Research paper

The R941L mutation in MYH14 disrupts mitochondrial fission and associates with peripheral neuropathy

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ABSTRACT

Background: Peripheral neuropathies are often caused by disruption of genes responsible for myelination or axonal transport. In particular, impairment in mitochondrial fission and fusion are known causes of peripheral neuropathies. However, the causal mechanisms for peripheral neuropathy gene mutations are not always known. While loss of function mutations in MYH14 typically cause non-syndromic hearing loss, the recently described R941L mutation in MYH14, encoding the non-muscle myosin protein isoform NMIIC, leads to a complex clinical presentation with an unexplained peripheral neuropathy phenotype.

Methods: Confocal microscopy was used to examine mitochondrial dynamics in MYH14 patient fibroblast cells, as well as U2OS and M17 cells overexpressing NMIIC. The consequence of the R941L mutation on myosin activity was modeled in C. elegans.

Findings: We describe the third family carrying the R941L mutation in MYH14, and demonstrate that the R941L mutation impairs non-muscle myosin protein function. To better understand the molecular basis of the peripheral neuropathy phenotype associated with the R941L mutation, we have established a previously unrecognized biological role for NMIIC in mediating mitochondrial fission in human cells. Notably, the R941L mutation acts in a dominant-negative fashion to inhibit mitochondrial fission, especially in the cell periphery. In addition, we observed alterations to the organization of the mitochondrial genome.

Interpretation: As impairments in mitochondrial fission cause peripheral neuropathy, this insight into the function of NMIIC likely explains the peripheral neuropathy phenotype associated with the R941L mutation.

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1. Introduction

Peripheral neuropathies are generally caused by defects affecting myelination and/or axonal transport. Mutations in genes regulating mitochondrial fission or fusion are a known cause of Charcot-Marie-Tooth (CMT) disease, a heterogeneous group of inherited peripheral neuropathies [1]. It is hypothesized that peripheral neuropathy is a consequence of mitochondria that are too fragmented or too fused, such that mitochondria are not efficiently transported down the long axons of peripheral neurons [2]. A key regulator of mitochondrial fission is the
Research in context

Evidence before this study

While most pathogenic variants in MYH14, encoding non-muscle myosin IIIC (NMIIIC), are associated with non-syndromic hearing loss, the R941L pathogenic variant was reported to segregate with a peripheral neuropathy phenotype in two separate families. Although many types of cellular dysfunction can cause peripheral neuropathies, the molecular mechanism underlying this phenotype in patients with the R941L variant in MYH14 was completely unknown. While, other non-muscle myosin proteins related to MYH14 have been implicated in mediating fission, a process that when impaired can cause peripheral neuropathy, a role for NMIIIC in fission had not been established previously, nor had pathogenic variants in non-muscle-myosin proteins been associated with a peripheral neuropathy phenotype.

Added value of this study

The current study characterized a third family where the R941L mutation segregates with a peripheral neuropathy phenotype. A completely novel role in mitochondrial fission is established for the NMIIIC protein encoded by MYH14, with the R941L mutation having a dominant negative effect that inhibits mitochondrial fission, especially at the cell periphery. Notably, the impaired fission in MYH14 patient fibroblasts also affects distribution of the mitochondrial genome within the mitochondrial network.

Implications of all the available evidence

With three unrelated families showing the R941L mutation segregates with peripheral neuropathy, there is increased confidence that this variant causes the phenotype. The novel role of MYH14 in mitochondrial fission, which is impaired by the R941L variant, provides insight into the underlying biological mechanism likely causing peripheral neuropathy in these patients.

dynamin-related protein DRP1 (encoded by DNM1L), which is recruited from the cytosol to mitochondrial tubules, where it forms a ring to constrict mitochondria. While Drp1 is generally regarded as the major protein mediating mitochondrial fission, recent studies have shown that additional factors, such as the endoplasmic reticulum (ER) and Dynamin2 (DNM2), also regulate key steps in fission [3–5].

We now appreciate that a pre-constriction step, mediated by the ER, is required to reduce the mitochondrial circumference such that DRP1 rings can encircle mitochondria [3]. Non-muscle myosin family II proteins A and B (NMIIA/B) are ATP-dependent molecular motors that interact with actin and provide the necessary mechanical force for this pre-constriction, and promote DRP1 recruitment to mitochondria [6–8]. In contrast to muscle myosins, which form the contractile filaments in smooth, skeletal, and cardiac muscle, NMII proteins are present in all cells, where they regulate intracellular processes. The human genome contains three NMII isoforms: NMIIA, NMIB and NMIIIC, encoded by the genes MYH9, MYH10, and MYH14, respectively. These NMII isoforms are thought to be partially redundant. However, while NMIIA/B are known to regulate cellular processes such as cytokinesis, cell motility, cell polarity, mtDNA regulation, and mitochondrial fission, the cellular functions of NMIIIC have not been thoroughly investigated [9–11]. Additionally, the interplay and redundancy of NMII proteins regarding their role in mitochondrial fission is unknown.

Recently, the R941L mutation in NMIIIC has been linked to a complex phenotype including peripheral neuropathy and hearing loss, and has been reported in two distinct families from different geographic regions, and in a single individual from a larger study [12–14]. The role of NMIIA/B in mediating mitochondrial fission [10] led us to hypothesize that NMIIIC is also involved in mitochondrial fission, and that the pathogenic R941L mutation would impair fission. While several other pathogenic variants in MYH14 are known to cause autosomal dominant hearing loss via haploinsufficiency [15–20], the peripheral neuropathy phenotype is unique to the R941L mutation. Moreover, MYH14 null mice are reported to have increased susceptibility to hearing loss [21], but no peripheral neuropathy phenotype has been reported.

