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

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\textbf{Abstract}

\textbf{Background:} Pathogenic variants in \textit{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 \textit{MFN2} variants can also lead to other phenotypes such as optic atrophy, hearing loss and lipodystrophy. Despite the clear link between \textit{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 \textit{MFN2} variants have been described to date, only a few have been characterized functionally, and even then, often only for one or two functions.

\textbf{Method:} Several MFN2-mediated functions were characterized in...
fibroblast cells from a patient presenting with cerebellar ataxia, 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
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. Mitochondrial fusion is a multistep process mediated by several essential proteins and regulators. 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). Highlighting the importance of mitochondrial fusion is the fact that knockout of MFN1, MFN2 or OPA1 genes is embryonic lethal. 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). 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

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In response to the reviewers’ comments several changes were made to the manuscript. Major changes involve the addition of new data. First, western blot analysis of protein expression for MFN2, MFN1, and OPA1 was added (Fig. 1E). Second, mtDNA copy number was reanalyzed, and now includes normalization to both 18S and Beta-2-Microglobulin (Fig. 3D). This new work was performed by Mashiat Zaman, who was added as an author. Based on the new western blot data, which revealed changes in expression levels of MFN1 and MFN2, and changes in the pattern of OPA1 isoforms new text was also added to the discussion. In addition, new text was added in the discussion regarding previous work describing ataxia as a CMT2A pathology; links between mitochondrial fusion and mtDNA maintenance; and interactions between mitochondria and lipid droplets. Finally, several smaller changes to the text were made throughout for clarification.

Any further responses from the reviewers can be found at the end of the article
been generated for MFNs, 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. 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. Impairments to mitochondrial fusion can lead to reduced copy number and increased nucleoid size. While the reduced copy number is thought to be a result of decreased distribution of the replication machinery, it is unclear exactly why nucleoid size might change. Meanwhile, mitochondrial respiration can also be compromised upon loss of fusion.

Notably, MFN2 has several functions in addition to its role in mitochondrial fusion. For example, MFN2 mediates mitochondrial autophagy (mitophagy) and transport of mitochondria. Meanwhile, MFN2 also localizes to the endoplasmic reticulum, where it mediates interactions between the ER and mitochondria. 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, as well as mtDNA replication. Notably, MFN2 has been implicated in directly binding and transferring phosphatidylserine from ER to mitochondria. While there is some debate whether MFN2 promotes or inhibits MERCs, 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.

Pathogenic variants in genes regulating the opposing forces of mitochondrial fusion and fission can be associated with peripheral neuropathy, suggesting that impairment in the balance of these processes contributes to the disease phenotype. To date, more than 100 pathogenic variants in MFN2 have been associated with peripheral neuropathy. However, despite the general assumption that impaired mitochondrial fusion causes the peripheral neuropathy phenotype, only a few pathogenic MFN2 variants have been investigated for their effects on MFN2 functions. While some pathogenic MFN2 variants do impair fusion, unexpectedly, other pathogenic variants seem to increase fusion, while several pathogenic variants do not appear to affect fusion at all. 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, lipid metabolism, mitochondrial respiration, mtDNA copy number, and mitochondrial transport, 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. Central nervous system involvement is also rarely described, with periventricular and subcortical white matter lesions in 8 of 21 patients from one series. A small minority of cases had transient neurological deficits such as dysarthria or paraesthesiae. 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. Additional atypical features including severe neuropathy with hearing loss has been described with biallelic mutations. We are also aware of a report of a large pedigree including optic atrophy and sensory ataxia associated with a heterozygous D210V substitution in MFN2.

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 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). Similar analysis can be performed using an alternative bioinformatic pipeline as described previously. Patient VCFs were annotated for predicted variant consequence, gnomAD allele frequency, CADD score, and OMIM phenotypes, in addition to default parameters with Ensembl’s command line Variant Effect Predictor (VEP). We additionally aligned off-target reads from exome sequencing to identify any mtDNA variants, as previously described.

