described here with the *MFN2* D414V variant are reminiscent of variants in *OPA1*, where patients also have optic atrophy, hearing loss and ataxia. This similarity suggests that impaired mitochondrial fusion may be the underlying mechanism driving these specific phenotypes.

To date, only a few CMT2A *MFN2* variants have been investigated for their effects on the various functions of MFN2, making it difficult to generalize which MFN2 function(s) may be causative for which phenotype. Nonetheless, the conflicting findings of pathogenic CMT2A *MFN2* variants that have been studied with respect to their consequences on mitochondrial fusion, mtDNA depletion, and bioenergetics suggest that these functions may not be the primary mechanisms underlying the peripheral neuropathy phenotype. However, impairment of these other functions could certainly contribute to disease and may explain some of the additional features sometimes associated with CMT2A

(e.g. hearing loss, optic atrophy or lipomatosis). It is also important to note that many of these conflicting reports are from distinct studies by different groups that do not always use the same methods, making direct comparisons difficult.

In summary, our study establishes that *MFN2*-D414V variant is incompetent in carrying multiple *MFN2* functions, arguing for the importance of the HR1 domain. One of the key cellular differences between D414V and CMT2A variants pertains to lipid droplets abundance, as well as a previously unreported perinuclear lipid droplet distribution. Furthermore, the fact that nearby variants also cause distinct phenotypes compared to ‘classic’ CMT2A variants when modelled in flies supports the notion that alterations to the HR1 domain could have different patient phenotypes in humans. However, as only a single D414V patient has been described to date, we cannot infer definitively on genotype/phenotype correlation. At the same time, the cellular phenotypes we describe are all consistent with impaired function of *MFN2*. Combined with bioinformatic predictions that this variant is likely to be deleterious, we believe the homozygous D414V variant to be the cause of the patient phenotypes, thus expanding the clinical spectrum *MFN2*-associated mitochondrial diseases.

Data availability statement

The underlying data, including DNA sequencing chromatograms, confocal images, uncropped agarose gel image, as well as raw data for quantification of oxygen consumption, quantitative PCR, and image analysis, are available from Harvard Dataverse as follows:

Harvard Dataverse: “Replication Data for: Characterization of a novel variant in the HR1 domain of *MFN2* in a patient with ataxia, optic atrophy and sensorineural hearing loss”, <https://doi.org/10.7910/DVN/0R3C7S>. CC0 license.

This project contains the following extended data:

Fig 2/A/Control Fibroblast

Confocal images of Control fibroblasts, immunostained with anti-TOMM20 antibody

Fig 2/A/*MFN2* D414V Fibroblasts

Confocal images of *MFN2* D414V Fibroblasts, immunostained with anti-TOMM20 antibody

Fig 2/B

Raw data for mitochondrial morphology quantification

Fig 2/CD

Raw data for mitochondrial oxygen consumption

Fig 3A/Control Fibroblasts

Confocal images of Control fibroblasts, immunostained with anti-dsDNA antibody

Fig 3A/*MFN2* D414V Fibroblasts

Confocal images of *MFN2* D414V fibroblasts, immunostained with anti-dsDNA antibody

Fig 3B/Nucleoid Number

Raw data for mitochondrial nucleoid number quantification

Fig 3C/Nucleoid Size

Raw data for mitochondrial nucleoid size quantification

Fig 3D/mtDNA copy number

Raw data for quantitative PCR quantification of mtDNA copy number

Fig 3E/mtDNA Deletions

Images of agarose gel for mtDNA deletions

Fig 4/A/Control Fibroblasts PLA

Confocal images of Control fibroblasts, Proximity Ligation Assay to visualize mito-ER contact sites

Fig 4/B/MFN2 D414V Fibroblasts PLA

Confocal images of MFN2 D414V fibroblasts, Proximity Ligation Assay to visualize mito-ER contact sites