Prior to this study, the cellular functions of NMIIIC, and the molecular mechanism by which the R941L mutation might cause peripheral neuropathy were unknown. Here, in addition to describing a novel family pedigree harbouring the R941L mutation, we address two unanswered questions, namely how the R941L mutation causes peripheral neuropathy, and how this mutation has appeared independently in multiple families. Mechanistically, we describe a previously uncharacterized role for the NMIIIC as a mediator of mitochondrial fission, which is impaired by the pathogenic R941L mutation.

2. Materials and methods

2.1. Ethics statement

Informed consent for enrollment in the Care4Rare project and for publication was obtained by the examined members of this 3 generation pedigree. This study was approved by the conjoint ethics board of the University of Calgary.

2.2. Genetic analysis

Whole exome sequencing was performed at the McGill Genome Centre as part of the Care4Rare Canada initiative. Details of this project including case selection and analytic pipeline are described elsewhere [22,23]. Whole Exome Sequencing (WES) using a SureSelect (Agilent, Santa Clara, California, USA) exome capture kit was performed on the Illumina sequencing platform.

2.3. CpG methylation detection

CpG methylation was assayed using the Fau enzyme (New England Biosystems), which does not cut methylated DNA. Briefly, 50 ng of genomic DNA was digested at 55 °C for 60 min, followed by heat inactivation at 80 °C for 20 min. Next, DNA was amplified using primers specific to MYH14 (MYH14_CpG_For, GCGACGCGGGGGAGGC, and MYH14_CpG_Rev, CTGCACAGGTTGGCTGC), and 10 μL of the PCR product was visualized via agarose gel electrophoresis.

2.4. In silico protein modeling

The amino acid sequence of human MYH14 isoform 1 (NP_001070654.1) was submitted to the Swiss Model in silico protein modeling tool [24]. Though a crystal structure for the head domain of the human NMIIIC protein has been solved (RCsb:2YCU), no structure has been published for the tail domain containing the R941L mutation. Thus, a predicted protein structure was created based on the structure of Gallus gallus meromyosin (RCsb:1884) [25], as this homologous structure contains the protein tail domain, which is not present in the crystal structure of human NMIIIC protein. The resulting structure prediction and mutations were visualized using UCSF Chimera [26].

2.5. Genetic modeling in C. elegans

C. elegans were cultured under standard conditions [27] at the indicated temperatures. Complete broods of at least four hermaphrodites were determined, with a total of 450–1300 progeny scored. The following mutations were used: the temperature-sensitive allele mel-11(t26),
the null allele nmy-1(sb113) [28] and the R915L CRISPR allele nmy-1(sb139) described here. To introduce the R915L mutation into nmy-1, left-3:Cas9, pJW58(dpy-10(cn644) gRNA) and a dpy-10(cn644) repair single-stranded oligonucleotide (University of Calgary Core DNA Services) were used as described for “Co-CRISPR” [29]. CRISPR targeting sequence and primers used in the generation of the mutant nmy-1(sb113) allele are provided in the additional methods (Supplemental Table 1). Two nmy-1 gRNA PCR products were created using PCR “stitching”. PCR’s using oJW1787 + nmy1sgRNA1rev and oJW1790 + nmy1sgRNA1fw were stitched together, substituting nmy-1 sequences for those of pha-1 and gel-purified to make nmy-1 gRNA-1 (oligonucleotides and nmy-1 gRNA target sequence are listed below). Similarly, PCR’s using oJW1787 + nmy1sgRNA2rev and oJW1790 + nmy1sgRNA2fw were stitched together and gel purified to make nmy-1 gRNA-2. The single-stranded repair oligonucleotide nmy-1-R915L-rescueoligo (IDT), was designed to have 60 base pair homology arms, a (GC-TC) mutation resulting in R915L and two silent mutations in R911 (G-A) and N916 (C-T) to disrupt nmy-1 gRNA targeting of the new allele and to introduce SalI/Msel restriction enzyme sites respectively. Gravid wild-type hermaphrodites were injected with 50 ng/μL left-3:Cas9, 25 ng/μL pJW58(dpy-10(cn644) gRNA), 500 nM dpy-10 (cn644) oligonucleotide (Arribere et al. 2014), 25 ng/μL nmy-1 gRNA-1, 25 ng/μL nmy-1 gRNA-2, and 500 nM nmy-1-R915L-rescue oligo. Dpy and Rol F1 progeny were screened by PCR and restriction enzyme digestion for insertions. One allele was identified, nmy-1(sb139) and confirmed by sequencing.

2.6. Cloning and plasmids

The wild-type NMIIC-EGFP plasmid was a gift from Dr. John Hammer at the National Institutes of Health, which was originally created in the lab of Dr. Adelstein of the National Institutes of Health. The NMIIC R941L mutant was generated by overlapping PCR mutagenesis and verified by Sanger sequencing. The wild-type mCh-Drp-1 plasmid was purchased from Addgene (plasmid# 49152), which was originally created by Dr. Gia Voeltz.

