RESEARCH ARTICLE

# Parkin Deficiency Impairs Mitochondrial DNA Dynamics and Propagates Inflammation

Kobi Wasner, PhD,[1] Semra Smajic, MSc,[1] Jenny Ghelfi, BSc,[1] Sylvie Delcambre, PhD,[1] Cesar A. Prada-Medina, PhD,[2] Evelyn Knappe, MSc,[3] Giuseppe Arena, PhD,[1] Patrycja Mulica, MSc,[1] Gideon Agyeah, MSc,[1] Aleksandar Rakovic, PhD,[3] Ibrahim Boussaad, PhD,[1,4] Katja Badanjak, MSc,[1] Jochen Ohnmacht, PhD,[1,5] Jean-Jacques Gérardy, BSc,[6] Masashi Takanashi, MD,[7] Joanne Trinh, PhD,[3] Michel Mittelbronn, MD,[1,6,8,9] Nobutaka Hattori, MD, PhD,[7] Christine Klein, MD,[3] Paul Antony, PhD,[1,4] Philip Seibler, PhD,[3] Malte Spielmann, MD,[2,10] Sandro L. Pereira, PhD,[1,7] and Anne Grünewald, PhD[1,3*]

[1]*Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Esch-sur-Alzette*
[2]*Max Planck Institute for Molecular Genetics, Berlin, Germany*
[3]*Institute of Neurogenetics, University of Lübeck, Lübeck, Germany*
[4]*Disease Modeling and Screening Platform, Luxembourg Centre of Systems Biomedicine, University of Luxembourg & Luxembourg Institute of Health, Luxembourg*
[5]*Department of Life Science and Medicine, University of Luxembourg, Esch-sur-Alzette, Luxembourg*
[6]*National Center of Pathology, Laboratoire National de Santé, Dudelange, Luxembourg*
[7]*Department of Neurology, Juntendo University, Tokyo, Japan*
[8]*Luxembourg Center of Neuropathology, Dudelange, Luxembourg*
[9]*Department of Cancer Research, Luxembourg Institute of Health, Luxembourg, Luxembourg*
[10]*Institute of Human Genetics, University of Lübeck, Lübeck, Germany*

**ABSTRACT: Background:** Mutations in the E3 ubiquitin ligase parkin cause autosomal recessive Parkinson's disease (PD). Together with PTEN-induced kinase 1 (*PINK1*), parkin regulates the clearance of dysfunctional mitochondria. New mitochondria are generated through an interplay of nuclear- and mitochondrial-encoded proteins, and recent studies suggest that parkin influences this process at both levels. In addition, parkin was shown to prevent mitochondrial membrane permeability, impeding mitochondrial DNA (mtDNA) escape and subsequent neuroinflammation. However, parkin's regulatory roles independent of mitophagy are not well described in patient-derived neurons.

**Objectives:** We sought to investigate parkin's role in preventing neuronal mtDNA dyshomeostasis, release, and glial activation at the endogenous level.

**Methods:** We generated induced pluripotent stem cell (iPSC)–derived midbrain neurons from PD patients with parkin (*PRKN*) mutations and healthy controls. Live-cell imaging, proteomic, mtDNA integrity, and gene expression analyses were employed to investigate mitochondrial biogenesis and genome maintenance. To assess neuroinflammation, we performed single-nuclei RNA sequencing in postmortem tissue and quantified interleukin expression in mtDNA/lipopolysaccharides (LPS)-treated iPSC-derived neuron–microglia co-cultures.

**Results:** Neurons from patients with *PRKN* mutations revealed deficits in the mitochondrial biogenesis pathway, resulting in mtDNA dyshomeostasis. Moreover, the energy sensor sirtuin 1, which controls mitochondrial biogenesis and clearance, was downregulated in parkin-deficient cells. Linking mtDNA disintegration to neuroinflammation, in postmortem midbrain with *PRKN* mutations, we confirmed mtDNA dyshomeostasis and detected an upregulation of microglia overexpressing proinflammatory cytokines. Finally, parkin-deficient neuron–microglia co-cultures elicited an enhanced immune response when exposed to mtDNA/LPS.

**Conclusions:** Our findings suggest that parkin coregulates mitophagy, mitochondrial biogenesis, and mtDNA maintenance pathways, thereby protecting midbrain neurons from neuroinflammation and degeneration.


**Key Words:** Parkinson's disease; mitochondrial DNA; induced pluripotent stem cells; parkin; neuroinflammation



***Correspondence to:** Dr. Anne Grünewald, Luxembourg Centre for Systems Biomedicine, University of Luxembourg, 6 Avenue du Swing, L-4367 Belvaux. E-mail: anne.gruenewald@uni.lu

Semra Smajic and Jenny Ghelfi contributed equally.

**Relevant conflicts of interest/financial disclosures:** The authors report no conflicts of interest.

**Received:** 22 December 2021; **Revised:** 7 March 2022; **Accepted:** 27 March 2022

**Published online in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/mds.29025**

## Introduction

Dopaminergic neurons (DANs) of the substantia nigra pars compacta (SNpc) in the midbrain are critically involved in the regulation of movement.[1] Loss of DANs results in clinical motor disturbances of patients with Parkinson's disease (PD)—the second most common neurodegenerative disorder.[2] Although the underlying biological mechanisms causing neuronal loss are still under investigation, mitochondrial dysfunction has been well implicated in PD pathology.[3]

The majority of patients with PD are sporadic, with individuals manifesting the disease at ≥65 years of age. The remainder are caused by genetic mutations, of which many are linked to mitochondrial dysfunction. Roughly 50% of patients with early-onset PD harbor mutations in parkin (*PRKN*).[2,4]

*PRKN* encodes the E3 ubiquitin ligase parkin—an established regulator of mitochondrial clearance.[5] However, parkin's substrates are involved in several fundamental cellular processes. For instance, parkin targets parkin-interacting substrate (PARIS)—an inhibitor of the mitochondrial biogenesis regulator peroxisome gamma coactivator 1-alpha (PGC1-α).[6] Moreover, *PRKN* overexpression in cell models revealed an association with the mitochondrial genome and a direct interaction with mitochondral transcription factor A (TFAM)—the main transcription factor of mitochondrial DNA (mtDNA).

mtDNA has gained recent interest as a determinant of aging and age-associated diseases, including PD.[7] Improper mtDNA maintenance has been shown to allow its escape from the mitochondrial compartment, triggering an immune response.[8,9] This phenomenon was furthermore demonstrated in parkin-knockout (KO) "mutator" mice, which harbor an error-prone version of DNA polymerase γ (POLG). These animals show elevated extracellular mtDNA levels and cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) signaling under stress conditions.[10] However, parkin's involvement in these cellular processes has yet to be investigated in patient-derived neurons.

To explore the role of parkin in mtDNA maintenance endogenously, we generated induced pluripotent stem cell (iPSC)–derived midbrain neurons from patients with PD with biallelic *PRKN* mutations. We found that parkin-deficient neurons exhibit impaired mitochondrial biogenesis, mtDNA dynamics, and increased cytosolic mtDNA levels. Parkin knockdown during mutagenic stress mirrored these phenotypes and evidenced upregulations of the cGAS protein and extracellular mtDNA. Moreover, treatment with lipopolysaccharides (LPS) and mtDNA elicited a stronger inflammatory response in *PRKN*-mutant compared with control neuron–microglia co-cultures. Finally, single-nuclei RNA sequencing (snRNAseq) of postmortem midbrain sections from a patient with *PRKN*-PD revealed microgliosis and proinflammatory signaling. Our findings elucidate novel parkin-regulated mitophagy-independent mechanisms contributing toward mitochondrial quality control. We show that parkin coordinates mitochondrial biogenesis and mtDNA maintenance and is essential to prevent neuroinflammation and neurodegeneration.