Fig 4/C/MERC numbers

Raw data for quantification of mito-ER contact site numbers

Fig 4/D/MERC size

Raw data for quantification of mito-ER contact site size

Fig 5/A/Control Fibroblasts LD

Confocal images of Control fibroblasts, stained with LipidTox Green to visualize lipid droplets

Fig 5/B/MFN2 D414V Fibroblasts LD

Confocal images of MFN2 D414V fibroblasts, stained with LipidTox Green to visualize lipid droplets

Fig 5/C/LD number

Raw data for quantification of lipid droplet numbers

Fig 5/D/LD content

Raw data for quantification of lipid droplet content

Fig 5/E.F/LD distance from nucleus

Raw data for quantification of distribution of lipid droplets

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

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Author contributions

Conceptualization: G.S., R.S., G.P., T.S.; Methodology: G.S., R.S., K.M.; Formal analysis: K.M. M.J., A. dK., G.P.; Investigation: G.S., R.S. M.J.; Resources: D.M., G.P.; Data Curation: M.J.; Writing – original draft: G.S., G.P., T.S.; Writing – review & editing: G.S., R.S., D.M., G.P., T.S.; Visualization: G.S.; Supervision: A. dK., G.P., T.S.; Project Administration: G.P., T.S.; Funding acquisition: T.S.

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The manuscript by Sharma et al. describes the studies on a novel *MFN2* variant (p. D414V), identified in a patient with ataxia, optic atrophy and deafness.

Our first objection concerns the phenotypic expansion to include cerebellar ataxia. It has been already reported in subjects with *MFN2* variants (Rouzier *et al.* 2012¹), and, in fact, this gene was present in the panel for ataxic syndromes used by the authors.

As a general thought, in a single patient harboring a putative - causative homozygous variant (hence with high probability of consanguinity for the parents) and presenting with atypical features, it is important to consider the possibility of dual molecular diagnosis to explain the unexpected clinical signs. Even deep functional studies cannot demonstrate that the whole set of clinical presentations are due to a single mutation. Only additional cases with the same phenotype can confirm the genotype-phenotype link.

Indeed, even with WES, some mutations (e.g. intronic ones) can be missed. Moreover, it is not clear if the sequencing of the whole mtDNA has been performed.

Regarding the statement that "These findings raise the possibility that impaired fusion does not lead to peripheral neuropathy per se", it is worthy to mention that mutations in genes involved in mitochondrial fission, i.e. the opposite process to fusion, may be associated with peripheral neuropathy (e.g. *MFF*) or optic atrophy (e.g. *DNM1L*). It is possible to speculate that impairment of the fine regulation of mitodynamics, in either direction, contributes to the pathological phenotype.

The characterization in patient's fibroblasts is very detailed and with interesting findings, but also with some flaws.

1. It would have been extremely useful to perform the same experimental set in fibroblasts carrying other *MFN2* mutations, in order to define what defects are specific for each mutation.
2. The strong reduction in oxygen consumption is unexpected since most of the cases with mutations in mitodynamics genes have poor or no biochemical defect. The very limited decrease in mtDNA amount cannot explain this result. Were the control cell lines aged-matched? and at the same sub-cultural passage?
3. For mtDNA copy number evaluation, authors use 18S as nuclear-encoded housekeeping gene. The small subunit 18S rRNA gene is one of the most frequently used genes in phylogenetic studies. In general, rRNA gene are organized in cluster, and each cluster consists of multiple rDNA repeat units that vary in number among individuals and chromosomes, so their repetitive arrangement within the genome provides excessive amounts of template DNA for PCR with total diploid copy number estimates ranging from 60 to >800 repeat units in a human genome. Instead, single copy genes (e.g. RNASEH, β 2M) are widely used control for qRT-PCR analyses because of its invariant expression across tissues, cells, and subjects.
4. Analysis on the protein is missing. There are numerous well-functioning antibodies against MFN2 and given the availability of the patient's fibroblasts it would be useful to check the amount of the protein.
5. Experiments for mito-network evaluation were conducted in fixed cells. The fixing process could alter the result; it would be recommended to perform these experiments using live imaging. Furthermore, since fragmented mitochondrial networks is not evident (by looking at the images), we suggest to try to stress mitochondrial dynamics in fibroblasts (e.g. by culturing them in galactose) to see if there is any clear alteration in morphology.

