Objective: Defective mitochondrial function attributed to optic atrophy 1 (OPA1) mutations causes primarily optic atrophy and, less commonly, neurodegenerative syndromes. The pathomechanism by which OPA1 mutations trigger diffuse loss of neurons in some, but not all, patients is unknown. Here, we used a tractable induced pluripotent stem cell (iPSC)-based model to capture the biology of OPA1 haploinsufficiency in cases presenting with classic eye disease versus syndromic parkinsonism.

Methods: iPSCs were generated from 2 patients with OPA1 haploinsufficiency and 2 controls and differentiated into dopaminergic neurons. Metabolic profile was determined by extracellular flux analysis, respiratory complex levels using immunoblotting, and complex I activity by a colorimetric assay. Mitochondria were examined by transmission electron microscopy. Mitochondrial DNA copy number and deletions were assayed using long-range PCR. Mitochondrial membrane potential was measured by tetramethylrhodamine methyl ester uptake, and mitochondrial fragmentation was assessed by confocal microscopy. Exome sequencing was used to screen for pathogenic variants.

Results: OPA1 haploinsufficient iPSCs differentiated into dopaminergic neurons and exhibited marked reduction in OPA1 protein levels. Loss of OPA1 caused a late defect in oxidative phosphorylation, reduced complex I levels, and activity without a significant change in the ultrastructure of mitochondria. Loss of neurons in culture recapitulated dopaminergic degeneration in syndromic disease and correlated with mitochondrial fragmentation.

Interpretation: OPA1 levels maintain oxidative phosphorylation in iPSC-derived neurons, at least in part, by regulating the stability of complex I. Severity of OPA1 disease associates primarily with the extent of OPA1-mediated fusion, suggesting that activation of this mechanism or identification of its genetic modifiers may have therapeutic or prognostic value.
core functions of OPA1 appear to be critical for adaptation to bioenergetic demands, the control of apoptosis by limiting cytochrome c mobilization from the cristae interior, and, possibly, mitochondrial DNA (mtDNA) stability. Despite these essential functions in model systems, OPA1 mutations in humans cause primarily a selective degeneration of retinal ganglion cells and optic atrophy, but 20% of patients develop a diffuse neurological syndrome. Most human OPA1 mutations are nonsense and frameshift mutations that encode truncated mRNA decay and could therefore produce haploinsufficiency. The extent of mutant OPA1 transcript depletion in patients is variable, ranging from no change to an apparent ~60% loss.

Recently, missense mutations in OPA1 causing decreased protein expression were associated with parkinsonism and cognitive decline, suggesting that under certain conditions, loss of OPA1 function may compromise dopaminergic cell viability. The mechanism by which loss of OPA1 leads to cellular dysfunction in human neurons and whether any of these are relevant to disease severity is unknown. Here, we investigated how loss of OPA1 attributed to haploinsufficiency impacts on induced pluripotent stem cell (iPSC)-derived dopaminergic neurons from patients with syndromic parkinsonism and pure optic atrophy and asked in this tractable model whether specific functions of OPA1 may associate with syndromic disease.

Materials and Methods

Reprogramming Donor Fibroblasts to iPSCs
Participants were recruited to this study having given signed informed consent, which included mutation screening and derivation of human iPSC lines from skin biopsies (Ethics Committee: National Health Service, Health Research Authority, NRES Committee South Central, Berkshire, UK, who specifically approved this part of the study [REC 10/H0505/71]). The control iPSC lines, AH017-7 and OX3-9, were previously published using the SeVdp(KOSM)302L Sendai virus system. iPSC lines tested negative for mycoplasma using MycoAlert (Lonza, Basel, Switzerland).

Assessment of Genome Integrity and Tracking
Genome integrity was assessed by Illumina Human CytoSNP-12v2.1 beadchip array (300,000 markers), analyzed using KaryoStudio and GenomeStudio software (Illumina, San Diego, CA). Single-nucleotide polymorphism (SNP) profiles in the iPSC lines were compared to the corresponding fibroblasts to confirm their origin and identity.

PluriTest
RNA was extracted from iPSCs using an RNasea kit (Qiagen, Hilden, Germany) for Illumina HT12v4 transcriptome array analysis. Image data files were uploaded to www.pluritest.org and scored for pluripotency as previously described.