## Materials and Methods

Generation of iPSCs was performed as described,[11] and DANs and microglia were derived using established protocols.[12-14] To isolate tyrosine hydroxylase (TH)–positive cells, iPSC-derived neurons were subjected to fluorescence-activated cell sorting (FACS) using an adapted protocol.[15] Production of lentiviral vectors expressing short hairpin RNA (shRNA) against human *PRKN* or a control plasmid was performed as described.[16] SH-SY5Y neuroblastoma cells were treated with 200 μM cobalt chloride ($CoCl_2$).[17] Nicotinamide adenine dinucleotide:nicotinamide adenine dinucleotide hydrogen ($NAD^+$:NADH) ratios were determined using a kit (Sigma, St. Louis, MO). Respiratory chain complex I (CI) and citrate synthase activities were assessed in mitochondrial fractions by means of spectrophotometry.[18,19] Isolated extracellular mtDNA was quantified using a Digital PCR System (Applied Biosystems, Waltham, MA) and TaqMan probes specific for mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 1 (*MT-ND1*) and beta-2-microglobulin (*B2M*).[20,21] Polar metabolites from 30 day-old neurons were extracted and then derivatized and measured as published.[22] All experiments using iPSC-derived neurons and SH-SY5Y cells were performed with at least three biological replicates. Unpaired two-tailed Student's *t* tests or one-way analysis of variance followed by post hoc Tukey tests were used to determine statistical significance ($P < 0.05$).

Midbrain sections were immunostained, and TH-positive neurons were isolated through laser capture microdissection (LCM) using the PALM MicroBeam (Zeiss, Oberkochen, Germany).[20] Nuclei isolation, snRNAseq, and data analysis of the *PRKN*-mutant midbrain was carried out as described.[23]

A detailed description of the materials and methods can be found in the supplement.

## Results

### Parkin Deficiency Impairs Mitochondrial Biogenesis in the Neurons of Patients with PD

We generated iPSC-derived midbrain neurons from healthy controls and *PRKN* mutation carriers (Fig. S1A, B), which lack the parkin protein (Fig. S1C). Furthermore, we employed SH-SY5Y wild-type (WT) and

isogenic parkin-KO cells, which were generated using clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR/Cas9) technology (Fig. S1D).

Parkin targets a plethora of proteins,[24] with mounting evidence supporting regulatory roles in diverse cellular mechanisms beyond mitophagy. Given that mitophagy and mitochondrial biogenesis are tightly linked to preserve bioenergetic homeostasis,[25] we sought to investigate possible alterations in mitochondrial biogenesis in our models. Previous research demonstrated that parkin overexpression enhances mitochondrial biogenesis through PGC1-α, either directly or via PARIS, its transcriptional repressor.[6,26,27] We found significantly lower levels of PGC1-α protein in parkin-deficient neurons and SH-SY5Y cells compared with controls (Fig. 1A,B). Interestingly, neither cell model showed differences in the PARIS protein under basal conditions (Fig. 1A,C).

Conversely, parkin deficiency substantially reduced sirtuin 1 (SIRT1) levels (Fig. 1A,D), an $NAD^+$-dependent energy sensor acting on mitochondrial biogenesis through regulation of PGC1-α gene expression and protein deacetylation.[28,29] Moreover, in parkin-deficient neurons, we detected higher lactate:pyruvate ratios (Fig. 1E), which suggests a lack of free $NAD^+$ based on the chemical equilibrium principle.[30]

PGC1-α regulates the transcription of nuclear respiratory factors (NRFs), which in turn mediates gene expressions of mtDNA transcription factors and replication activators TFAM and mitochondrial transcription factor B2 (TFB2M).[31] In agreement with reduced PGC1-α abundance, we found that parkin deficiency resulted in decreased NRF1 protein levels (Fig. 1A,F) and reduced *TFAM* and *TFB2M* expression (Fig. 1G, H). In addition, we detected significantly downregulated mRNA levels of twinkle mtDNA helicase (*TWNK*), a factor mainly involved in mtDNA replication (Fig. 1I).

## Parkin Influences mtDNA Dynamics and Respiratory Chain Function

Mitochondrial biogenesis may be defined as the division and growth of preexisting mitochondria and is accomplished by the import of nuclear-encoded proteins and transcription and replication of the mitochondrial genome, which contains genes encoding subunits