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neurogenetics, mitochondrial diseases

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 30 Jul 2022

Timothy Shutt

>We would like to thank the reviewers for their interest in the manuscript and their suggestions, which we have taken to heart and have addressed to the best of our ability. We would also like to apologize for the extended period of time for the revisions. The first author left the lab shortly after the paper was initially submitted, and it took some time to get a new person up and running on the project after the reviews were all received. In addition to several clarifications in the text, the revised version of the manuscript contains new data showing alterations to the expression of mitochondrial fusion proteins, as well as new mtDNA copy number data normalized to B-tubulin. We hope that the revisions answer the questions and issues raised by the reviewers. Below are the specific point by point response to the reviews.

Reviewer Expertise

I have worked on mitochondrial genetics for 3 decades, and now on mitochondrial dynamics and mitophagy.

The manuscript by Sharma et al. describes the studies on a novel *MFN2* variant (p. D414V), identified in a patient with ataxia, optic atrophy and deafness.

Our first objection concerns the phenotypic expansion to include cerebellar ataxia. It has been already reported in subjects with *MFN2* variants (Rouzier et al. 2012¹), and, in fact, this gene was present in the panel for ataxic syndromes used by the authors.

*>Thank you for bringing this important article to our attention. This work characterises in detail a family with a heterozygous variant in *MFN2*, c.629A>T (p.D210V). The family is described to have variable clinical severity, penetrance, and optic atrophy. The index case is reported as having "sensory and cerebellar ataxia" without providing details about the clinical examination. Neuroimaging showed cerebral atrophy but there was no mention of cerebellar structural change and representative images did not include the cerebellum. Eleven other family members were described as having sensory ataxic neuropathy, but not cerebellar ataxia. This being the case, it seems the phenotype being described with the D210V substitution is sensory ataxia, which implies*

a different anatomic localisation than cerebellar ataxia. It does remain possible cerebellar ataxia was present in the index case, but the examination findings were not described, MRI findings did not show cerebellar abnormalities, and none of the 11 other affected members in that pedigree had cerebellar findings. In contrast, our case is the first to demonstrate clear-cut cerebellar ataxia, with cerebellar examination findings, absence of major sensory findings, and presence of cerebellar atrophy on neuroimaging. To provide this important additional information we bring attention to this article in the introduction. We have also included additional text at the beginning of the discussion:

"There is one described report of an MFN2 patient having neuropathy with sensory ataxia and optic atrophy. The index case was actually described as having "sensory and cerebellar ataxia" however the presence of cerebellar dysfunction in this case is uncertain because physical findings were not described, the MRI did not have cerebellar abnormalities, and none of the other 11 cases in the pedigree were considered to have cerebellar findings. In contrast, our case is the first to demonstrate clear-cut cerebellar ataxia, with cerebellar examination findings, absence of major sensory findings, and presence of cerebellar atrophy on neuroimaging."

With regard to MFN2 being present on the ataxia panel, genetic testing companies will frequently include a broad list of genes associated with the relevant phenotypes or potential diagnostic mimickers. The genetic testing company does not publish justifications for the content of their panels, but we think it is likely MFN2 was included because of the report by Rouzier and colleagues showing sensory ataxia in several patients, and sensory ataxia can be difficult to distinguish clinically from cerebellar ataxia in some cases.