2.7. Cell culture conditions

Fibroblast cells were generated from skin biopsies and cultured in DMEM containing 10% fetal bovine serum (FBS), supplemented with Penicillin/Streptomycin (100 IU/mL/100 μg/mL). U2OS human osteosarcoma cells were grown in McCoy’s 5A (modified) media containing 10% FBS and supplemented with Penicillin/Streptomycin. For transfection, 8 x 10⁴ U2OS cells were initially seeded on glass bottom dishes (MatTek, P35G-1.5-14-C). The following day, cells were transfected with 0.5 μg of plasmid DNA using Lipofectamine 3000 according to the manufacturer’s recommended protocol. Finally, U2OS cells were imaged 24 hours after transfection. BE(2):M17 neuroblastoma cells (hereafter M17) were cultured in a 1:1 mixture of Eagle’s Minimum Essential Medium and F12 medium with 10% FBS, and supplemented with non-essential amino acids and Penicillin/Streptomycin. For M17 transfection, cells were first differentiated as previously described [30]. Briefly, 1 x 10⁶ M17 cells were seeded on glass bottom dishes pre-coated with poly-D-lysine for 24 h. Retinoic acid at concentration of 15 μM was added and the cells incubated for 72 h. Following 72 h, M17 cells were transfected with 2 μg of plasmid DNA (wild-type or R941L mutant NMIIC-EGFP, or empty pcDNA vector as a control) using Lipofectamine 3000. Twelve hours after transfection, the media was changed and M17 cells were imaged the following day.

2.8. Microscopy

Cells were imaged as indicated with either of the following two microscope setups. A Zeiss LSM 710 confocal microscope (plan-Apochromat 63x/1.4 oil objective) with image capture and processing performed with Zen Blue and Black software (Carl Zeiss, Jena, Germany). For live cell imaging with the Zeiss microscope, cells were supplemented with 50 mM HEPES and incubated at 37 °C. Alternatively an Olympus spinning disc confocal system (Olympus SD-OSR) (UAPON 100XOTIRF/1.49 oil objective) operated by Metamorph software was utilized. The SD-OSR was equipped with a cellVivo incubation module to maintain cells at 37 °C and 5% CO₂ during live cell imaging.

2.9. Live cell imaging

Approximately 24 h prior to imaging, cells were seeded at 8 x 10⁴ cells/mL onto glass bottom dishes. Immediately prior to imaging, cells were treated with the indicated dyes as follows. To label mitochondria, cells were stained for 30 min with 50 nM MitoTracker Red (Thermo Fisher Scientific, M7512), or MitoTracker Deep Red (Thermo Fisher Scientific, M22426), as indicated. To label mDNA nucleoids, cells were co-stained with the DNA dye PicoGreen (Thermo Fisher Scientific, P11495) (3 μL/mL), as previously described [31], in addition to MitoTracker Red. After staining, cells were washed 3 times with pre-warmed 1x phosphate buffered saline (PBS), and normal growth media was added prior to imaging. For the phototoxic-dependent mitochondrial fission assay using the Olympus SD-OSR microscope, MitoTrackerRed-labelled fibroblasts were repeatedly exposed (100 ms at 1 frame per second intervals) to higher levels of the 561 nm excitation laser (100 mW at 4% power), for a total of 5 min.

2.10. Immunofluorescence

Cells were grown on glass coverslips (no. 1.5), then fixed in prewarmed 4% formaldehyde for 10 min at 37 °C, permeabilized in 0.25% Triton X-100, and quenched with 50 mM ammonium chloride. After blocking with 5% FBS in PBS, cells were incubated for 1 h at 37°C with primary antibodies to TOMM20 (rabbit anti-TOMM20, Santa Cruz Biotechnology, FL-145) diluted 1:1000 in 5% FBS in PBS, and subsequently incubated for 1 h at room temperature with appropriate AlexaFluor secondary antibodies (Themo Fisher Scientific) diluted 1:1000 in 5% FBS in PBS. Coverslips were then mounted on glass slides with Dako mounting media (Agilent, S3023) and imaged.

2.11. Image analysis

Mitochondrial length was quantified from 30 cells from each patient as described previously [32], using the Tubenness plug from Fiji for mitochondrial segmentation. For nucleoid analysis, images were cropped, globally adjusted for contrast and brightness, and median filtered using ImageJ (https://imagej.nih.gov/ij/index.html). Mitochondrial DNA nucleoid size and number were analyzed in 10 fibroblast cells from each group using the particle analysis tool on ImageJ Fiji [33]. Briefly, images were scaled and binarized. A region of interest engulfing the entire cell was selected and the particle analysis tool was used to generate surface area and total nucleoid count measurements. Nuclear and nongeneric signal outside the mitochondrial network were manually excluded from the analysis.

2.12. Mitochondrial membrane potential and mitochondrial mass analyses

Mitochondrial membrane potential and mitochondrial mass were assessed in control and patient fibroblasts by flow cytometry, as described previously [34]. Briefly, fibroblasts were seeded at 1 x 10⁵ cells in 6 well plates and allowed to grow for 2 days. Prior to analysis, cells were stained with TMRE (Tetramethylrhodamine, ethyl ester) (50 nM, 20 min) (T669; Life Technologies) or MitoTracker Green (50 nM, 20 min) (M7514; Life Technologies). Cells were subsequently washed with 1x PBS, trypsinized and harvested. Cell pellets were resuspended in media and analyzed using the BD LSR II flow cytometer (BD Fisher Scientific, M22426), as indicated. To label mDNA nucleoids, cells were co-stained with the DNA dye PicoGreen (Thermo Fisher Scientific, P11495) (3 μL/mL), as previously described [31], in addition to MitoTracker Red. After staining, cells were washed 3 times with pre-warmed 1x phosphate buffered saline (PBS), and normal growth media was added prior to imaging. For the phototoxic-dependent mitochondrial fission assay using the Olympus SD-OSR microscope, MitoTrackerRed-labelled fibroblasts were repeatedly exposed (100 ms at 1 frame per second intervals) to higher levels of the 561 nm excitation laser (100 mW at 4% power), for a total of 5 min.
Bioscience) and the BD FACSDiva software. Signal intensity was recorded from at least 20,000 events and data is presented as percent control. Analyses were performed on triplicates for each fibroblast line.