Cell maintenance
Control and patient fibroblast cultures were generated from skin biopsies and cultured in low glucose (5.56 mM) 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 × 10⁴ cells per well, and incubated for 1–2 days. As described previously, 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 labeled with a mouse monoclonal anti-dsDNA antibody (Developmental Studies Hybridoma Bank, AB_10805293, (RRID:AB_10805293)), and visualized with a polyclonal goat anti-mouse 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. 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: HP011562-100U (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 fluoro 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_1384040)). 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

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. 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 ± 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. 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 ± 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 ± 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 × 10⁵ 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. Briefly, the 20 μL quantitative PCR (qPCR) reaction contained 10 μL 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 and β-2-Microglobulin was analyzed using the delta delta Ct method and represented as percent control. Reactions were performed in triplicate technical replicates. The following primers were used to amplify β-2-Microglobulin: F: TGCTGTCCTCCATTTTGGATATATC; R: TCTCTGCTCCCCACCTCTAAGT. Data is presented as mean ± 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. The following primers were used to amplify nearly full length mtDNA (16.3 kb),

1482–1516 F: ACCGCCGTCACCCTCCTCAAGTATACTTCAAAGG;
1180–1146 R: ACCGCCAGGTCCTTTGAGTTTTAAGCTGTGGCTCG.

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. Briefly, cells were seeded in an XF24 microplate (3.75 × 10⁴/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 1-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).

Western blot analyses

Cells at the same confluency were collected and RIPA buffer (Thermo Scientific™, 89900) complimented with protease inhibitors was used to lyse and collect the protein extracts. Following quantification of protein concentrations, 50 μg of total protein from control and patient fibroblasts were loaded on an SDS-PAGE gel. Subsequently, PVDF membranes were used for overnight transfer of the blots. The blots were probed with the following antibodies: anti-MFN2 (Abnova, H00009927-M03; 1:1000), anti-MFN1 (Cell Signaling Technology, D6E2S; 1:1000), anti-OPA1 (BD Transduction Laboratories, 612607; 1:1000), anti-Actin (Sigma, A5316; 1:1000), anti- VDAC1 (abcam, ab14734; 1:1000), anti- HSP60 (Cell Signaling Technology, D6F1; 1:1000). Complimentary horseradish peroxidase conjugated antibodies were used: goat anti rabbit IgG, HRP linked Antibody (Cell Signaling Technology, 7074S) or goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2055). Blots were treated with the SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific™, 34095), and luminescence visualized with an Amersham Imager AI600.

Statistical analysis

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

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 ongoing use of a wheelchair and required 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 ATP1A3), 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).

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. Coverage of the mitochondrial genome was highly limited (6% coverage at ≥20X, which is considered the minimum read depth to accurately call mtDNA variants from off-target reads). Within these limitations we did not identify any pathogenic mtDNA variants.

Characterization of patient fibroblasts
We obtained skin fibroblast cells from the patient 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 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. 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.

Expression of mitochondrial fusion proteins
We examined the expression of MFN2, and two other mitochondrial fusion proteins, MFN1 and OPA1 (Figure 1E). We observed marked decreases in the protein levels of MFN1 and MFN2. In addition, two other mitochondrial proteins, HSP60 and VDAC1 were also decreased, suggesting an overall decrease in mitochondrial abundance in the patient fibroblasts, rather than a specific defect in MFN2 expression. Meanwhile, we also observed differential expression of OPA1 protein isoforms, with one band increasing, and other bands decreasing.

Impact of the MFN2-D414V variant on mitochondrial morphology
We compared mitochondrial morphology in patient and control fibroblasts 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.
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 derived (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. (E) Western blot analysis of control and MFN2-D414V patient fibroblasts for expression of MFN2, MFN1, OPA1, β-Actin, VDAC and HSP60 proteins. The graphs indicate ratio of MFN2, MFN1, or OPA1 normalized to cellular protein loading control β-Actin as ratio of percentage of control fibroblasts. n = 3 biological replicates. Error bars indicate mean ± SD, unpaired t-test.
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).