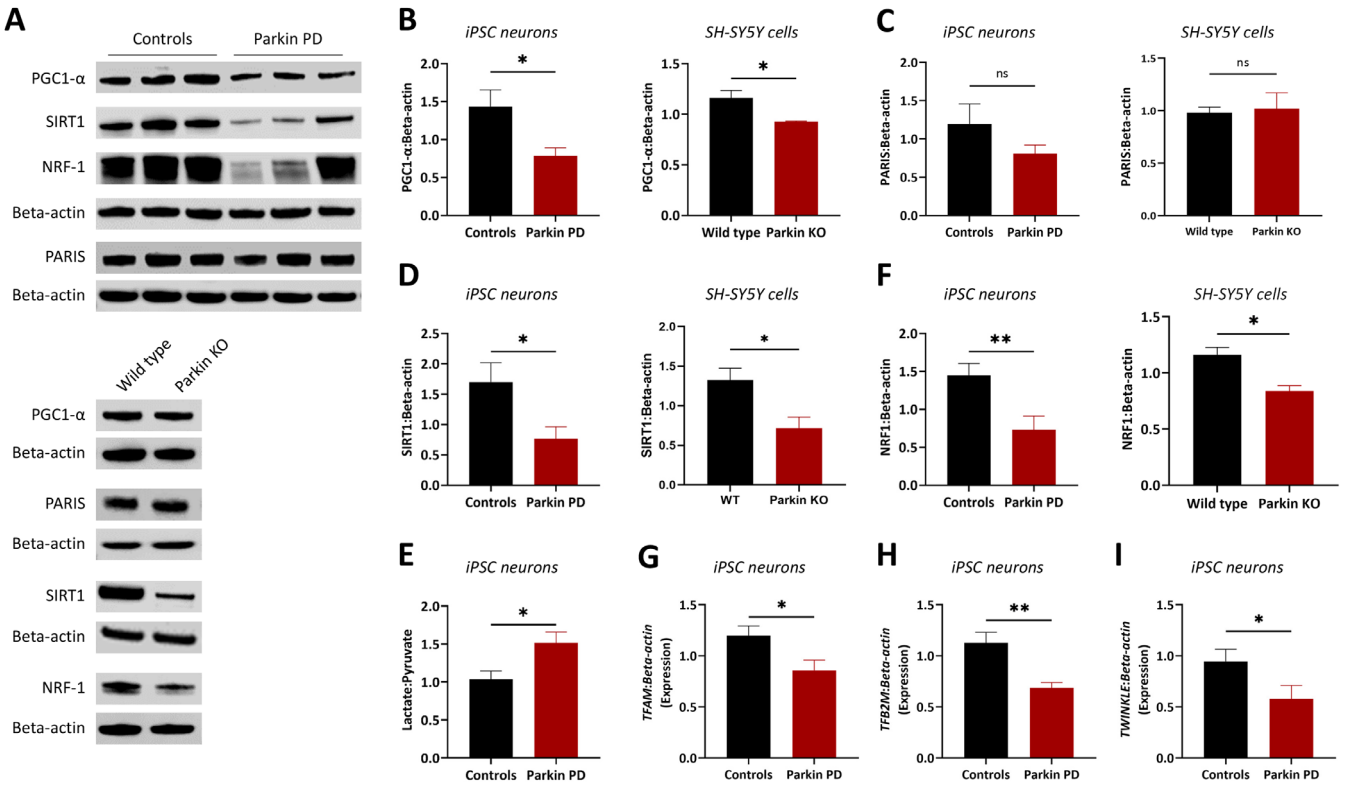


**FIG. 1.** Mitochondrial biogenesis is impaired in parkin-deficient cells. (**A**) Representative cropped Western blot images of total cell lysates from induced pluripotent stem cell (iPSC)–derived neurons from controls and patients with parkin-associated Parkinson's disease (Parkin PD) as well as wild-type and parkin-knockout (KO) SH-SY5Y neuroblastoma cells. (**B**-**E**) Quantifications from (**A**) peroxisome gamma coactivator 1-alpha (PGC1-α) (**B**), parkin-interacting substrate (PARIS) (**C**), sirtuin 1 (SIRT1) (**D**) and nuclear respiratory factor 1 (NRF1) (**F**) protein levels normalized to beta-actin. (**E**) Lactate-to-pyruvate ratios served as a proxy measure for free nicotinamide adenine dinucleotide:nicotinamide adenine dinucleotide hydrogen ($NAD^+$/NADH) ratios. (**G**-**I**) Quantitative polymerase chain reaction (qPCR) was used to quantify gene expression of mitochondrial transcription factor A (*TFAM*) (**G**), mitochondrial transcription factor B2 (*TFB2M*) (**H**) and twinkle mtDNA helicase (*TWNK*) (**I**) in iPSC-derived neurons from controls and patients with parkin-associated PD normalized to beta-actin. Data are presented as the mean ± SEM. SEM = standard error of the mean, $^*P < 0.05$, $^{**}P < 0.01$; ns = not significant as determined by Student's *t* test.

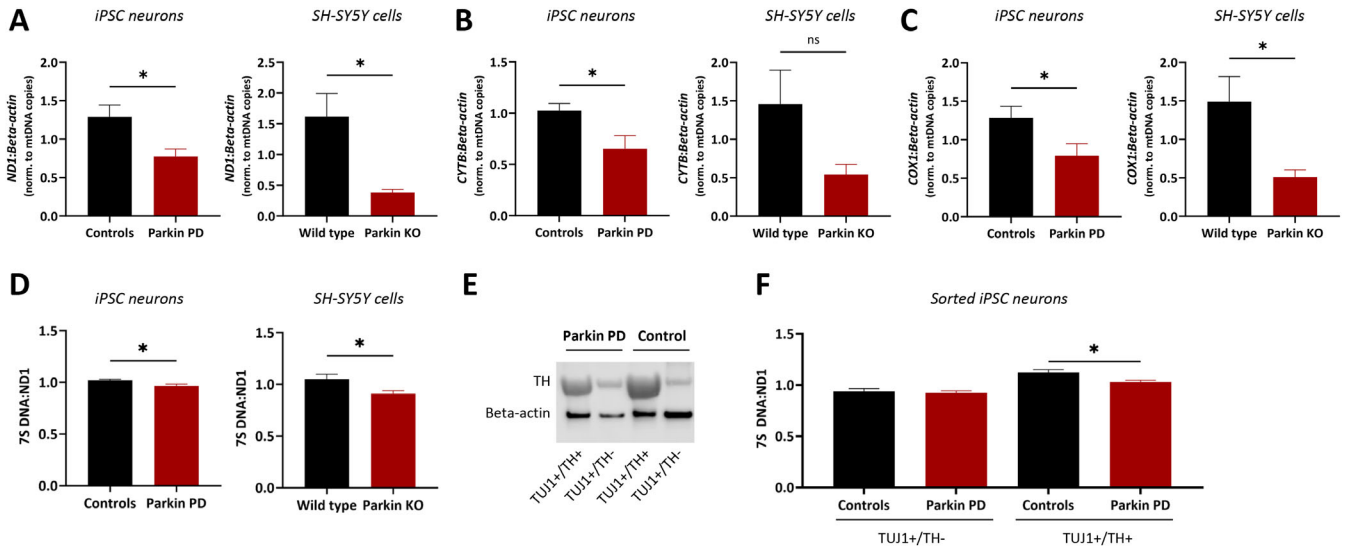


**FIG. 2.** Parkin influences mitochondrial DNA (mtDNA) dynamics. (**A-C**) Quantitative polymerase chain reaction (qPCR) was used to measure expression of mtDNA-encoded genes NADH:ubiquinone oxidoreductase core subunit 1 (*ND1*) (**A**), cytochrome B (*CYTB*) (**B**), and (**C**) cytochrome C oxidase I *(COX1)* in induced pluripotent stem cell (iPSC)–derived neurons from controls and Parkinson's disease patients with mutations in parkin (Parkin PD) and wild-type and parkin-knockout (KO) SH-SY5Y neuroblastoma cells. Gene expression was normalized to beta-actin and mtDNA copy number. (**D**) A multiplex real-time polymerase chain reaction (RT-PCR) assay was used to quantify transcription-associated 7S DNA per mtDNA molecule (with probes targeting *ND1*) in iPSC-derived neurons and SH-SY5Y cells. (**E, F**) Neuronal cultures from three controls and three *PRKN* mutation carriers were sorted using the pan-neuronal marker β-Tubulin 3 (TUJ1) and the dopaminergic neuron (DAN) marker tyrosine hydroxylase (TH) and subjected to Western blotting and RT-PCR analyses. (**E**) Representative cropped Western blotting images of TH and TUJ1 protein abundances in sorted control and patient neurons. (**F**) TUJ1/TH double-positive iPSC-derived neurons were separated from TUJ1-positive/TH-negative neurons derived from controls and patients with *PRKN* mutations. The resulting cell populations underwent multiplex RT-PCR to quantify the abundance of 7S DNA per mtDNA molecule (with a probe targeting *ND1*). RT-PCR results are from two technical replicates. n = 3 biological replicates; data are presented as the mean ± SEM; SEM = standard error of the mean, $^*P < 0.05$, ns = not significant as determined by Student's *t* test.

of the electron transport chain (ETC).[32] Our experiments suggest decreases in nuclear-encoded factors controlling mitochondrial biogenesis. We next sought to assess mtDNA dynamics by measuring the expression of mtDNA-encoded genes. The expression of mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 1 (*MT-ND1*), mitochondrially encoded cythochrome B (*MT-CYTB*), and mitochondrially encoded cytochrome C oxidase I (*MT-COX1*) (encoding subunits of complexes I, III, and IV, respectively) per mtDNA molecule was significantly downregulated in our parkin-deficient models (Fig. 2A–C). In line with this, both parkin-deficient iPSC-derived neurons and SH-SY5Y cells showed diminished 7S DNA:*MT-ND1* ratios, suggestive of fewer transcription initiation events (Fig. 2D).[20] Moreover, we used FACS to isolate TH-positive neurons and found that the 7S DNA phenotype is specific to DANs (Fig. 2E,F).

