**Supplementary data**

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5. **Table e-1**

**Oligonucleotide sequences used for this study.** All sequences are listed 5′-3′.

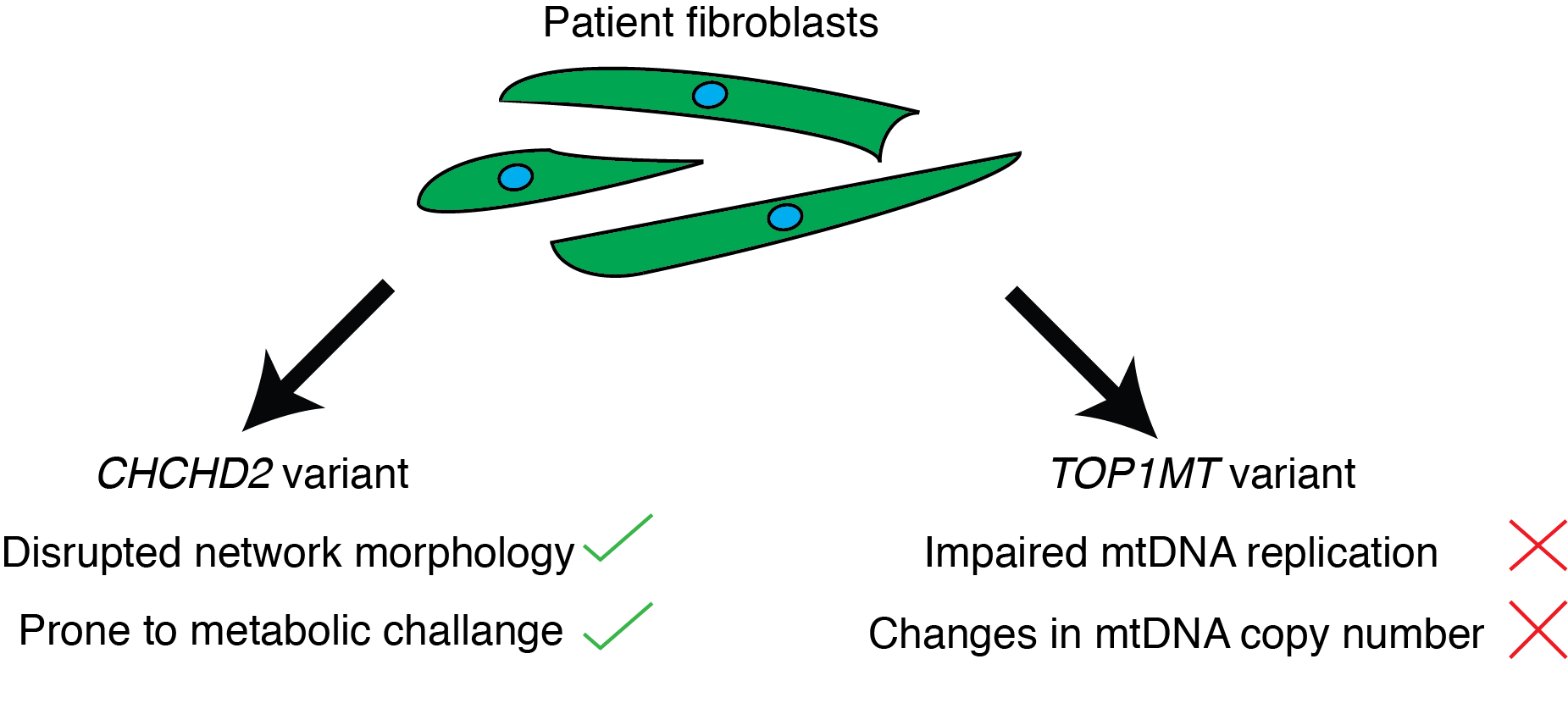
**Primers used for long range PCR**

|  |  |
| --- | --- |
| Mt16426 Fwd | CCGCACAAGAGTGCTACTCTCCTC |
| Mt16425 Rev | GATATTGATTTCACGGAGGATGGTG |

**Primers used for mitochondrial DNA copy number RT-PCR**

|  |  |
| --- | --- |
| hCytB Fwd | TAGCCATGCACTACTCACCAGA |
| hCytB Rev | AAGCAGGAGGATAATGCCGATC |
| hBglobin Fwd | AGAAGTCTGCCGTTACTGCC |
| hBglobin Rev | CAAGGGTAGACCACCAGCAG |
|  |  |

1. **Figure e-1**

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**CHCHD2 alone contributes to mitochondrial dysfunction and PD pathology.** Model of the observed phenotypes in patient fibroblasts and how it was determined that CHCHD2 contributed to mitochondrial dysfunction and Parkinson’s disease (PD) pathology. Mitochondrial dysfunction has been implicated in PD pathogenesis in knockout models for PD candidate genes, like *PINK1,* MPTP and rotenone-induced toxicity models, as well as studies on patient cells and tissues 1–7. The role of CHCHD2 in mitochondrial morphology, as well as the disrupted morphology and metabolic sensitivities of *PINK1-/-* cells lead us to examine the mitochondrial morphology of the patient fibroblast in both non-metabolic stress (glucose growth) and metabolic stress (galactose growth) conditions 7,8. The previously established role of *TOP1MT* in the regulation of mitochondrial DNA negative supercoiling lead us to investigate the mitochondrial DNA copy number and integrity9.