Flow Cytometry
iPSC lines were assessed for expression of pluripotency markers by flow cytometry. Cells were lifted with TrypLE (Life Technologies, Carlsbad, CA), fixed with 4% paraformaldehyde (PFA), permeabilized in methanol at -20°C, washed and stained in flow cytometry buffer with antibody, measured using fluorescent-activated cell sorting (FACS) Calibur (Becton Dickinson, Franklin Lakes, NJ), and analyzed using FlowJo software (TreeStar, Inc., Ashland, OR). Antibodies used were TRA-1-60 (B119983, IgM-488; BioLegend, San Diego, CA) and NANOG (D73G4, IgG-647; Cell Signaling Technology, Beverly, MA).

Exome Sequencing and Analysis
DNA was extracted from freshly collected blood samples and sequenced on Illumina HiSeq 2000. Reads (100 base pair [bp], paired-end) were mapped to the reference genome (HG19) using BWA, and variants were called using the Genome Analyzer Toolkit (GATK) best practice guidelines. Variants were annotated using SnpEFF and filtered using SnpSift and GATK. We searched for genes that showed nonsynonymous coding variants, with a minor allele frequency (MAF) <1% in public databases (<1% in the 1000 Genomes Project [1KG] and in the Exome Aggregation Consortium [ExAC] that were present only in the index case, but not his mother. We further filtered the candidate genes on the basis of the predicted impact of each variant by combined annotation-dependent depletion score (>10) and the number of damaging mutations found in healthy individuals for each gene by gene-damaging index. Candidate modifiers were annotated with their expression level in brain using the Gtex database and their haploinsufficiency score (http://www.nature.com/articles/ncb3192). Genes with known mitochondrial function were annotated using the MitoCarta database.

Accession Numbers
SNP data sets and Illumina HT12v4 expression array data sets for the iPSC lines have been deposited in GEO, under Accession number GSE94433.

Generation of iPSC-Derived Midbrain Dopaminergic Neurons
Dopaminergic neurons were derived using a previously published protocol with some modifications. iPSCs were seeded onto Gelrex coated six-well plates, expanded until >80% confluence, and mTeSR1 media was changed to day 0 (D0) media (2 μM of A83-01, 100nM of LDN in neural induction base medium). D1-4 media contained 2 μM of A83-01, 100nM of LDN, 300ng/ml of SHH C25II, 2 μM of purmorphamine, and 200ng/ml of FGF8a in neural induction base medium. CHIR-99021 (3 μM) was added from day 3 until day 12. D5-6 media
immunofluorescence staining

iPSC-derived neurons were fixed in 4% PFA, permeabilized in 0.3% Triton-X100 in 2% bovine serum albumin, 3% goat serum containing phosphate-buffered saline, and incubated with primary antibodies and fluorescently labeled secondary antibodies. The following primary antibodies were used: anti-Tom20 (FL145, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), anti-TH (mouse monoclonal; Millipore, Billerica, MA), anti-βIII tubulin/TUJ1 (mouse monoclonal, BioLegend; rabbit polyclonal from Abcam [Cambridge, MA]), anti-FOXa2 (A12 clone, Santa Cruz Biotech). Images were obtained using UltraVIEW VoX Spinning Disk Confocal Microscope (PerkinElmer, Waltham, MA) and analyzed using CellProfiler. For dopaminergic markers, 30 images (63× magnification) were obtained per clone per differentiation. Between 15 and 30 images per clone per differentiation were scored using ImageJ (NIH, Bethesda, MD) software. Between 15 and 30 images per clone per differentiation were scored using ImageJ (NIH, Bethesda, MD) software.