2.13. mtDNA copy number analysis

Genomic DNA (gDNA) from control and patient fibroblasts was extracted using PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, K182001) according to manufacturer’s instructions. Relative mtDNA copy number was assessed by real-time quantitative PCR (qPCR) using the QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific). Primer sequences to amplify mtDNA, the nuclear-encoded housekeeping gene 18S, and thermocycling conditions were exactly as described previously [35]. The 20 μL reactions contained 10 μL PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, A25742), 100 ng gDNA and a final concentration of 500 nM forward and 500 nM reverse primers.

2.14. Long-range PCR

To examine mtDNA deletions, the following primers were used to amplify nearly full length mtDNA (16.3 kb), (1482–1516 F: ACCGCC CTCACCCCTCTCAGATCACTCCTAAGG; 1180–1146 R: ACCGGCAG GTCTTGGATTTAAGCTGTGGCTCG) as reported previously [36]. Long range PCR reactions were performed using the Takara LA Taq polymerase (Takara Bio, RR002M), with 250 ng genomic DNA, 200 nM forward and reverse primers. The PCR cycling conditions 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. PCR products were visualized by electrophoresis on a 0-6% agarose gel, run for approximately 12 h at 20 V.

2.15. Statistical analysis

Mitochondrial morphology was assessed in a blinded fashion by qualitatively assigning morphology into three or four categories. Quantification was performed in at least 50 cells from multiple technical and experimental replicates, as indicated. In order to quantify the peripheral hyper-connectivity in fibroblasts cells, the presence/absence of hyperconnected mitochondrial networks at the cell periphery were scored from three independent experiments. At least 70 cells were assessed per replica. MitDNA nucleoid sizes are presented as the average size of all nucleoids per cell ± SD. Nucleoid counts are presented as mean ± SD. Experimental replicates, as indicated. In order to quantify the peripheral hyper-connectivity in fibroblasts cells, the presence/absence of hyperconnected mitochondrial networks at the cell periphery were scored from three independent experiments. At least 70 cells were assessed per replica. MitDNA nucleoid sizes are presented as the average size of all nucleoids per cell ± SD. Nucleoid counts are presented as mean ± SD. Experimental replicates, as indicated. In order to quantify the peripheral hyper-connectivity in fibroblasts cells, the presence/absence of hyperconnected mitochondrial networks at the cell periphery were scored from three independent experiments. At least 70 cells were assessed per replica. MitDNA nucleoid sizes are presented as the average size of all nucleoids per cell ± SD. Nucleoid counts are presented as mean ± SD. Experimental replicates, as indicated. In order to quantify the peripheral hyper-connectivity in fibroblasts cells, the presence/absence of hyperconnected mitochondrial networks at the cell periphery were scored from three independent experiments. At least 70 cells were assessed per replica. MitDNA nucleoid sizes are presented as the average size of all nucleoids per cell ± SD. Nucleoid counts are presented as mean ± SD. Experimental replicates, as indicated. In order to quantify the peripheral hyper-connectivity in fibroblasts cells, the presence/absence of hyperconnected mitochondrial networks at the cell periphery were scored from three independent experiments. At least 70 cells were assessed per replica. MitDNA nucleoid sizes are presented as the average size of all nucleoids per cell ± SD. Nucleoid counts are presented as mean ± SD. Experimental replicates, as indicated. In order to quantify the peripheral hyper-connectivity in fibroblasts cells, the presence/absence of hyperconnected mitochondrial networks at the cell periphery were scored from three independent experiments. At least 70 cells were assessed per replica. MitDNA nucleoid sizes are presented as the average size of all nucleoids per cell ± SD. Nucleoid counts are presented as mean ± SD. Experimental replicates, as indicated.
predicted to engage in hydrophilic interactions that could potentially influence myosin dimerization (Fig. 1e). Our in silico modeling suggests that the introduction of a leucine residue at position 941 eliminates a predicted hydrophilic interaction with residues on an opposite alpha helix. We thus speculate that this substitution could alter the formation of a functional dimer, which would be consistent with a possible dominant-negative effect, where a defective subunit poisons the complex.