Because parkin overexpression was reported to enhance the selective removal of mitochondria harboring deleterious mtDNA mutations,[33] we explored the abundance of somatic major arc deletions. By contrast, we did not find differences between groups (data not shown). We further evaluated the mtDNA copy number and detected significantly higher mtDNA levels in parkin-deficient cells using both real-time polymerase chain reaction (RT-PCR) (Fig. 3A) and immunocytochemistry (Fig. 3B). Finally, we assessed respiratory chain function in SH-SY5Y cells and found that parkin-KO cells exhibited significantly reduced CI activity compared with WT cells (Fig. 3C). These results suggest that although parkin deficiency leads to an accumulation of mtDNA molecules, it also hinders the mtDNA transcription process, which likely contributes to ETC dysfunction.

## Parkin Mitigates Cytosolic mtDNA Infiltration

Consistent with its evolutionary bacterial origin, mtDNA has been identified as a damage-associated molecular pattern (DAMP). Cytosolic mtDNA molecules can activate the innate immune system via the cGAS-STING pathway.[8] Implicating mtDNA release in the pathogenesis of *PRKN*-PD, our previous research showed increased levels of circulating cell-free mtDNA (ccf-mtDNA) and inflammatory cytokines in serum from *PRKN* mutation carriers.[34] Several mechanisms have been proposed to facilitate mtDNA release into the cytosol, including TFAM depletion.[8] We next confirmed that reduced *TFAM* gene expression detected in parkin-deficient neurons resulted in diminished TFAM (protein):mtDNA ratios (Fig. 3D, Fig. S2A). TFAM also acts as a packaging factor compacting the mtDNA molecule to form the mitochondrial nucleoid, and disruption of this process is associated with mtDNA extrusion from mitochondria. To investigate if impaired TFAM:mtDNA ratios coupled to disrupted mtDNA

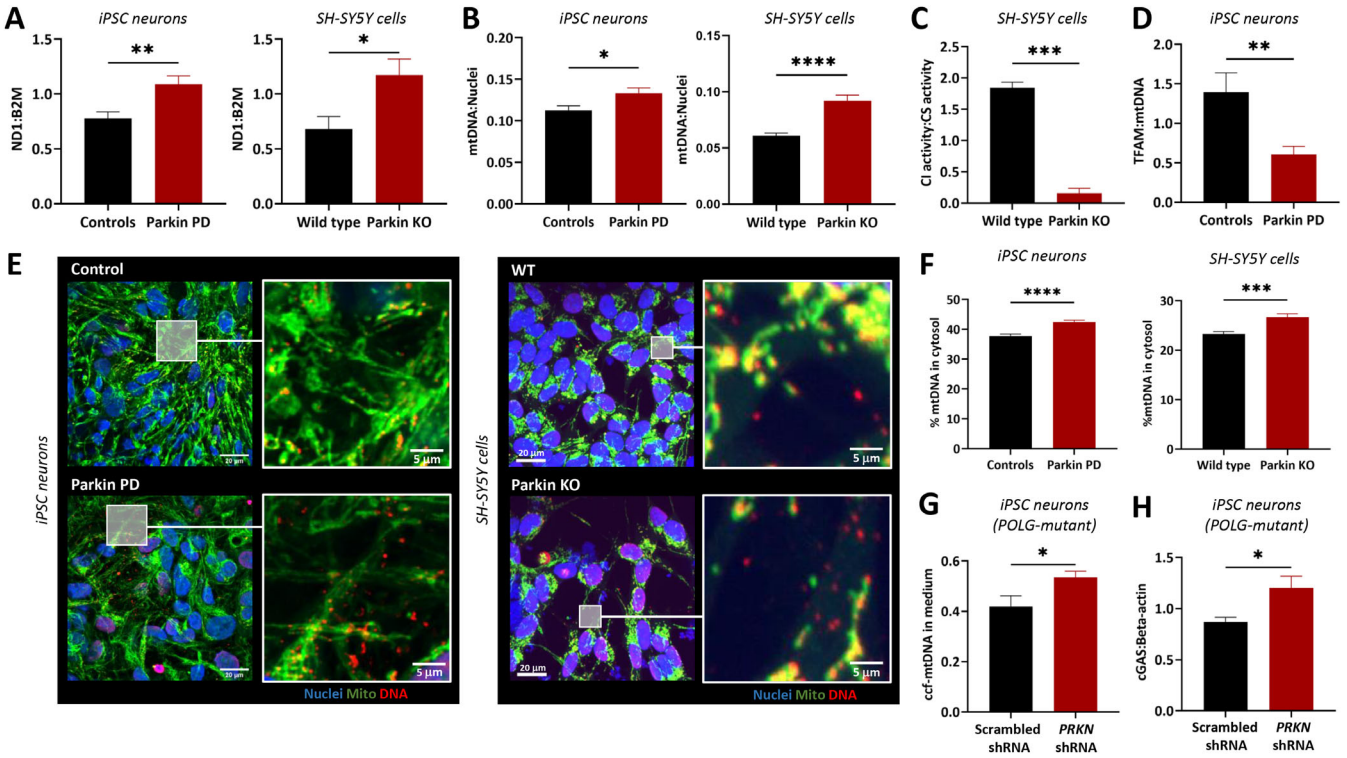


**FIG. 3.** Parkin attenuates cytosolic mitochondrial DNA (mtDNA) infiltration at baseline and extracellular mtDNA release during mutagenic stress. (**A**) A real-time polymerase chain reaction (RT-PCR) assay was used to quantify mtDNA copy number (with a probe targeting NADH:ubiquinone oxidoreductase core subunit 1 [*ND1*]) relative to the nuclear single copy gene beta-1-microglobulin (*B2M*) in induced pluripotent stem cell (iPSC)–derived neurons and SH-SY5Y neuroblastoma cells in healthy controls and patients with parkin-associated Parkinson's disease (Parkin PD) as well as wild-type (WT) and parkin-knockout (KO) SH-SY5Y cells. (**B**) Immunocytochemistry analysis of mtDNA copy number in iPSC-derived neurons and SH-SY5Y cells. Copy number was determined by mtDNA volume:nuclei volume. (**C**) Quantification of mitochondrial complex I activity normalized to citrate synthase activity in SH-SY5Y cells. (**D**) Quantification of mitochondrial transcription factor A (TFAM) protein abundance normalized to mtDNA copy number in iPSC-derived neurons. (**E**) Representative cropped immunocytochemistry images used to assess mtDNA subcellular location by targeting mtDNA, nuclei (Hoechst 33342), the mitochondrial marker translocase of outer mitochondrial membrane 20 (TOM20) and cytosol (high content screening CellMask Orange Stain [ThermoFisher Scientific, Waltham, MA]). (**F**) Analysis of mtDNA localization in iPSC-derived neurons and SH-SY5Y cells from (**E**). Percentage of mtDNA in cytosol was calculated by dividing the mtDNA volume outside mitochondria by total mtDNA volume. (**G**) Extracellular circulating cell-free mtDNA (ccf-mtDNA) in medium from polymerase γ (POLG)–mutant iPSC-derived neurons transduced with scrambled or *PRKN* short hairpin RNA (shRNA). Quantification by means of multiplex digital PCR (dPCR) targeting the mtDNA fragment *ND1* and the nuclear single-copy gene *B2M*. ccf-mtDNA was calculated as the ratio of extracellular *ND1* normalized to extracellular *B2M* copies to the sum of intra- and extracellular *ND1* normalized by their respective intra- and extracellular *B2M* copies. (**H**) cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) signaling in *POLG*-mutant iPSC-derived neurons transduced with scrambled or *PRKN* shRNA. Quantification of cGAS protein levels relative to beta-actin from Figure S2E. n = 3 or 5 biological replicates; data are presented as the mean ± SEM; SEM = standard error of the mean, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ as determined by Student's *t* test.