Electrophysiology

Voltage-clamp recordings were obtained from iPSC-derived neurons using an Axopatch 200B amplifier (Molecular Devices, San Jose, CA). For the current clamp recordings 130mM of KCl, 1mM of MgCl2, 5mM of MgATP, 10mM of HEPES, and 0.5mM of EGTA (pH 7.3) solution was used. Recordings were obtained at room temperature, with a sampling rate of 5KHz, using the pClamp 10 acquisition software (Molecular Devices). Data were analyzed using MatLab R2015A (The MathWorks, Inc., Natick, MA).

mtDNA Copy Number and Deletion Levels Determination and Long-Range PCR

mtDNA was amplified in two fragments of approximately 9.9 kilobases (kb) and 15.4 kb in length using Takara PrimeSTAR GXL DNA polymerase (Takara Bio Inc., Kusatsu, Japan). Primer sequences were as follows: 1F: CCCTCTCTCCTACTCTCTG (bp 6,222–6,239), 1R: CAGGTGTCAG ATATTATGG (bp 16,133–16,153) and 2F: TTTAAATCAGGACCTGGC (bp 1,157–1,177), 2R AGGGTATAGACCTGTGAC (bp 19–1). qPCR was used to amplify mitochondrial genes MTND1 and MTND4 and the nuclear gene, B2M. Control samples with or without mtDNA deletions were included in all assays. All samples were assayed in triplicates per qPCR run. mtDNA copy number (CN) was calculated based on the relative abundance of MTND1 gene compared to the nuclear gene, B2M. mtDNA deletion levels were calculated using the comparative threshold cycle (Ct) method of MTND4 and MTND1 genes.

Statistical Analysis

The statistical analysis was performed using Prism (GraphPad Software Inc., La Jolla, CA). Data were analyzed using one-way analysis of variance (ANOVA). Biological replicates (n) are defined as differentiations performed at least one cell-split apart, which is generally 1 week. The number of replicates included two clones per subject each differentiated three times.

Results

Clinical and Genetic Information

The proband is a 62-year-old male who presented at the age of 46 with 1-year history of micrographia and
unilateral tremor on a background of dominantly inherited optic atrophy since his mid-thirties. On examination at presentation in 2001, he had a mild resting tremor, moderate rigidity, and bradykinesia on the right upper limb without any corticospinal, cerebellar features or autonomic involvement. He had a positive DaTScan showing asymmetric left-sided dopaminergic loss (Fig 1). He did not respond to dopamine agonists or levodopa therapy and progressed significantly within 6 months with inability to write and unsteadiness attributed to a rigid right leg, requiring support with a stick. His examination revealed progression of the extrapyramidal signs with bilateral asymmetric involvement of both upper and lower limbs, worse on the right. Within 2 years of his initial diagnosis, he developed frequent falls, walked with a broad-based festinant gait, and had impaired postural reflexes. There was no objective improvement with subcutaneous apomorphine. He became wheelchair bound within 6 years from the initial presentation. He stopped working at the age of 48. His estimated premorbid IQ was 110 and his cognitive profile, recognition memory, and non-verbal memory as documented at 10 years postdiagnosis were in line with estimates of his premorbid ability. He developed some swallowing difficulties, occasional visual hallucinations, and suffered from low mood when assessed 14 years postdiagnosis. His brain magnetic resonance imaging (MRI) at presentation was normal. He had normal motor and sensory nerve conduction on neurophysiology. His mother suffered with progressive visual loss since the second decade of her life with documented optic atrophy and passed away at the age of 87. When assessed at the age of 84, she did not have any other neurological deficits and was very independent in her daily activities living alone despite her visual problems. During the last 3 years of her life, her condition deteriorated with balance problems and memory decline. She was using the wheelchair only during the last 12 months of her life. Genetic testing in both cases revealed a novel insertion in exon 2 of 3 in-frame codons (c.33-34ins9), the second of which is a stop codon predicted to result in OPA1 haploinsufficiency (Fig 1A). Common POLG and mtDNA mutations were negative in blood, and no mutations were identified in genes implicated in familial parkinsonism. The second OPA1 allele was sequenced in both cases and was normal without any of the intronic OPA1 mutations that have been reported to act as modifiers in some cases with syndromic OPA1 disease.10 To identify candidate modifier genes, we performed whole-exome sequencing searching for genes that showed nonsynonymous coding variants, with a MAF that were present only in the index case but not his mother. We identified a total of 54 variants in 54 genes that met these criteria. Nine of the genes identified (ATG4C, CENPJ, ERCC3, SH3GLB2, TSC1, HNRNPU, PIN1, NT5M, and HSPBP1) are expressed in neuronal tissues, but none of the variants identified are known to be pathogenic.
Generation and Characterization of Human iPSC Lines