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 fibroblasts from control (n = 24 cells) or MFN2-D414V patient (n = 27 cells). Error bars indicate mean ± SD. p < 0.0001, unpaired t-test. (C) Oxygen consumption rate (OCR) traces in fibroblasts from control or MFN2-D414V patient measured using the Seahorse XF24 extracellular flux analyzer (n = 12 replicates). (D) Basal (upper) and maximum (lower) OCR in control and MFN2-D414V fibroblasts calculated from C. Error bars indicate mean ± SD. p < 0.0001, unpaired t-test.

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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.
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 in fibroblast cells from control (n = 35) or MFN2-D414V patient (n = 49 cells). Error bars indicate SD. p = 0.0002, unpaired t-test. (D) Quantification of the average size of MERCs 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.
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 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.

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 μm. (C) Quantification of the number of LDs. Each data point indicates the total number of LDs in fibroblast cells from control (n = 35) or MFN2-D414V patient (n = 49). 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 for fibroblast cells from control (n = 35) or MFN2-D414V patient (n = 45). 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 fibroblasts from control (n = 8303 LD from 24 cells) and MFN2-D414V (n = 5259 LD from 25 cells). 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 fibroblast cells from control (n = 24) or MFN2-D414V patient (n = 25). Error bar indicates SD. p = 0.0006, unpaired t-test.
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.60,67 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 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,48,49 cerebellar ataxia has not. There is one described report of an MFN2 patient having neuropathy with sensory ataxia and optic atrophy.47 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. As pathogenic 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 from the D414V variant is whether different mechanisms underlying MFN2 dysfunction might explain the distinct patient phenotypes. 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. 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.68,69 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,70 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,66 C390F,71 R400X,71 R400P,72 R418Q71 and R418X.71 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.65 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.

An initial investigation in the expression of MFN2 and other mitochondrial fusion proteins MFN1 and OPA1 provided interesting findings. The observation of reduced MFN2 protein expression in our patient fibroblasts likely reflects a total
loss of mitochondrial mass as the expression of several other mitochondrial proteins is also reduced. While this reduction could be due to reduced biogenesis or increased turnover, we favour the later explanation as MFN2 has a known role in mediating mitochondrial mitophagy. Notably, reduced MFN2 protein expression has also been reported for MFN2 variants linked to mtDNA depletion, though total mitochondrial abundance was not investigated in that study.

The observation of altered abundance of OPA1 isoforms in the D414V fibroblasts is also intriguing in the context of the functional link between MFN2 and OPA1. While some MFN2 variants linked to mtDNA instability have reduced total OPA1 expression, to our knowledge, a link between pathogenic MFN2 variants and alterations in OPA1 isoform abundance has not been reported previously. Elucidating the exact cause of the changes in the relative abundance of OPA1 protein bands in our patient fibroblasts is beyond the scope of the current work. However, based on previous work examining OPA1 isoforms we hypothesize the following explanations for the observed pattern of OPA1 bands in the patient fibroblasts: 1) the loss of OPA1 bands a and c is likely due to reduced expression of OPA1 isoform 7; 2) band d may be the d’ band described recently for OPA1 isoforms containing exon 4b (i.e. isoforms 3,5,6,8), which are cleaved by YME1L; 3) the increased abundance of band b and reduced abundance of band e is likely due to reduced OMA1 processing of isoform 1.

While it is uncertain how changes in MFN2 function might impact OPA1 expression and processing, it is worth noting that MFN2 interacts SLP2 (STOML2), which is an IMM protein that regulates OMA1 cleavage of OPA1 and mediates stress-induced mitochondrial hyperfusion. Meanwhile, MFN2 has also been implicated in additional processing of OPA1 linked to starvation. Additionally, while OMM and IMM fusion events are sequential and can be uncoupled, direct interaction between MFNs and OPA1 have been observed in yeast, fly, and humans. Regardless of the mechanism by which MFN2 impacts OPA1, it is possible that alterations in the OPA1 isoform expression also contribute to the mitochondrial dysfunction observed in these cells.