Fig. 1. Genetic analysis of patients and modeling of the R941L mutation. (a) Pedigree of a three generation family with peripheral neuropathy and hearing loss. Shaded sections indicate the presence of both CMT and hearing loss, which were always seen together in the pedigree. Exome sequencing was performed on individuals II-5, III-2 and IV-2. The c.2822G>T p.Arg941Leu mutation was identified in individuals II-5, III-2, IV-1 and IV-2. Note that for subsequent fibroblast studies individuals III-2, IV-1, and IV-2 are patients 1–3, respectively. (b) Sanger sequencing confirms the presence of the c.2822G>T p.Arg941Leu mutation in individuals II-5, III-2, IV-1 and IV-2. (c) Alignment of the human and worm genes showing the conservation in the region surrounding MYH14 R941, which corresponds to R915 of the worm gene. (d) The R915L mutation in C. elegans NMY-1 decreases nonmuscle myosin function. nmy-1(sb139) acts semidominantly at all three temperatures to increase the hatching rate of mel-11 mutations, indicating that sb139/R195L compromises NMY-1 function (the 2.6% hatching at 25° was significant as it represented 25/936 vs. 2/938 for the control). The 27% viability of nmy-1(sb139)/+; mel-11/mel-11 embryos at 15 °C was inferred from the hatching rates of the progeny of mothers of that genotype. These animals segregate a mixture of nmy-1(sb139) homozygotes and heterozygotes, as well as nmy-1(+) homozygotes in a Mendelian 1:2:1 ratio. The hatching rates of homozygous nmy-1(sb139) and nmy-1(+) embryos in the mel-11/mel-11 background are 68% and 19%, respectively. We observed 36% hatching from nmy-1(sb139)/+; mel-11/mel-11 mothers and this corresponds to 27% hatching of their heterozygous offspring, an increase above the 19% hatching nmy-1(+) nmy-1(+) mel-11/mel-11. Similar calculations demonstrated the semidominance at 20 °C (e). In silico protein modeling suggests that the wild-type arginine residue at position 941 (left) has hydrophilic interactions with residues on an adjacent alpha-helix during dimerization. The mutant leucine residue (right) is unable to form the same hydrophilic interactions with the opposite myosin chain.
3.2. A role for NMIIC in mitochondrial fission

3.2.1. Characterization of R941L patient fibroblasts

Given the established link between NMIIA/B isoforms and mitochondrial fission, and the fact that impaired mitochondrial fission can cause peripheral neuropathies, we hypothesized that the R941L mutation might impair mitochondrial fission. Thus, we examined mitochondrial morphology in fibroblast cells obtained from our R941L patients. We observed a significant shift towards a more fused mitochondrial network in all three patient fibroblast lines, consistent with impaired mitochondrial fission (Fig. 2a, b). Notably, the mitochondrial morphology in the control fibroblast line we used is similar to other control fibroblast lines (Supplemental Fig. 2). We confirmed these changes in mitochondrial morphology by quantifying the length of individual mitochondria, which were also longer in patient fibroblasts (Fig. 2c, d).

As changes in mitochondrial morphology are sometimes linked to alterations in mitochondrial function (e.g. more fragmented mitochondrial tend to be less active), we looked at whether there were any

Fig. 2. R941L Patient Fibroblasts have altered mitochondrial morphology. (a) Representative confocal images of mitochondrial networks taken with an Olympus SD-OSR microscope. Mitochondria in fixed control and patient cells were stained via immunofluorescence using a TOMM20 antibody. Scale bars indicate 10 μm. (b) Quantification of mitochondrial morphology in control fibroblast and R941L patient fibroblast cells. One hundred cells were quantified in three technical replicates for two independent experimental replicates. Error bars indicate standard deviations, while p-values (Student’s t-test) were determined by comparison to the number of cells with hyperfused mitochondria in control. (c) Quantification of average mitochondrial length. Data represents automated analyses from at least 30 cells per fibroblast line. (d) Mitochondrial length profile from cells in (c), binned into 0–3 and 6+ μm bins and presented as % total mitochondria. (e) Mitochondrial membrane potential in control and patient fibroblasts measured with flow cytometry analysis of TMRE-stained cells. (f) Mitochondrial mass measured in MitoTracker Green stained samples by flow cytometry. Data for (e) and (f) are presented as % control. Error bars indicate standard deviations, and p-values (Student’s t-test) determined by comparison to the control.
functional consequences of reduced fission in MYH14 patient fibroblasts. To this end, we measured mitochondrial membrane potential with the potentiometric dye TMRE, and mitochondrial mass with the Mitotracker Green dye. Although we observed a slight ~20% increase in TMRE signal in MYH14 fibroblasts (Fig. 2e), we also observed a corresponding ~20% increase in Mitotracker Green signal (Fig. 2f), suggesting that the increased TMRE signal is due to increased mitochondrial mass rather than membrane potential.

Given that there were no major global changes in mitochondrial function, combined with the notion that peripheral neuropathy linked to impaired mitochondrial fission and fusion are thought to be due to impaired mitochondrial transport, rather than decreased function per se [2], we decided to look at the distribution of mitochondria in MYH14 patient fibroblasts. Surprisingly, a close examination of mitochondrial morphology in patient fibroblasts also revealed an enrichment of hyper-connected mitochondrial networks in the periphery of greater than 60% of cells, compared approximately 20% of control fibroblasts (Fig. 3a). This observation led us to hypothesize that fission may be more impaired at the cell periphery.

To test this notion, we devised a fission assay that allowed us to induce mitochondrial fission and monitor the mitochondrial network throughout the cell. When mitochondrial fission was induced with a phototoxic stress, we found that these peripheral hyper-connected regions remained intact in R941L patient fibroblasts, even though central mitochondrial networks in the same cell were able to fragment (Fig. 3b, Supplemental Video 1). In comparison, mitochondrial networks in control fibroblasts were able to undergo fission throughout the entire cell, even in the few hyper-connected peripheral regions that could be found. Thus, this peripheral mitochondrial fission defect phenotype is unique to R941L patient fibroblasts.