We generated two human iPSC clonal lines from fibroblasts obtained from each of the 2 patients with OPA1 haploinsufficiency and compared them to iPSC from 2 healthy controls (Table). Detailed characterization of the OPA1 lines and the newly derived healthy lines used in this study is shown in Figure 2. Genome integrity and
FIGURE 3: iPSC-derived dopaminergic neurons from syndromic OPA1 parkinsonism recapitulate disease severity. (A) Immunostaining for TH, FOXA2, and TUJ1 was readily detected across controls and patient lines, scale bar 30 μm. (B) Quantification at D45 revealed equal percentage of FOXA2-, TUJ1-, and TH-positive cells across lines. (C) Representative example of iPSC derived dopaminergic neuron at DIV65, showing repetitive spiking in response to a 400 ms depolarizing pulse (D) Quantification of immunoblotted OPA1 protein revealed marked reduction in both patient lines at all time-points tested. (E) Accelerated cell death was detected in clones of iPSC-derived dopaminergic neurons from the patient with syndromic disease when cultured for D45 and D65, scale bar 150 μm. (F) Quantification of the percentage of cells remaining at D45 and D65 compared to D25. Data are mean ± SEM and biological replicates are defined as individual clone differentiations using two clones per subject each differentiated three times. HC = healthy control. Analysis was done using one-way ANOVA (**p < 0.01; ***p < 0.001; ****p < 0.0001). ANOVA = analysis of variance; iPSC = induced pluripotent stem cell; OPA1 = optic atrophy 1; SEM = standard error of the mean.
tracking to the original patient sample was confirmed by Illumina SNP array (Fig 2A). All iPSC lines displayed embryonic stem cell–like morphology and expressed the pluripotency-associated proteins, TRA-1-60 and Nanog (Fig 2B). Conformity to a pluripotent expression profile was shown by PluriTest (Fig 2C).

**Progressive Neuronal Loss in syndromic OPA1 Disease Is Recapitulated in iPSC-Derived Cultures**

To study the effect of the OPA1 haploinsufficiency in the context of parkinsonism, two iPSC clones from each of the 2 controls and the 2 patients were each differentiated three times into dopaminergic neuronal cultures. Results from lines derived from the patient with syndromic OPA1 parkinsonism are denoted as Opa1P, whereas lines derived from the patient with optic atrophy are represented as Opa1. We confirmed by immunofluorescence that in all lines, dopaminergic neurons coexpressed the floor plate marker, FOXA2, with TH or β-3 tubulin (TUJ1) with TH (Fig 3A). Differentiation efficiency, as assessed by the immunofluorescence for FOXA2, β-3 tubulin, and TH, was similar across the genotypes used with approximately

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**FIGURE 4:** OPA1 haploinsufficiency causes progressive OXPHOS defect in iPSC-derived neurons. (A) Extracellular flux analysis at D25 and D65 revealed a progressive decline in OCR, maximal respiration and ATP production in both patient lines. (B) Immunoblotting for mitochondrial proteins revealed a progressive decline in complex I in OPA1 haploinsufficient lines, which was quantified at D65 as shown in panel (C). (D) Complex I activity was reduced in OPA1 haploinsufficient lines compared to controls at D65. (E) Morphometric analysis of the ratio of surface area of cristae to corresponding mitochondrial segment or mitochondrial width at D65. Each dot represents the average of at least 15 randomly selected images from each clone. Quantification in all panels is based on two clones per subject each differentiated three times. Data are mean ± SEM and analysed using One-way ANOVA (*p < 0.05; **p < 0.01; ****p < 0.0001). ANOVA = analysis of variance; HC = healthy control; iPSC = induced pluripotent stem cell; OCR = oxygen consumption rate; OPA1 = optic atrophy 1; SEM = standard error of the mean.
80% of cells expressing β-3 tubulin and 70% expressing TH (Fig 3B). At day in vitro (DIV) 65, current-clamp recordings showed that neurons were able to fire repetitive action potentials in response to current injection (Fig 3C). Both patient lines exhibited markedly reduced OPA1 protein levels when compared to controls (Fig 3D). We found accelerated neuronal death in the Opa1P patient when compared to Opa1 or control lines at DIV 45 and 65 (Fig 3E,F), recapitulating the degeneration of neurons detected by DaTScan in the patient. The absence of a secondarily pathogenic mutation on exome sequencing of patient DNA to explain this phenotypic difference between Opa1P and Opa1 lines suggests that syndromic OPA1 parkinsonism may arise from a modifier effect of the primary mitochondrial defect. We therefore asked how functions that were previously ascribed to OPA1 are manifested in Opa1 and Opa1P lines.