1. **e-Methods**

*DNA isolation*

Extraction of genomic DNA was performed from whole blood from three family members using DNeasy Blood & Tissue kit (Qiagen, Hilden Germany), according to the manufacturer’s instructions.

*Genetic analysis*

*Next Generation sequencing*

*Neuromuscular sub-exomic sequencing (NSES)*

NSES was performed on DNA from the patient and her unaffected sister, using the Ion ProtonTM sequencer (Life Technologies), as previously described 10,11. The NSES panel comprised of 336 known and candidate neuromuscular disease genes, including those listed within the December 2012 freeze of Neuromuscular Disorders gene table 12. NSES data were analysed using the Cartegenia software package (Agilent Technologies). Only variants with a minor allele frequency of <2% in control population databases ExAC, 1000 Genome Project and dbSNP 13 were retained.

*Whole exome sequencing (WES)*

WES was performed on DNA from one affected and one unaffected individual in three families with hereditary PD, as previously described 14. Briefly, target enrichment was performed with 3 μg genomic DNA using the SureSelectXT Human All Exon Kit version 5 (Agilent Technologies, Santa Clara, CA, USA) to generate barcoded whole-exome sequencing libraries. Libraries were sequenced on the HiSeq2000 platform (Illumina, San Diego, CA, USA) as paired-end 2 ×100-bp reads with 60x coverage. Quality assessment of the sequence reads was performed by generating QC statistics with FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). Read alignment to the reference human genome (hg19, UCSC assembly, February 2009) was done using BWA 15 with default parameters. After removal of PCR duplicates (Picard tools, http://picard.sourceforge.net) and file conversion (SAMtools) 16, quality score recalibration, indel realignment and variant calling were performed with the HaplotypeCaller algorithm in the GATK package 17 based on established best practices 18.

*Variant annotation and selection*

Variants were annotated with ANNOVAR 19 using a wide range of databases such as dbSNP build 135, dbNSFP, KEGG, the Gene Ontology project and tracks from the UCSC. A filtering strategy, directed to disease gene candidates, was performed by QIAGEN’s Ingenuity® Variant Analysis™ software (www.qiagen.com/ingenuity). Ingenuity Variant Analysis combines analytical tools which annotates variants and displays data including Polyphen, SIFT and CADD scores. We focused initially on coding variants in known neurogenetic disease genes, e.g. where the mutation produced a missense change, stop gain or stop loss, frameshift or essential splicing change. For these family with known consanguinity, the initial filtering strategy was also focused on homozygous variants. Only those changes that were predicted to be damaging or with unknown impact were analysed. We excluded variants that were frequent in control datasets (>1% in dbSNP 4, the Exome Variant Server (EVS) (NHLBI) (<http://evs.gs.washington.edu/EVS/>), the 1000 Genome Project Database (<http://browser.1000genomes.org/index.html)>, the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/) and the Human Background Variant Database (<http://neotek.scilifelab.se/hbvdb/>).

The number of reads, and the proportion of reads in forward and reverse orientations using Integrative Genomics Viewer (IGV) were considered for quality controls to eliminate false positive variants. In addition, WES data from control samples as well as the unaffected sister were analyzed in parallel.

*Polymerase chain reaction (PCR) and Sanger sequencing*

PCR and bi-directional Sanger sequencing of *CHCHD2* and *TOP1MT* was performed on patients and relatives. Primer details and conditions are available upon request.

*Multiple sequence alignment*

Evolutionary conservation of the altered residues of *CHCHD2* (p.Ala71Pro) and *TOP1MT* (p.Asp221Asn) was assessed by alignment of regions encompassing affected amino acids from *CHCHD2* or *TOP1MT* encoded proteins in human and other species, using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

*Cell culture and transfection*

Fibroblast cells were cultured at 37°C in humidified 95% air/ 5% CO2 in Dulbecco’s modified essential medium (DMEM) containing: glucose (4.5 g/L), L-glutamine (2 mM), foetal bovine serum (FBS) (10%, v/v), penicillin (100 U/ml), and streptomycin sulphate (100 µg/ml) (Gibco, Life Technologies). For galactose analyses, fibroblasts were grown in DMEM containing the above supplements with glucose replaced with galactose (1 g/L). Cells were equilibrated to galactose media for seven days before performing experiments. For transfections, cells were seeded at 80% confluency and transfected with 263 ng/cm2 of wild-type *CHCHD2* (OHu08881D)or *TOP1MT* (OHu10035D) (GenScript) in a pcDNA3.1+ vector (GenScript) using FuGene® HD (Promega) + Lipofectamine LTX (Invitrogen) (1:1) in OptiMEM (Invitrogen) and incubated for 48 hours. For fluorescence microscopy, transfection of individual cells was confirmed by fluorescence from co-transfection with GFP.