dynamics could result in elevated mtDNA release, we assessed the subcellular localization of mtDNA molecules with an imaging approach. Indeed, patient neurons and parkin-KO SH-SY5Y cells harbored significantly more mtDNA molecules in the cytosol compared with controls (Fig. 3E,F). This mtDNA shift from mitochondria to the cytosol was independently validated by applying an RT-PCR approach to cellular fractions from control and parkin-KO SH-SY5Y cells (Fig. S2B).

## Hypoxic Conditions Mirror mtDNA Phenotypes Observed in Parkin-Deficient Cells

To test our hypothesis of metabolic remodeling as the underlying cause of mtDNA dyshomeostasis in *PRKN*-PD, we exposed WT SH-SY5Y cells to the hypoxia-inducing agent $CoCl_2$.[17] $CoCl_2$ treatment triggered an upregulation of the hypoxia-inducible factor 1-α (Fig. 4A) and a shift from oxidative phosphorylation to glycolysis as indicated by increased $NAD^+$:NADH ratios (Fig. 4B). In line with the cellular function of SIRT1, the protein was less abundant under hypoxic conditions (Fig. 4A,C). We then investigated the SIRT1 target PGC1-α, which was also downregulated in $CoCl_2$-treated cells (Fig. 4A,D). To explore the impact of PGC1-α depletion on mtDNA maintenance, we next determined the protein levels of NRF1 and TFAM, which were both downregulated in response to $CoCl_2$ exposure (Fig. 4A,E,F). In addition, the treatment reduced the 7S DNA:*MT-ND1* ratio (Fig. 4G) and *MT-ND1* mRNA levels (Fig. 4H).

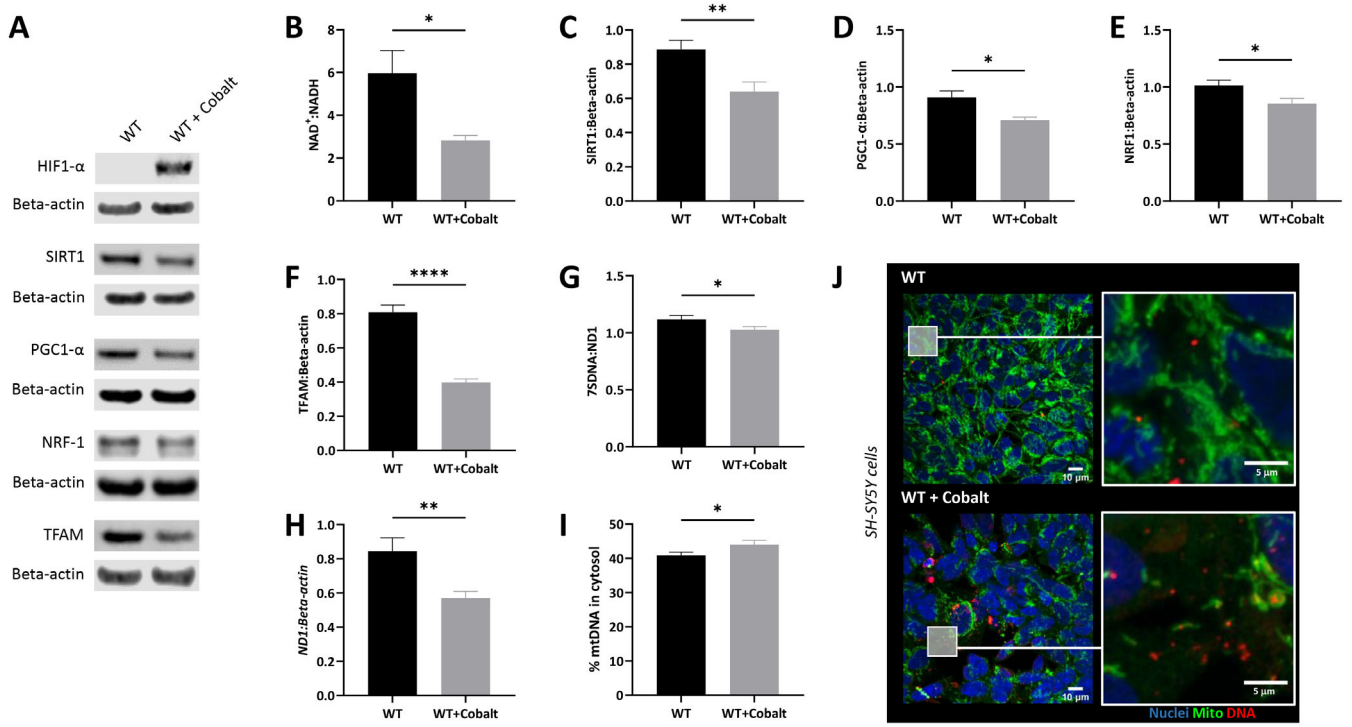


**FIG. 4.** Hypoxia-mediated metabolic alterations induce mitochondrial DNA (mtDNA) dyshomeostasis. (**A**) Representative cropped Western blot images of total cell lysates from untreated and cobalt chloride ($CoCl_2$)-treated wild-type (WT) SH-SY5Y neuroblastoma cells. (**B**) Nicotinamide adenine dinucleotide:nicotinamide adenine dinucleotide hydrogen ($NAD^+$/NADH) ratios measured in cellular extracts from untreated and $CoCl_2$-treated WT SH-SY5Y cells. Quantifications from (**A**) sirtuin 1 (SIRT1) (**C**), peroxisome gamma coactivator 1-alpha (PGC1-α) (**D**), nuclear respiratory factor 1 (NRF1) (**E**), mitochondrial transcription factor A (TFAM) (**F**) protein levels normalized to Beta-actin. (**G**) Real-time polymerase chain reaction quantification of transcription-associated 7S DNA per mtDNA molecule. (**H**) qPCR was used to quantify gene expression of the mtDNA gene NADH:ubiquinone oxidoreductase core subunit 1(*ND1*). (**I**) Analysis of mtDNA localization in untreated and $CoCl_2$-treated WT SH-SY5Y cells (**J**). Percentage of mtDNA in cytosol was calculated by dividing the mtDNA volume outside mitochondria by total mtDNA volume. (**J**) Representative cropped immunocytochemistry images used to assess mtDNA subcellular location by targeting mtDNA, nuclei (Hoechst 33342), the mitochondrial marker translocase of outer mitochondrial membrane 20 (TOM20), and cytosol (high content screening CellMask Orange Stain [ThermoFisher Scientific, Waltham, MA]). n ≤ 3 biological replicates; data are presented as the mean ± SEM; SEM = standard error of the mean, $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ as determined by Student's *t* test.