As an independent approach to look at whether mitochondrial fission was impaired in R941L patient fibroblasts cells, we also examined mitochondrial genomes, as mitochondrial fission is required for mtDNA maintenance [40–42]. Specifically, loss of fission leads to

Fig. 3. Hyperconnected mitochondrial networks at the cell periphery in R941L patient fibroblasts are resistant to phototoxicity-induced fission. (a) Quantification of control and patient cells containing hyperconnected mitochondrial networks at the cell periphery. At least 70 cells were quantified from two independent replicates. Error bars indicate standard deviations, and p-values (Student’s t-test) were determined by comparison to the number of control cells with hyperfused mitochondria. (b) Representative confocal images of mitochondrial networks taken with an Olympus SD-OSR microscope. Control and patient cells stained with MitoTracker Red were imaged continuously over 5 min with high laser power to induce fission. Inset zoomed boxes represent regions with fragmented mitochondria (green hashed boxes) or resistant to fission (magenta hashed boxes) when imaging commenced, and at the end of 5 min (full video available as Supplemental Video 1). Signal intensity was enhanced for later frames to adjust for photobleaching. Scale bars indicate 10 μm.
enlarged mtDNA nucleoid structures [43]. Moreover, NMIIA and B isoforms have also been implicated in regulating mtDNA [11]. Using live-cell imaging to visualize nucleoid size and distribution, we observed that patient fibroblast cells had fewer, but larger nucleoids than controls (Fig. 4a). Moreover, the distribution of mtDNA nucleoids throughout the mitochondrial network was different in MYH14 fibroblasts. In control fibroblasts, mtDNA nucleoids are distributed evenly throughout the network, even in hyper-connected peripheral regions. In contrast, when we looked at the nucleoid distribution in the hyper-connected mitochondria in the cell periphery of R941L patient fibroblasts, we observed a marked lack of nucleoids within these regions (Fig. 4b).

Next, we quantified the average size and number of mtDNA nucleoids and found that MYH14 fibroblasts has fewer, but larger mtDNA nucleoids (Fig. 4c, d). As decreases in the total number of nucleoids can be due to either loss of mtDNA or clumping of several smaller nucleoids, we also looked at the total amount of mtDNA in these cells. Quantification of the mtDNA copy number by qPCR showed that there were no significant changes in R941L patient fibroblast cells compared to control (Fig. 4e). Finally, we performed long-range PCR to rule out the possibility of mtDNA deletions in MYH14 fibroblasts (Fig. 4f). Altogether, these observations are consistent with impaired mitochondrial fission leading to mtDNA nucleoid alterations.

### 3.2.3. NMIIC localizes to future sites of mitochondrial fragmentation

Next, we quantified the average size and number of mtDNA nucleoids and found that MYH14 fibroblasts has fewer, but larger mtDNA nucleoids (Fig. 4c, d). As decreases in the total number of nucleoids can be due to either loss of mtDNA or clumping of several smaller nucleoids, we also looked at the total amount of mtDNA in these cells. Quantification of the mtDNA copy number by qPCR showed that there were no significant changes in R941L patient fibroblast cells compared to control (Fig. 4e). Finally, we performed long-range PCR to rule out the possibility of mtDNA deletions in MYH14 fibroblasts (Fig. 4f). Altogether, these observations are consistent with impaired mitochondrial fission leading to mtDNA nucleoid alterations.

### 3.2.4. The R941L mutation disrupts neuronal mitochondrial fission

Given the peripheral neuropathy phenotype in patients, which can be caused by impaired axonal mitochondrial transport in other peripheral neuropathies due to alterations to mitochondrial fission, we decided to look at the effects of R941L overexpression in a neuronal cell type. Thus, we turned to M17 neuroblastoma cells, which are amenable to transfection, and which can be differentiated into neuronal-like cells [30,45]. Similar to U2OS cell, and consistent with a role for NMIIC in fission, overexpression of WT-NMIIC promoted mitochondrial fragmentation. Meanwhile, the R941L led to more hyperfused mitochondrial networks, again indicating a dominant-negative function (Fig. 6a, b).

### 4. Discussion

Prior to this study, although it was shown that the R941L mutation in NMIIC was associated with a peripheral neuropathy phenotype, there was little known about the molecular function of the NMIIC protein, and no insight into the underlying mechanism of the pathology. Here, we describe a third family where the R941L mutation segregates with a peripheral neuropathy phenotype, further strengthening the causal link between the mutation and the phenotype. Identifying a causal gene ends a diagnostic odyssey for patients and can provide families answers, counselling and prognosis. Moreover, diagnosis is also the first step towards therapy, with the next step involving mechanistic studies to understand the molecular basis of the disease. In this context, we also demonstrate a previously unrecognized role for NMIIC in regulating mitochondrial fission, which is impaired by the R941L mutation, and which may explain the peripheral neuropathy phenotype.

Two previous reports of the R941L mutation occurring de novo in independent families exhibiting deafness and peripheral neuropathy have been described in the literature [12,14]. In addition, this mutation has also been reported as part of a larger study of peripheral neuropathies [13]. However, the reason for reoccurrence of the same base pair change was unexplained. In the current report, we describe a new family pedigree harbouring the c.2822G–T mutation resulting in the R941L amino acid substitution in the protein sequence. Thus, the c.2822G–T mutation in MYH14 appears to have occurred de novo four times. Normally, it would be exceedingly rare for the exact same mutation to arise independently. However, our data support existing evidence that the locus in question is a methylated CpG site, increasing the chance of a mutation, and explaining the recurrence of this de novo mutation. Consistent with this notion, there are multiple different nucleotide substitutions at this position in The Exome Aggregation Consortium (ExAC) [46] (Supplemental Fig. 3), suggesting an increased sensitivity to mutations. Though of note, the R941L variant itself does not appear in the ExAC database.