*Fluorescence Microscopy*

Fibroblasts were seeded on 22x22 mm coverslips and allowed to attach overnight. Cells were stained with 100 nM MitoTracker Orange (Molecular Probes, Thermofisher) in FBS-free DMEM for 15 minutes at 37°C, washed in DMEM, and fixed in 4% paraformaldehyde (w/v) in PBS for 30 minutes at room temperature. Cells were washed in PBS and mounted in 1,4-diazabicyclo[2.2.2]octane/polyvinyl alcohol (DABCP/PVA) medium. Images were acquired using a DeltaVision fluorescent microscope (GE Healthcare) with a 60x objective; with all images presented as deconvoluted maximum projections of 0.2 µm optical sections. Maximum projections and deconvolution was performed using softWoRx software (GE Healthcare).

*Long range PCR and mitochondrial DNA copy number quantitative PCR*

Fibroblast cell DNA was extracted using a GeneJET Genomic DNA purification kit according to the manufacturer’s instructions (Fermentas). The mitochondrial genome was amplified using primers against the human mitochondrial genome (GeneWorks) from 100 ng of DNA using a TaKaRa LA PCR Kit version 2.1 (Cat. No. RR013A) according to the manufacturer’s instructions. DNA was separated on a 1% agarose gel and visualised using a Gel Doc XR+ Gel Documentation System (Bio-Rad). Real time PCR was conducted on 100 ng of DNA using primers for cytochrome b and β-globin to determine mitochondrial and nuclear DNA levels respectively (GeneWorks). Amplification was conducted using a Rotor-Gene Q (Qiagen) using SensiMix SYBR mix (Bioline). All primer sequences are listed in Supplementary Table 1.

*Immunoblotting*

Specific proteins were detected using mouse antibodies against: SDHA (ab14715), total OXPHOS rodent WB antibody cocktail (ab110413), COXIII (ab110259) (Abcam; diluted 1:1000), and APOO (OAEC04484) (Sapphire Biosciences; diluted 1:1000); and rabbit antibodies against: CHCHD2 (19424-1-AP; diluted 1:1000), TOP1MT (16540-1-AP), COXII (55070-1-AP; diluted 1:500) (Proteintech Group), CHCHD3 (ARP57040) and APOOL (OAAF03292) (Sapphire Biosciences; diluted 1:1000) in 20% odyssey blocking buffer (Li-COR Biosciences) in tris-buffered saline and Tween 20 (TBST). IRDye 680LT goat anti-mouse IgG and IRDye 800CW goat anti-rabbit secondary antibodies (Li-COR Biosciences, diluted 1:10000) were used to detected primary antibodies. Blots were imaged using an Odyssey infrared imaging system (Li-COR Biosciences).

*Respiration*

Complex I, II+III, and IV respiration was measured using: 10 mM glutamate/2 mM malate, 10 mM succinate/0.5 μM rotenone, and 2.1 μM ascorbate/0.75 μM N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) (Sigma) respectively. For respiratory control ratio (RCR) measurements, cells were supplemented with 10 mM succinate/0.5 μM rotenone (Sigma) and stage 3 respiration was measured after addition of 1 mM ADP (Sigma). Stage 4 respiration was measured after addition of 2.5 μM oligomycin (Sigma).

*JC-1 assay*

Fibroblasts were seeded in black 96-well plates and allowed to attach overnight. Cells were stained with 33 µM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes, Thermofisher) in FBS-free DMEM and incubated for 60 minutes at 37°C. Cells were incubated in 5% fatty acid free BSA (w/v) (Sigma) in PBS for five minutes at 37°C, which was replaced with PBS. Control treatments were carried out with 50 µM FCCP added 10 minutes prior to JC-1 staining. Fluorescence readings were taken using a CLARIOstar (BMG Labtech). Data is presented as a ratio of 590 nm:520 nm values.

*Mitochondrial mass measurement*

Fibroblasts cells were seeded in black 96-well plates and allowed to attach overnight. Cells were stained with 5 μM nonyl acridine orange (NAO) (Molecular Probes, Thermofisher) in FBS-free DMEM and incubated for 30 minutes at 37˚C. Cells were washed in PBS and fluorescence readings were made in PBS at 530 nm using a CLARIOstar.

*Dihydroethidium measurements*

Fibroblasts were seeded in black 96-well plates and allowed to attach overnight. Cells were stained with 10 µM dihydroethidium (DHE) (Molecular Probes, Thermofisher) in FBS-free DMEM and incubated for 45 minutes at 37˚C. Cells were washed in PBS and 0.1% Triton X-100 (v/v) in PBS was added to cells. Fluorescence readings were taken at 590 nm using a CLARIOstar.

*MTS assay*

Fibroblasts were seeded in 96-well plates and allowed to attach overnight. 20 µl of CellTiter 96 AQueous One Solution Reagent (Promega), containing [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate (PES), was added to cells and incubated in normal growth conditions for four hours. Absorbance was measured at 490 nm using a Powerwave XS2 spectrophotometer (Millennium Science).

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