Finally, we tested whether the $CoCl_2$-induced metabolic shift was sufficient to trigger mtDNA release. Indeed, high-throughput imaging revealed elevated cytosolic mtDNA levels in treated SH-SY5Y cells (Fig. 4I,J). These data indicate that metabolic impairments can interfere with mtDNA dynamics.

## Mutagenic Stress Exacerbates Parkin-Mediated Mitochondrial Biogenesis and mtDNA Transcription Deficits

Next, we further explored the downstream effects of parkin deficiency–induced mtDNA dyshomeostasis. Although we observed an increase of cytosolic mtDNA in parkin-mutant neurons, this mtDNA release from the mitochondria was not accompanied by an upregulation of extracellular mtDNA or immune-related factors (Fig. S2C,D), which may be explained by the inability of iPSC-derived cultures to model age-associated phenotypes such as inflammation.[35] To overcome the rejuvenation-associated limitations of iPSC-derived neurons, we adapted a mitochondrial aging strategy from mice. Recently, an innovative mouse strain was generated that, contrary to most established rodent PD models, recapitulates motor phenotypes during the short lifetime of the animals.[10] To simulate mitochondrial aging, parkin-KO mice were crossed with animals harboring an error-prone version of POLG, which causes mtDNA mutagenic stress. The resulting parkin-KO "mutator" mice showed increased serum levels of ccf-mtDNA and inflammatory cytokines mediated by cGAS-STING signaling.[9] Inspired by this work, we differentiated iPSCs from a patient with compound-heterozygous mutations in *POLG* and subjected the resulting neurons to shRNA to reduce parkin expression (Fig. S3A).

Compared with *POLG*-mutant cells transduced with scrambled shRNA, parkin shRNA reduced 7S DNA:*MT-ND1* ratios (Fig. S3B) and showed significantly lower levels of mitochondrial biogenesis factors PGC1-α and NRF1 (Fig. S3A,C,D), strengthening our findings in *PRKN*-mutant neurons. Next, we quantified ccf-mtDNA in the extracellular medium and found a

significant upregulation, suggesting that parkin reduction in the presence of mtDNA stress elevated the release of mtDNA from mitochondria into the extracellular space (Fig. 3G). Furthermore, we found an increase in protein abundance of the cytosolic DNA sensor cGAS (Fig. 3H). Finally, we assessed the expression of key cytokines that were previously shown to be upregulated in serum from parkin-KO "mutator" mice and patients with *PRKN* mutations in response to cGas/STING signaling.[9] By contrast, RT-PCR analyses still revealed very low levels of interleukin 6 (*IL6*) and interleukin-1-beta (*IL1B*) in any of the investigated neurons (Fig. S3E), likely attributed to the absence of microglia in the cultures.

## mtDNA Maintenance Impairments Propagate Neuroinflammation in *PRKN*-PD Tissue

Although iPSC-derived neurons allow the study of parkin-related mitochondrial functions, the cultures do not reflect the cellular diversity of the midbrain. Recent publications implicate glia-mediated inflammation in the pathogenesis of *PRKN*-associated PD.[9,34] In light of these findings, we next used postmortem tissue from a patient with PD with compound-heterozygous *PRKN* mutations and two healthy controls to assess the extent and possible consequences of mtDNA disintegration in a more comprehensive environment (Fig. 5A).

We first sought to validate our findings concerning mtDNA maintenance in human brain tissue. We