As a general thought, in a single patient harboring a putative - causative homozygous variant (hence with high probability of consanguinity for the parents) and presenting with atypical features, it is important to consider the possibility of dual molecular diagnosis to explain the unexpected clinical signs. Even deep functional studies cannot demonstrate that the whole set of clinical presentations are due to a single mutation. Only additional cases with the same phenotype can confirm the genotype-phenotype link.

>Thank you for raising this important and very thoughtful point. It is agreed that consanguinity is likely for the parents, although not proven. The possibility of a dual molecular diagnosis was considered as a possibility early on, and this was the main reason that we proceeded with exome sequencing after identifying the MFN2 variant with panel testing. Findings from exome sequencing did not identify any additional diagnosis. We agree that definitively associating this complex phenotype to this homozygous variant will only be possible by identifying additional cases. In the meantime, we believe that the extensive functional characterisation provides strong evidence connecting this variant to the clinical presentation. To address this comment the discussion includes the following comment:

"However, as only a single D414V patient has been described to date, we cannot infer definitively on genotype/phenotype correlation."

Indeed, even with WES, some mutations (e.g. intronic ones) can be missed. Moreover, it is not clear if the sequencing of the whole mtDNA has been performed.

>Exome sequencing has coverage for intron-exon boundaries but it is agreed that deeper intronic variants would not be detectable. The patient's muscle biopsy was done when he lived in a different country, so we did not have access to muscle tissues for mtDNA sequencing. Nonetheless, we did attempt to identify mtDNA variants from the off-target reads of our exome sequencing. In general, this approach is only considered accurate if read depth is 20X or greater (PMID: 24901348), which was the case for only 6% of the mitochondrial genome. Within the limitations of this approach, we did not identify pathogenic variants. We have updated the manuscript with this information and the caveat in limitations that a mtDNA variant remains possible. Added to discussion:

"Other limitations of this study include the possibility that another clinically relevant intronic variant was missed due to the use of exome sequencing. We did not perform mitochondrial genome sequencing (and coverage of mtDNA from off-target reads was very low in our exome analysis), so it is also possible a relevant mtDNA variant was missed."

Regarding the statement that "These findings raise the possibility that impaired fusion does not lead to peripheral neuropathy per se", it is worthy to mention that mutations in genes involved in mitochondrial fission, i.e. the opposite process to fusion, may be associated with peripheral neuropathy (e.g. *MFF*) or optic atrophy (e.g. *DNM1L*). It is possible to speculate that impairment of the fine regulation of mitodynamics, in either direction, contributes to the pathological phenotype.

>As suggested, we have added this notion to the revised introduction.

The characterization in patient's fibroblasts is very detailed and with interesting findings, but also with some flaws.

1. It would have been extremely useful to perform the same experimental set in fibroblasts carrying other *MFN2* mutations, in order to define what defects are specific for each mutation.

>We agree with the reviewer that looking at other MFN2 variants would be of value. Unfortunately, we don't have access to other patient cell lines carrying 'classic' MFN2 variants to make such comparisons. Notably, this is an issue that is also present in many other reports of MFN2 variants, which often only look at one or two molecular functions (if that) for a single MFN2 variant. Notably, since the initial submission of this manuscript, we have published a review paper that tries to summarize what is known about many different MFN2 variants with respect to the various MFN2 functions (PMID: 35399520).

1. The strong reduction in oxygen consumption is unexpected since most of the cases with mutations in mitodynamics genes have poor or no biochemical defect. The very limited decrease in mtDNA amount cannot explain this result. Were the control cell lines aged-matched? and at the same sub-cultural passage?

>Yes, the control cells were age-matched and analyzed at the same passage. We note that while reduced oxygen consumption is not always observed with MFN2 variants, there are several examples (also linked to mtDNA depletion) where this is the case (reviewed in PMID: 35399520). We also note that our new data suggest a significant decrease in mitochondrial abundance, which likely contributes to the reduced oxygen consumption.