**OPA1 Haploinsufficiency Leads to Defective Oxidative Phosphorylation and Reduced Complex I Function in iPSC-Derived Neurons**

To assess whether OPA1 haploinsufficiency impairs bioenergetic demand, we performed extracellular flux analysis of OCR. This revealed that loss of OPA1 in iPSC-derived neurons led to lower basal and maximal OCR compared to controls at a late (DIV65), but not early (DIV25), time point, mechanistically suggesting a progressive defect in electron transport in both Opa1P and Opa1 lines (Fig 4A). Bioenergetic failure was associated with loss of complex I as assessed by NDUFV2 levels relative to actin (Fig 4B,C) and a defect in complex I activity (Fig 4D) in both patients compared to controls. Notably, mitochondrial mass based on Tom20 levels relative to actin and complex V based on ATP5A levels relative to actin were unchanged across time points and lines (Fig 4B). Despite this clear functional deficit, we did not detect overt changes in the morphology of the
mitochondria or cristae when assessed by TEM at either early or late time points (DIV25/45/85, not shown) and illustrated here at DIV65 (Fig 4E). mtDNA analysis did not reveal any deletions in the patient-derived neurons (Fig 5) as previously reported in skeletal muscle.5,6 Thus, reduced OPA1 protein levels in iPSC-derived neurons cause a delayed defect in oxidative phosphorylation, which is most likely at the level of complex I.

Mitochondrial Fragmentation in iPSC-Derived Neurons Correlates With Clinical Severity in Patients

To investigate the relevance of OPA1-mediated mitochondrial fusion in our model, we assessed the mitochondrial network by quantifying the percentage of neurons with fragmented or filamentous Tom20-positive mitochondria. This analysis showed that by DIV65, fragmentation was increased in the Opa1P lines compared to either Opa1 or controls (Fig 6A,B). Accordingly, the abundance of the cleaved (short) S-OPA1 isoform, which typically accumulates in cells with fragmented mitochondria, was increased in Opa1P neurons (Fig 6C). When assessed as individual networks using the ratio of mitotracker Red to Tom20, the fragmented mitochondrial segments in Opa1P neurons exhibited reduced uptake and thus impaired function at DIV65 (Fig 6D,E).

Discussion

Despite the multifactorial role of OPA1 in mitochondrial health, loss of its function commonly causes autosomal-dominant optic atrophy and, only under certain conditions, leads to a systemic mitochondrial disease. What is its critical function in human neurons and how this is linked to neuronal vulnerability to cell death is currently unclear. Using an iPSC-based model, we showed that loss of OPA1 causes a late-onset deficit in oxidative phosphorylation and a differential loss of mitochondrial fusion, which associates with disease severity. Unlike earlier OPA1 knockdown studies in cell lines,3,17 we did not observe marked disorganization of the morphology of the