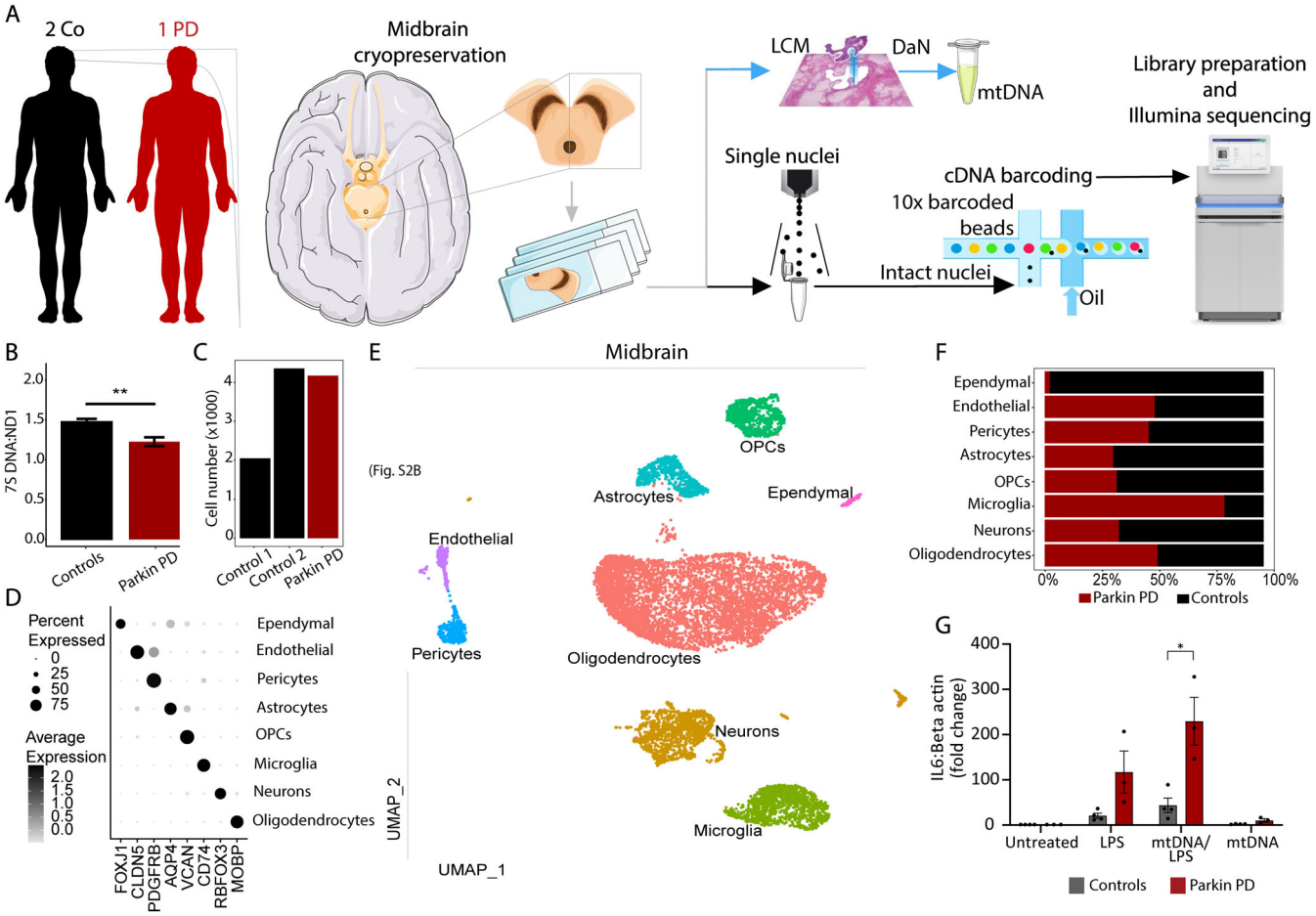


**FIG. 5.** Cell-type differences in human *PRKN*-mutant and control midbrains. (**A**) Handling of the midbrain tissue for single-cell studies. Midbrain sections were used for (1) laser capture microdissection (LCM) of dopaminergic neurons (DAN) and (2) nuclei isolation, 10X Genomics platform processing, and Illumina sequencing. (**B**) 7S DNA:mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 1 (7SDNA:*MT-ND1*) ratios quantified via multiplex real-time polymerase chain reaction from single postmortem DANs of the substantia nigra pars compacta. (**C**) Number of nuclei per sample. The analyzed population is composed of 4173 *PRKN*-mutant nuclei and 6405 control nuclei, making a population of 10,578 nuclei. (**D**) Representative cell-type specific marker genes. (**E**) The population of 10,578 nuclei projected onto a uniform manifold approximation and projection (UMAP) space. (**F**) Percentage of *PRKN*-mutant and control cell-type specific nuclei. (**G**) Fold change of interleukin 6 (*IL6*) expression at baseline (NT = nontreated) or after treatment with mitochondrial DNA (mtDNA), lipopolysaccharides (LPS), or both. n = 3 patients and n = 4 controls; data are presented as the mean ± SEM; SEM = standard error of the mean, *P < 0.05, **P < 0.01, ns = not significant as determined by two-way analysis of variance and Sidak's multiple comparisons test. Co = healty control subjects, PD/Parkin PD = Parkinson's disease patient with mutations in parkin, OPCs = oligodendrocyte precursor cells, *FOXj1* = forkhead box J1, *CLDN5* = claudin 5, *PDGFRB* = platelet derived growth factor receptor beta, *AQP4* = aquaporin 4, *VCAN* = versican, *RBFOX3* = RNA binding fox-1 homolog 3, *MOBP* = myelin associated oligodendrocyte basic protein.

applied LCM to midbrain tissue to isolate single neuromelanin- and TH-positive neurons (n = 10 per sample) from the SNpc, which were then subjected to RT-PCR. In line with our previous results, we found significantly reduced 7S DNA:*MT-ND1* ratios in postmortem patient DANs (Fig. 5B). However, we did not detect any differences in mtDNA deletion levels or in mtDNA copy number (data not shown).

Next, to determine whether *PRKN* mutation carriers suffer from neuroinflammation, we performed snRNAseq from postmortem ventral midbrain sections (Fig. 5A). A total of 10,578 high-quality nuclei (patient = 4173, controls = 2047 and 4358) were projected into two dimensions with the uniform manifold approximation and projection (UMAP) algorithm (Fig. 5C,E). We found eight major cell types including neurons and glial cells (Fig. 5D,E). Each cell cluster was annotated based on the expression of marker genes (Fig. 5D, Table S1). The most abundant cell type making ≈52.5% of the midbrain population are oligodendrocytes, followed by neurons (≈14.6%), microglia (≈11.6%), oligodendrocyte precursor cells (≈7.3%), and astrocytes (≈6.6%). Residual abundances were detected for other cell types. To assess neuroinflammation, we first compared cell density distributions in UMAP and observed a large increase in the microglial population and a decreased fraction of neurons and astrocytes (Fig. 5F) in the *PRKN*-mutant midbrain. Our results confirm the alteration in glial cell populations in the *PRKN* mutation carriers reported previously.[36] This shift infers an incorrect immune response, which likely leads to neuroinflammation. The resulting decrease of the *PRKN* midbrain neuronal population (Fig. 5F) compared with controls may be a trigger or downstream effect of the aforementioned immune response. Furthermore, the proinflammatory cytokines *IL1B* and tumor necrosis factor (*TNF*) were differentially expressed and upregulated in *PRKN*-mutant microglia compared with controls (Table S2). Accordingly, the immune response pathways, primarily the interleukin signaling pathways, were most perturbed in microglia from the *PRKN* mutation carrier (Tables S3 and S4).

Finally, to assess the causal link between mtDNA dyshomeostasis and neuroinflammation in *PRKN*-associated PD, we generated iPSC-derived neuron–microglia co-cultures from controls and patients with *PRKN* mutations and exposed them to LPS, mtDNA isolated from patients with *PRKN*-PD, or both. The composition of the co-cultures with regard to DANs and microglia was not affected by the different treatments (Fig. S4A,B). Moreover, stimulation by mtDNA or LPS alone did not reveal any differences in the expression of cytokines in cells lacking parkin. However, when mtDNA was added as a secondary trigger after LPS priming, we observed that patient-derived co-cultures showed a greater response in the expression of *IL6* (Fig. 5G). Although *IL1B* was equally upregulated after LPS/mtDNA treatment, the difference between *PRKN*-PD and control co-cultures was not significant (Fig. S4C).

## Discussion

Using a multimodal approach, we explored novel mechanisms of mitochondrial quality control exerted by parkin. Although most parkin studies focused on its role in mitochondrial clearance, the wide range of parkin substrates suggests that the protein functions in cellular processes beyond mitophagy.[24] Thus, we decided to investigate the mitochondrial biogenesis and mtDNA maintenance pathways in parkin-deficient cells.

Mitochondrial biogenesis is synchronized in the nucleus, with PGC1-α acting as the master regulator.[27] Studies found a direct interaction between parkin and PGC1-α,[27] whereas others have shown an interaction with PARIS—a PGC1-α transcriptional repressor.[6] In the current study, parkin-depleted cells showed reduced PGC1-α protein levels while the abundance of PARIS remained unchanged, insinuating an alternative mechanism of PGC1-α reduction. We looked upstream and found that the SIRT1 protein was reduced in parkin-deficient cells. *PGC1-α* gene expression and protein deacetylation is regulated by the $NAD^+$-dependent energy sensor SIRT1.[28,29] Moreover, our and a published metabolic study in parkin-deficient neurons revealed increased lactate:pyruvate ratios, suggesting lower levels of freely accessible $NAD^+$ as a result of metabolic remodeling from oxidative phosphorylation to glycolysis.[30,37]

PGC1-α can activate the NRFs, which control the expression of the mitochondrial transcription factors *TFAM* and *TFB2M*.[31] Our gene expression and protein analyses confirmed disruptions of this pathway at each level in cells lacking parkin. The sole semiautonomous organelle of human cells, mitochondria, encompass their own system to coordinate mtDNA transcription, replication, translation, and repair. Because of its dynamic nature and proximity to the ETC, mtDNA maintenance is exceptionally important for ensuring homogeneity and preventing the expansion of aberrant mtDNA molecules. Studies in mitotically active parkin-overexpressing cells have shown a direct interaction of parkin with TFAM and an association with the mitochondrial genome.[26,38]

Focusing on mtDNA integrity, we found significantly less 7S DNA per mtDNA molecule in iPSC-derived neurons, SH-SY5Y cells, and postmortem DANs lacking parkin. This is in agreement with previous findings in postmortem DANs isolated from patients with idiopathic PD (IPD).[20] The D-loop region of the

mitochondrial genome serves as the initiation site for its replication and transcription. The synthesis of 7S DNA in the D-loop is stimulated by TFAM, consistent with evidence showing that elevated TFAM matrix levels increase the rate of 7S DNA synthesis.[39] Indeed, calculating TFAM:mtDNA ratios, parkin-deficient neurons exhibited a significant reduction. Moreover, we observed decreased mtDNA gene expression that coincided with diminished ETC CI activity in parkin-KO SH-SY5Y cells. Of note, we previously reported ETC CI impairments in iPSC-derived neurons from our *PRKN*-mutant samples.[40] Interestingly, experiments in purified TH-positive neurons from patients with *PRKN* mutations highlighted that the 7S DNA phenotype is more pronounced in DANs, suggesting a link between mtDNA homeostasis and dopamine signaling. Because of their high energy requirements and the autoxidation properties of the neurotransmitter, DANs are particularly vulnerable to metabolic changes.[41,42] Interestingly, hypoxic conditions mimicked the mtDNA phenotypes observed in parkin-deficient cells. These findings further strengthened our hypothesis that a shift from oxidative phosphorylation to glycolysis triggers mtDNA dyshomeostasis in the absence of parkin.