1. For mtDNA copy number evaluation, authors use 18S as nuclear-encoded

housekeeping gene. The small subunit 18S rRNA gene is one of the most frequently used genes in phylogenetic studies. In general, rRNA gene are organized in cluster, and each cluster consists of multiple rDNA repeat units that vary in number among individuals and chromosomes, so their repetitive arrangement within the genome provides excessive amounts of template DNA for PCR with total diploid copy number estimates ranging from 60 to >800 repeat units in a human genome. Instead, single copy genes (e.g. RNASEH, β 2M) are widely used control for qRT-PCR analyses because of its invariant expression across tissues, cells, and subjects.

>We have reanalyzed the mtDNA copy number compared to both the multicopy 18S and the single copy B-tubulin (new fig 3D). Notably, copy number is decreased significant with respect to for both controls.

1. Analysis on the protein is missing. There are numerous well-functioning antibodies against MFN2 and given the availability of the patient's fibroblasts it would be useful to check the amount of the protein.

>We investigated the levels of MFN2 in our patient fibroblasts, and discovered some interesting differences. Notably, we see decreased protein levels for MFN2, as well as several other mitochondrial proteins, suggesting that overall mitochondrial abundance is reduced in the patient cells. These new data and a discussion of these novel results has been added to the revised manuscript.

1. Experiments for mito-network evaluation were conducted in fixed cells. The fixing process could alter the result; it would be recommended to perform these experiments using live imaging. Furthermore, since fragmented mitochondrial networks is not evident (by looking at the images), we suggest to try to stress mitochondrial dynamics in fibroblasts (e.g. by culturing them in galactose) to see if there is any clear alteration in morphology.

>We have extensive experience in imaging mitochondria, and are confident that our fixation methods, which are widely used in the field for this purpose, do not introduce artefacts. There are also potential artefactual issues with live-cell imaging, as lasers can induce mitochondrial stress, so there is no perfect way to image mitochondrial networks. Our quantitative analysis of the mitochondrial size does in fact show significant differences between control on patient mitochondrial networks when many cells are examined. Though they may not seem fragmented to the reviewer, these differences are evident to the practiced eye in the representative images. As we already see significant differences in the cells when grown in normal glucose containing media, we did not culture our cells in galactose.

Competing Interests: No competing interests were disclosed.

Reviewer Report 12 August 2021

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Thank you for asking me to review this paper characterizing a mutation in MFN2 that results in an unusual phenotype. I think that this paper is ready for indexing provided they can confirm some additional details including the number of controls studied.

Main points:

1. Neither the methods nor the results clearly spell out the N for each experiment. Figure 2B suggests that data was generated from 24 control and 27 patient fibroblasts. How many controls were used? The study would not be ready for publication if they only used one control. They should state how many repetitions of each of the experiments there were (their single statement, "...three independent biological replicates" on page 6 is not sufficiently detailed). These additional data would provide important support for the findings. Even though the findings are relevant and interesting, that only one patient has been used should be stated more clearly along with the need to study more patients/relative with the novel D414V MFN2 variant.
2. The loss of mitochondria ER contacts (MERCs) is very beautiful and interesting. This may well be linked to the slight reduction in mitochondrial DNA copy number and in respiratory chain function. The authors discuss the hypothesis that mtDNA replication is impaired in MFN2 because of the effect of excessive mitochondrial fragmentation on the distribution of the replication machinery (ref 19). However, they barely discuss two other factors that are relevant to mtDNA replication: mitochondrial dynamics affect both nucleoid division and mitophagy (and hence mtDNA turnover). Interactions with the ER are thought to license mtDNA replication as well as initiating nucleoid division (ref 30) so having a reduced MERC area, and potentially having a smaller midzone (Kleele *et al.* (2021¹)) could have an important effect. Impaired mitochondrial fusion may increase (Liao *et al.* (2017²)) or decrease (Sebastián *et al.* (2016³)) mitophagy in impaired MFN2 expression. Increased mitophagy could decrease mtDNA copy number and slow recovery from mtDNA depletion (ref 19).
3. The decrease in lipid droplets is interesting and I think novel in MFN2 mutants. Examining the distribution of lipid droplets in the form of distance to the nucleus is also a nice addition to the data. They should mention that (i) the exact molecular mechanisms how MFN2 maintains lipid homeostasis is unknown (ii) perilipin 1 much more abundant in adipose tissue (ref 34) than fibroblasts. From the discussion it is not clear how perilipins can explain the altered distribution found in the fibroblasts in the present paper. The lipid droplets in figure 5 could follow either (i) the mitochondrial network which seems also more perinuclear in the representative figure or (ii) the microtubular network which could be clarified by drug treatments such as nocodazole.
4. Was the number of mitochondrial nucleoids or lipid droplets corrected for the cell size? Did the authors also measure the size of the lipid droplets in these cells?
5. The patient's presentation is interesting: impaired vision, poor motor function and deafness. Of these, unusual features included early loss of vision, cerebellar ataxia, and brain MRI