While several autosomal dominant loss of function mutations in MYH14 have been linked to non-syndromic hearing loss [15–20], the additional peripheral neuropathy phenotype in patients with the R941L mutation suggests that this neuronal phenotype is not likely to be due simply to haploinsufficiency. Nonetheless, it is possible that peripheral neuropathy was not closely examined in these other MYH14 affected individuals with hearing loss alone, or that the disease had not progressed sufficiently to exhibit peripheral neuropathy when hearing...
a) MitoTracker Red, NMIIC-GFP, Merge, Zoom

b) Mitochondrial Morphology in U2OS Cells

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loss was first diagnosed. However, the most likely explanation is that the non-syndromal hearing loss associated with other MYH14 mutations is due to haploinsufficiency from loss of function in these alleles. The fact that MYH14-null mice are also susceptible to hearing loss [21], but have not reported peripheral neuropathy phenotype supports this conclusion.

In contrast, our data suggest that the peripheral neuropathy phenotype associated with the R941L mutation is not simply due to loss of function. In this regard, in silico modeling of the R941L mutation suggests that the replacement of the arginine residue with a leucine could impair protein interactions required for dimer formation that could influence the functional activity of the NMIC protein. Such structural changes that alter dimerization are consistent with a dominant-negative effect where a dimer of mutant with wild-type protein would be inactive. Meanwhile, our modeling of the R941L mutation in C. elegans, where there is a highly sensitive functional assay of NMII protein function, shows that the correlating R915L mutation acts semidominantly, but retains some myosin activity. However, our data does not allow us to conclude if the R915L mutation also has dominant-negative properties in C. elegans. Nonetheless, as it pertains to mitochondrial fission in human cell culture models, we see that overexpression of the R941L mutant protein has the opposite effect on mitochondrial morphology as the wild-type NMIC protein. Taken together, these findings are consistent with a distinct dominant-negative mechanism underlying the peripheral neuropathy of the R941L mutation.

Critically, we demonstrate a role for NMIC as a mediator of mitochondrial fission, a cellular function that is known to cause peripheral neuropathy when impaired [1]. To characterize the role of the R941L mutation at endogenous levels, we examined mitochondria in patient fibroblasts. We found that patient fibroblast cells exhibit several phenotypes that are consistent with impaired mitochondrial fission, most notably hyperfused mitochondrial networks. Although such hyperfused mitochondrial networks could be caused by either increased fusion or decreased fission, our data clearly show that NMIC plays a previously unrecognized role in fission, which is impaired in patient fibroblasts harbouring the R941L mutation. Notably, impaired fission is even more prominent at the cell peripheral, as central mitochondrial fission in the same cell could still undergo fragmentation. This intriguing finding demonstrates a definitive defect in mitochondrial fission in the fibroblasts cells, and suggests that NMIC may play a more important role in mediating mitochondrial fission at the cell periphery. Such a role would be especially relevant in the context of the peripheral neuropathy phenotype in patients with the R941L mutation in MYH14.

A peripheral fission phenotype is also intriguing as it pertains to our understanding of how impaired fission leads to mitochondrial dysfunction and peripheral neuropathies. Rather than the proposed model where impaired mitochondrial fission in the cell body prevents the anterograde transport of large mitochondria down axons [2], impaired fission at the tips of axons could impact local mitochondrial quality control and function in a different fashion. Specifically, the fused state at the ends of axons could prevent the retrograde transport of mitochondria. Additionally, as fission is required for the autophagic removal of mitochondria [47], which has been reported to occur in axons [48,49], impaired peripheral mitochondrial fission could also inhibit mitochondrial autophagy and thus impair neuronal function. The slight increase in mitochondrial mass that we observe in MYH14 fibroblasts would also be consistent with decreased turnover of mitochondria due to reduced mitochondrial autophagy.

In addition to mitochondrial network changes, we observed fewer and larger mtDNA nucleoids in patient fibroblasts, alterations that are also consistent with impaired fission [43]. While the larger mtDNA nucleoids that we visualized could be due to clumping of multiple mtDNA genomes, it is notable that signal from the DNA intercalating dye picogreen can also reflect changes to the supercoiling or packaging state of the mtDNA, not just mtDNA abundance [50,51]. Meanwhile, the decrease in the number of nucleoids could also be due to clumping, or alternatively to loss of total mtDNA. As our quantification of the mtDNA copy number in patient fibroblasts shows there is no significant mtDNA loss, we conclude that the larger nucleoids are most likely due to clumping of multiple nucleoids. This result is consistent with a previous report where inhibition of fission via acute knockdown of DRP1 leads to fewer, but larger nucleoids composed of multiple genomes, without any change in copy number [43]. The notion that fission is playing a key role in the distribution of mtDNA throughout the network is also supported by the lack of mtDNA nucleoids that we observe in the hyper-connected mitochondrial networks in the cell periphery of R941L patient fibroblasts. The mtDNA nucleoid impairments we observed in R941L patient fibroblasts are also notable, as regulation of mtDNA is involved in peripheral neuropathies. For example, peripheral neuropathies can be due to mutations in the mtDNA or mutations in proteins that regulate mtDNA replication [52]. In addition, a peripheral neuropathy is a side-effect of the chemotherapeutic agent cisplatin, which preferentially damages mtDNA [53] and also impairs mitochondrial dynamics in peripheral nerves [54].