FIGURE 6: Mitochondrial fragmentation in iPSC-derived neurons associates with severity of clinical phenotype in patients. (A) Progressive fragmentation of Tom20-positive mitochondria was detected by D65 in Opa1P lines. (B) Quantification of the percentage of neurons with fragmented mitochondria networks. (C) The abundance of cleaved (short) S-OPA1 isoform was increased in Opa1P neurons. (D–E) Mitochondria in Opa1P exhibited reduced uptake of mitotracker. Quantification in all panels is based on two clones per subject each differentiated three times. Data are mean ± SEM and analysed using one-way ANOVA (*p < 0.05; **p < 0.01; ****p < 0.0001). Scale bar, 20 μm. ANOVA = analysis of variance; HC = healthy control; iPSC = induced pluripotent stem cell; Opa1 = optic atrophy 1; SEM = standard error of the mean.
mitochondria or their cristae in patient-derived neurons. This discrepancy is most likely attributed to the fact that previous studies in cell lines have examined the consequences of acute and/or complete depletion of OPA1 within 24 to 72 hours \(^3,17\) instead of pathophysiological relevance of OPA1 levels as in our patient-derived neuronal model, where the defect developed over 45 to 65 days. This scenario is in agreement with the slowly progressive focal neurological phenotype in most patients with OPA1 haploinsufficiency.\(^7\) It is possible that under conditions of neuronal stress, the ultrastructural morphology of OPA1 haploinsufficient mitochondria is disorganized before cell death and our TEM studies have not captured such events. Instead, our data suggest that a late defect in complex I may account for the reduction of electron transport across the respiratory chain as evidenced by the decreased maximal respiration in mutant neurons and the reduction of complex I levels and activity. Functional changes in complex I were previously reported in fibroblasts with OPA1 haploinsufficiency\(^18\) or the p.G488R mutation\(^9\) and in models of acute OPA1 depletion, where respiratory efficiency was impaired when mitochondria were energized specifically with the complex I substrates, glutamate/malate.\(^17\) Therefore, in human iPSC-derived neurons, OPA1 levels are important for the maintenance of oxidative phosphorylation, at least partly, by regulating the stability of complex I.

In the context of syndromic disease, impaired regulation of oxidative phosphorylation and complex I deficiency was not sufficient to explain the differential viability of dopaminergic neurons between Opa1P and Opa1 lines. Interestingly, clinicopathological studies in patients with POLG mutations also found that complex I deficiency did not correlate with parkinsonian features.\(^19\) Given that fragmentation has been associated with apoptosis,\(^20\) the cell death in culture could be, at least partly, determined by the exacerbated mitochondrial fragmentation in Opa1P lines, which also exhibited increased proteolytic processing of L-OPA1 to the short isoform. Proteolytic cleavage of L-OPA1 is regulated by either YME1L1, which is under metabolic control\(^21\) or OMA1, which is activated upon various stress insults, resulting in the complete degradation of L-OPA1 and mitochondrial fragmentation.\(^22,23\) Interestingly, in a mouse model of neurodegeneration, L-OPA1 promoted neuronal survival without affecting cristae shape, whereas stress-induced OMA1 activation and L-OPA1 cleavage increased mitochondrial fragmentation and promoted neuronal death.\(^24\)

Further investigation is required to determine which cellular stressors or adaptive mechanisms promote or oppose OPA1-mediated mitochondrial fragmentation in syndromic OPA1 disease and potentially other common diseases. Our initial whole-exome analysis did not identify a secondary mutation of known pathogenicity, but does not exclude the contribution of multiple minor genetic determinants such as effects on autophagy, which is activated in cells carrying a mutant \(OPA1\) allele\(^9,25\) or microtubule-associated transport, which is important for axonal mitochondrial motility. This withstanding, our finding that key cellular phenotypes are reproduced in vitro suggests, for the first time, that syndromic disease may arise from genetic modifiers that exacerbate the mitochondrial defect, which, at least in dopaminergic neurons, manifested as OPA1-mediated mitochondrial fragmentation. It is possible that different mechanisms operate in other subtypes of syndromic OPA1-mediated disease. Nevertheless, understanding how L-OPA1 processing varies in syndromic OPA1 and common diseases of dopaminergic cell loss such as Parkinson’s and promoting L-OPA1 stability (eg, by OMA1 inhibition) could pave the way for novel targeted therapies against mitochondrial dysfunction in neurodegeneration. More broadly, our study demonstrates how deep-phenotyping of a rare disease using iPSCs can help contextualize key functions of essential mitochondrial proteins in the study of disease severity, which is an important step toward personalized neurology, and suggests that mapping of genetic modifiers could have prognostic significance.
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Author Contributions
G.K.T. contributed to the conception and design of the study and drafting of the manuscript. M.J., M.M., A.M., T.Z., S.W., C.E.Z., M.K.-A., C.B. D.S., P.F.C, S.C., and G.K.T contributed to data acquisition and analysis and drafting of figures.

Potential Conflicts of Interest
Nothing to report.

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