abnormalities. However, some basic clinical details are missing.

- a) No segregation analysis is presented, presumably his parents may be no longer available. It would be useful to support recessive inheritance by identifying an asymptomatic individual in the pedigree who is heterozygous for the same mutation.
- b) Analysis of skeletal muscle for mitochondrial DNA copy number and variable deletions. Muscle is more relevant than these data that they present in fibroblasts, and should be available because the patient had a muscle biopsy.

6. The authors present a nice visualization of mitochondrial fusion. As MFN1 is also important in mitochondrial fusion and MFN2 might interact with OPA1, do the authors have any data in changes of MFN1 and OPA1 levels in this patient?

Minor points:

1. A cartoon to illustrate the main findings would be welcome.
2. Sentence on page 4, paragraph 4, line 3, please rephrase ("Central nervous ... of cases")
3. Please state the glucose content of the medium during cell maintenance.
4. Page 5, third paragraph, line 9, goat anti-rabbit, should be goat anti-mouse.
5. Please state the company of the DAPI labelling.
6. Please state which program is used to do the statistical testing and if a p-value of <0.05 was considered significant.

References

1. Kleele T, Rey T, Winter J, Zaganelli S, et al.: Distinct fission signatures predict mitochondrial degradation or biogenesis. *Nature*. 2021; **593** (7859): 435-439 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Liao C, Ashley N, Diot A, Morten K, et al.: Dysregulated mitophagy and mitochondrial organization in optic atrophy due to OPA1 mutations. *Neurology*. 2017; **88** (2): 131-142 [PubMed Abstract](#) | [Publisher Full Text](#)
3. Sebastián D, Zorzano A: When MFN2 (mitofusin 2) met autophagy: A new age for old muscles. *Autophagy*. **12** (11): 2250-2251 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I have worked on mitochondrial genetics for 3 decades, and now on mitochondrial dynamics and mitophagy.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 30 Jul 2022

Timothy Shutt

>We would like to thank the reviewers for their interest in the manuscript and their suggestions, which we have taken to heart and have addressed to the best of our ability. We would also like to apologize for the extended period of time for the revisions. The first author left the lab shortly after the paper was initially submitted, and it took some time to get a new person up and running on the project after the reviews were all received. In addition to several clarifications in the text, the revised version of the manuscript contains new data showing alterations to the expression of mitochondrial fusion proteins, as well as new mtDNA copy number data normalized to B-tubulin. We hope that the revisions answer the questions and issues raised by the reviewers. Below are the specific point by point response to the reviews.

Reviewer 1

Thank you for asking me to review this paper characterizing a mutation in MFN2 that results in an unusual phenotype. I think that this paper is ready for indexing provided they can confirm some additional details including the number of controls studied.

Main points:

1. Neither the methods nor the results clearly spell out the N for each experiment. Figure 2B suggests that data was generated from 24 control and 27 patient fibroblasts. How many controls were used?