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, which could be due to impaired MFN2 functionality and/or altered expression of OPA1 isoforms. 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, there are other CMT2A MFN2 variants that do not seem to affect mitochondrial morphology. Conversely, some CMT2A MFN2 variants actually enhance fusion. 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 could be a result of several abnormalities observed in these cells. First, reduced fusion could be involved, as inefficient fusion-dependent distribution of the mtDNA replication machinery is proposed to lead to reduced mtDNA copy number. Second, the decrease in MERCs could contribute, as the ER is thought to licence mtDNA replication. Third, alterations to the levels of OPA1 isoforms could also play a role, as OPA1 also mediates mtDNA. Finally, the reduced abundance of several mitochondrial proteins likely reflects a global loss of mitochondrial mass, which would also be consistent with decreased mtDNA copy number. In this light, it is relevant that mitophagy can be mediated by MFN2, MERC, and OPA1. To our knowledge, a link between pathogenic MFN2 variants and increased mitophagy has not been described. However, increased mitophagy has been noted in the context loss of FBXL4, another mitochondrial fusion protein, mutations in which cause a mtDNA depletion syndrome.

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. However, it should be noted that it is not clear why nucleoid size increases in response 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 is consistent with observations that a significant subset of nucleoids is actively undergoing replication. 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. Notably, cells where mitochondrial fission is
inhibited also exhibit large, clustered nucleoids, demonstrating that mitochondrial dynamics are important for the distribution of nucleoids.25,26 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.47,97

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,98 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,41,98,99 others cause an increase,100,101 while still more variants do not cause any change at all.25,38,42

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,26,38 disruption of contacts between mitochondria and ER seems to be a common theme underlying neuropathies.87,102,103 Further, a study in Drosophila indicated that MFN2 could regulate ER stress,104 and peripheral neuropathy in diabetic patients is also thought to be partly due to ER stress.105,106 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.38,97

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.36,107 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.38,108 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.38 Interestingly, we also discovered an unexpected perinuclear accumulation of lipid droplets in D414V 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,26,38 disruption of contacts between mitochondria and ER seems to be a common theme underlying neuropathies.87,102,103 Further, a study in Drosophila indicated that MFN2 could regulate ER stress,104 and peripheral neuropathy in diabetic patients is also thought to be partly due to ER stress.105,106 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.38,97

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,109,110 while adipocyte specific knockout of MFN2 leads to obesity in mice.34,111,112 Finally, the R707W MFN2 variant, which causes CMT2A when present heterozygously, also causes lipomatosis when present homozygously.51,52 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 1,1 an important mediator of lipid
droplet distribution.\textsuperscript{113} Though it should be noted that perilipin 1 is more abundant in adipose tissue than in fibroblasts.\textsuperscript{34} In contrast, the other CMT2A MFN2 variants investigated previously are proposed to increase lipid droplet signal via alterations to MERCs.\textsuperscript{36} 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 \textit{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. Other limitations of this study include the possibility that a 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.

In summary, our study establishes that the MFN2-D414V variant is incompetent in carrying multiple \textit{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 \textit{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 1B**

Primers used for sequencing and raw chromatogram files

**Fig 1E**

Uncropped western blot images used to generate Fig 1E
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
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).

Author contributions


Acknowledgements

We would like to thank the patient and their family for participation.

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71. HMDG. (retrieved on 21 June 2020).


93. Kakat C, Wurm CA, Spiesz H, et al.: Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA.


Open Peer Review

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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. 2012), 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, β2M) 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.

References

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.

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. (2021^1)) could have an important effect. Impaired mitochondrial fusion may increase (Liao et al. (2017^2)) or decrease (Sebastián et al. (2016^3)) 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

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.
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