Supporting the previously unrecognized role for NMIC in mediating mitochondrial fission, we found that overexpression of a GFP-tagged NMII shifted mitochondrial networks to a more fragmented state. While there are always potential concerns about protein overexpression artifacts, we note that the R941L mutation is not competent to promote mitochondrial fission, arguing against the increased mitochondrial fragmentation resulting from an accumulation of NMIC protein. In fact, overexpression of the R941L mutation shifted cells towards a more hyperfused network, consistent with a dominant-negative effect of the mutation. Moreover, we could directly visualize NMII-GFP puncta at mitochondrial fission sites preceding Drp1 recruitment, demonstrating that NMIC mediates fission in the same fashion as NMIIA and NMICB. Although we observed NMII-GFP at only 29% of total fission sites, it is important to remember that the U2OS cells express unlabeled NMII proteins [44], which likely mediate ER-mediated constriction at the remaining mitochondrial fission events.

The functional interplay and redundancy of the three NMII isoforms (A, B, and C) remains unknown in the context of regulating mitochondrial fission, but has important implications to the peripheral neuropathy phenotype in patients. In this regard, the situation of NMII isoforms is reminiscent to that of the fusion proteins MFN1 and MFN2. Though MFN1 and MFN2 are functionally redundant for fusion, mutations in MFN2 are thought to lead to a peripheral neuropathy phenotype because MFN1 is not highly expressed in neurons [55]. Thus, it is notable that neurons predominantly express NMIIA and NMIC [58]. As such, it
might be expected that neurons are more sensitive to mutations affecting NMIIC function than other cell types. It is intriguing that the phenotype associated with the R941L mutation is primarily a distal motor neuropathy phenotype, and few reported patients have any sensory symptoms or signs (Patient III-2 from this report and Patient III-8 from Choi et al. [12]). One possible explanation could be that the various NMII isoforms are differentially expressed in nerve fibers and sensory neurons.

In addition to expression differences, it is also possible that the cellular distribution and precise roles of the different NMII protein isoforms varies. Although little is known about the relative cellular functions of the NMII isoforms, based on our findings we would predict that NMIIC is more important for mediating fission at the cell extremities. Finally, it is notable that all three NMII isoforms can form homo or heterodimers with each other [44]. Though our structural modeling suggests that the R941L mutation could affect dimerization, we do not know how this might affect homo versus heterodimers, let alone the resulting functional outcomes on fission.

Due to the fact that we do not know all of the cellular functions of NMIIC, a limitation of this study is that we cannot say with certainty that the peripheral neuropathy is caused specifically by impaired mitochondrial fission. Future work using a peripheral neuron model expressing the R941L mutant would allow us to strengthen this connection. Nonetheless, the role of NMIIC in regulating mitochondrial

Fig. 6. The R941L NMIIC does not promote mitochondrial fission in differentiated M17 neuronal cells. (a) Representative confocal images of live M17 cells transfected with empty vector (control) or NMIIC-EGFP constructs (WTNMIIC = wild-type, R941L = mutant). Mitochondria were stained with MitoTracker Red, and the EGFP signal represents wild-type or R941L mutant NMIIC. Images were captured with a Zeiss LSM microscope. Scale bars indicate 10 μm. (b) Quantification of mitochondrial morphology in differentiated M17 cells as transfected as in (a). At least 40 cells were quantified in two independent experimental replicates. Error bars indicate standard deviations, and p-values (Student's t-test) were determined by comparison to the number of cells with fragmented or fused mitochondrial networks (as indicated) in the empty vector control.
fission, particularly at the cell periphery is likely to be relevant to the peripheral neuropathy phenotype in R941L patients. Notably, there is a growing list of peripheral neuropathy genes encoding proteins that regulate mitochondrial dynamics via fission (e.g. GDA1, DNM2, INE2, and MFF), fusion (e.g. MNF2, and OPA1), or transport (e.g. KIF1A, KIF1B, and KIF5A) [1,57,58]. Of particular relevance to the role of the NMIIIC protein in fission, the INE2 protein regulates actin, which interacts with NMII proteins during the ER-mediated mitochondrial constriction that initiates fission [59]. Thus, the R941L is likely a second example of how impairing the actin/myosin contraction required for fission can lead to a peripheral neuropathy phenotype. Collectively, our findings provide evidence that NMIIIC mediates mitochondrial fission. Moreover, the fact that the R941L mutation is deficient in the ability to promote mitochondrial fission provides additional evidence that impaired mitochondrial fission underlies the peripheral neuropathy in these patients. Thus, MYH14 can be added to the list of genes regulating mitochondrial dynamics, mutations in which lead to peripheral neuropathy.

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Declarations of interest

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Author contributions

Designed and/or performed and analyzed mitochondrial experiments, W.A., C.S., R.S., T.Z., and T.E.S.; Analyzed mitochondrial data, W.A., C.S., R.S., T.Z., H.S.I., J.D.G., M.G., T.E.S., R.W., and L.L.-G.; Designed, performed and analyzed C. elegans studies, R.B.S., and P.E.M.; Clinical evaluation of patients, O.S., G.P., and A.M.I.; Analyzed exome data, C.S., and J.S.P.; Performed skin biopsy from mitochondrial patients, G.P.; Drafted the manuscript or figures, W.A., C.S., R.S., G.P., A.M.I., T.E.S. All authors discussed the results and commented on the manuscript. W.A. and C.S. are joint first authors. T.E.S. and A.M.I. are co-responding authors.

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