>We have updated the figure legends to indicate the number of cells used for analysis in each experiment. Furthermore, all of the images analyzed are included in the extended data, should anyone wish to reanalyze the data.

The study would not be ready for publication if they only used one control.

>While we only used a single control line for these experiments, our previously published work has shown that this control line behaves similar to other control lines (PMID: 31231018). We have added the following text to make this clear to the reader:

“As a control fibroblast line, we used an age-matched cell line that we have shown previously to behave similar to other controls fibroblast lines with respect to mitochondrial function (PMID: 31231018).”

They should state how many repetitions of each of the experiments there were (their single statement, “...three independent biological replicates” on page 6 is not sufficiently detailed). These additional data would provide important support for the findings.

>We have clarified the text in the methods as follows:

“For all of the image analysis in this manuscript, the data shown in the figures are from a single biological replicate with three technical replicates (e.g. images used for analysis were gathered from three coverslips processed in parallel). Experiments were repeated independently at least twice, for a total of three biological replicates, and all reported trends were consistent with the representative data reported in the figures.”

Even though the findings are relevant and interesting, that only one patient has been used should be stated more clearly along with the need to study more patients/relative with the novel D414V MFN2 variant.

>We agree that more patients with the D414V variant would strengthen the study, unfortunately we are only aware of this single case with this clinical presentation. Other family members were not available for investigation and there was no family history of similar disease. We have clarified the text to highlight the fact that we have only one patient with the following addition to the discussion:

“Unfortunately, the D414V variant has not been described to date in any other patients. As such, we only have a single patient cell line in which to study this variant.”

2. The loss of mitochondria ER contacts (MERCs) is very beautiful and interesting. This may well be linked to the slight reduction in mitochondrial DNA copy number and in respiratory chain function. The authors discuss the hypothesis that mtDNA replication is impaired in MFN2 because of the effect of excessive mitochondrial fragmentation on the distribution of the replication machinery (ref 19). However, they barely discuss two other factors that are relevant to mtDNA replication: mitochondrial dynamics affect both nucleoid division and mitophagy (and hence mtDNA turnover). Interactions with the ER are thought to license mtDNA replication as well as initiating nucleoid division (ref 30) so having a reduced MERC area, and potentially having a smaller midzone (Kleele *et al.* (2021¹)) could have an important effect. Impaired mitochondrial fusion may increase (Liao *et al.* (2017²)) or decrease (Sebastián *et al.* (2016³)) mitophagy in impaired MFN2 expression. Increased mitophagy could decrease mtDNA copy number and slow recovery from mtDNA depletion (ref 19).

>As the initial discussion was already somewhat lengthy, we had not gone into details on these other possibilities. However, in light of the new data in the revised manuscript, which suggest that mtDNA copy number may be reduced in part by reduced levels of mitochondria, we have amended the discussion to further discuss other factors linking fusion to altered mtDNA.

3. The decrease in lipid droplets is interesting and I think novel in MFN2 mutants. Examining the distribution of lipid droplets in the form of distance to the nucleus is also a nice addition to the data. They should mention that (i) the exact molecular mechanisms how MFN2 maintains lipid homeostasis is unknown (ii) perilipin 1 much more abundant in adipose tissue (ref 34) than fibroblasts. From the discussion it is not clear how perilipins can explain the altered distribution found in the fibroblasts in the present paper. The lipid droplets in figure 5 could follow either (i) the mitochondrial network which seems also more perinuclear in the representative figure or (ii) the microtubular network which could be clarified by drug treatments such as nocodazole.

>We appreciate the comments from the reviewer regarding the interesting aspects of lipid droplet alterations. We agree that there is certainly much more to learn in this regard, however, we believe that it is beyond the scope of the current investigation. As suggested, we included the following statements in our discussion:

"Though it should be noted that perilipin 1 is more abundant in adipose tissue than in fibroblasts (34)."

"... it is clear that the roles of MFN2 in maintaining lipid homeostasis are important, though the exact molecular mechanisms remain undefined"

4. Was the number of mitochondrial nucleoids or lipid droplets corrected for the cell size?

>No, we did not correct for cell size, as we did not have a marker for the plasma membrane. Furthermore, the surface area covered by the cell does not necessarily correlate to the cell size if the cells do not attach and spread out to the same extent.

Did the authors also measure the size of the lipid droplets in these cells?

>We did not measure the size of the lipid droplets, as the image processing required to visualize individual lipid droplets did not preserve the size information, though it is still reliable to quantify the number and distribution of the lipid droplets. At least by eye, the lipid droplet size does not appear to change between the cell lines.

5. The patient's presentation is interesting: impaired vision, poor motor function and deafness. Of these, unusual features included early loss of vision, cerebellar ataxia, and brain MRI abnormalities. However, some basic clinical details are missing.

- a) No segregation analysis is presented, presumably his parents may be no longer available. It would be useful to support recessive inheritance by identifying an asymptomatic individual in the pedigree who is heterozygous for the same mutation.

>Indeed, the patient's parents are not available for research, which is unfortunately common when investigating patients in adulthood. The suggestion to study heterozygous family members

is of interest and we would have pursued this if other family members were available for research. Participation in research was offered to his sister who lives in the same city (who would have had a 50% chance of being a heterozygous carrier of this variant) but she declined participation.

- b) Analysis of skeletal muscle for mitochondrial DNA copy number and variable deletions. Muscle is more relevant than these data that they present in fibroblasts, and should be available because the patient had a muscle biopsy.

>Unfortunately, the muscle tissue was not available for mtDNA copy number analysis, as the patient's muscle biopsy was done when he lived in a different country.

6. The authors present a nice visualization of mitochondrial fusion. As MFN1 is also important in mitochondrial fusion and MFN2 might interact with OPA1, do the authors have any data in changes of MFN1 and OPA1 levels in this patient?

>At the reviewer's suggestion, we investigated the levels of MFN1 and OPA1 in our patient fibroblasts, and discovered some interesting differences (Fig 1E). Notably, we see decreased protein levels for both MFN1 and MFN2, as well as several other mitochondrial proteins, suggesting that overall mitochondrial abundance is reduced in the patient cells. In addition, we see unexpected changes to the abundance of OPA1 protein isoforms, which to our knowledge have not been noted for other pathogenic MFN2 variants. These new data and a discussion of these novel results has been added to the revised manuscript.

Minor points:

1. A cartoon to illustrate the main findings would be welcome.

>We decided against including a cartoon figure, as it is not simple to illustrate the various findings of this manuscript in a straightforward figure that is easy to interpret, and did not feel it would add significantly to the manuscript.

2. Sentence on page 4, paragraph 4, line 3, please rephrase ("Central nervous ... of cases")

*>We have revised the text to make the meaning clearer, as follows:
"Central nervous system involvement is also rarely described (50), with periventricular and subcortical white matter lesions in 8 out of 21 patients from one series. A small minority of cases had transient neurological deficits such as dysarthria or paraesthesiae."*

1. Please state the glucose content of the medium during cell maintenance.

> The medium contains low glucose (5.56mM).

2. Page 5, third paragraph, line 9, goat anti-rabbit, should be goat anti-mouse.

> Corrected

3. Please state the company of the DAPI labelling.

>The DAPI used in this study is included in the Prolong Glass Antifade Mountant with NucBlue Stain from Fisher Scientific (Thermo Scientific P36983). In this case, the NucBlue in

the commercial description is DAPI.

4. Please state which program is used to do the statistical testing and if a p-value of <0.05 was considered significant.

>All statistical analyses were performed using Prism, and yes, for all analyses the significant differences indicate $p<0.05$.

Competing Interests: No competing interests were disclosed.

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