Beyond alterations in the mitochondrial biogenesis pathway, we detected elevated mtDNA copy numbers but diminished TFAM abundance per mitochondrial genome in parkin-deficient neurons. TFAM reduction has previously been shown to allow mtDNA to escape from mitochondria into the cytosol, where it is recognized by the cytosolic DNA sensor cGAS, provoking activation of an innate immune response.[8] Indeed, we observed that neurons from patients with PD and parkin-KO SH-SY5Y cells incur higher levels of mtDNA in the cytosolic compartment compared with controls. We also quantified mtDNA molecules in the extracellular medium of our cellular samples, yet we did not detect any differences compared with controls or increases in inflammatory cytokines.

However, keeping in mind that reprogramming of postnatal cells can cause artificial rejuvenation,[35] we burdened parkin-deficient cells with mitochondrial mutagenic stress—a normal aging phenomenon[43]—to investigate mtDNA release and inflammation. To create a neuronal aging model of *PRKN*-PD, we adopted an approach by Pickrell and colleagues who crossed parkin-KO with *POLG*-mutant mice.[10] These animals exhibited levodopa-reversible motor deficits, a selective loss of nigral DANs, impaired mtDNA dynamics, and cGas/STING-mediated inflammation.[9,10] Accordingly, we generated iPSC-derived midbrain neurons from a patient with Alper's disease with compound-heterozygous *POLG* mutations and subjected the cultures to either scramble or parkin shRNA. Parkin knockdown in *POLG*-mutant neurons replicated the 7S DNA phenotype as well as disruptions of the mitochondrial biogenesis pathway. In addition, we detected elevated ccf-mtDNA levels in the cellular medium and an increase in cGAS protein abundance. In agreement with previous results in parkin-KO "mutator" mice, this suggests that parkin depletion combined with mitochondrial mutagenic stress triggers the release of mtDNA into the cytosol and extracellular space in patient neurons.

Microglia are considered the resident innate immune cells in the brain, which can be activated by various DAMPs. The lack of microglial cells in our neuronal cultures may explain the inability to detect inflammation in our samples. We therefore decided to use postmortem tissue from a patient with compound-heterozygous *PRKN*-PD and controls to investigate a potential link between parkin dysfunction and neuroinflammation using snRNAseq. Our results showed a strong infiltration of microglia in the *PRKN*-mutant midbrain and an upregulation of the proinflammatory cytokines *IL1B* and *TNF*. This dysregulation resulted in the perturbation of immune and oxidative stress response pathways.

To additionally evaluate the extent of the microglia phenotype in *PRKN*-PD, we made use of our recently published single-cell data set of five patients with IPD and six controls.[23] Comparing the cell-type distribution across the three groups (Fig. S5A-D), the *PRKN*-mutant midbrain showed an even larger percentage of microglia than the IPD tissue samples (Fig. S5D). Moreover, the expression of microglia activation markers heat shock protein 90 alpha family class A member 1 (*HSP90AA1*) and *IL1B* was the highest in the *PRKN*-mutant cell population (Fig. S5E). Thus, despite the limitation of examining brain tissue from a single mutant case, this cross-comparison further supports a role for microglia in the pathogenesis of *PRKN*-PD.

With regard to the distribution of other glial cells, we detected a reduction in astrocytes in *PRKN*-PD (Fig. S4D), which is in line with previous observations in the *PRKN*-mutant midbrain.[36] The resulting lack of astroglial neuron support likely perpetuates the inflammatory phenotypes in *PRKN*-PD as indicated by elevated expression of the astrocyte activation marker cluster of differentiation 44 (*CD44*) (Fig. S5F). Interestingly, despite lower overall astrocyte numbers, *CD44* levels in *PRKN*-PD astrocytes were even higher than in IPD astrocytes (Fig. S5F). Although additional rare *PRKN*-mutant midbrain samples need to be studied in the future, our snRNAseq analysis, which also considered our published results from IPD cases, provides valuable insights into the cell-type composition and transcriptomic landscape of the *PRKN*-PD midbrain.

Finally, to assess whether mtDNA dyshomeostasis and inflammation are causally linked in *PRKN*-PD, we generated iPSC-derived neuron–microglia co-cultures from controls and patients with *PRKN* mutations, which were treated with mtDNA isolated from *PRKN*-mutant cells. Our results showed that, when added as a secondary stimulus to LPS priming, mtDNA caused *IL6* overexpression

in cells lacking parkin, suggesting that parkin deficiency renders cells more responsive to proinflammatory stimuli.

Taken together, our study highlights parkin's involvement in mtDNA maintenance and supports a link between mtDNA dyshomeostasis and inflammation in human cellular models of PD. In iPSC-derived cultures from patients with *PRKN* mutations, we observed that mtDNA transcription-associated 7S DNA is preferentially depleted in DANs. This TFAM deficiency-mediated phenotype is likely the consequence of SIRT1 depletion and inactivation in response to a lack of free $NAD^+$ in the absence of parkin. However, the origin of the mitochondrial energy deficit in *PRKN*-mutant neurons currently remains elusive. Interestingly, recent studies suggest that parkin deficiency can be a driver of altered mitochondrial metabolism. Either via its function in mitochondrial clearance or through translational control of nuclear-encoded ETC mRNAs,[5,26] the mutant protein could trigger the respiratory complex impairments detected in patient neurons. With SIRT1 acting as a connector between cell metabolism, mitophagy, and biogenesis pathways, further research will be needed to determine the exact sequence of events triggering mitochondrial dysfunction in parkin-deficient DANs.[44] Moreover, additional analyses in iPSC-derived co-culture systems should aim at the identification of the immune signaling pathways activated in microglia in response to neuronal release of mitochondrial DAMPs, including mtDNA. Such investigations will pave the way for innovative anti-inflammatory treatment approaches in PD.

**Acknowledgments:** We express our gratitude to the tissue donors and their families for their generous participation. Postmortem tissue for this study was provided by the Newcastle Brain Tissue Resource and Juntendo University. Moreover, Prof. Dr. Doug Turnbull (Wellcome Trust Centre for Mitochondrial Research, Newcastle University) kindly provided fibroblasts for the study from a patient with Alper's disease. We are grateful to Thea Maria Van Wüllen (Luxembourg Centre for Systems Biomedicine) and Carola Dietrich (Max-Planck-Institute for Human Molecular Genetics, Berlin) for their assistance in postmortem sample preparation for single-cell RNA sequencing. We thank Nassima Ouzren and Ursula Heins-Marroquin for optimizing protocols for experiments used in the study. In addition, the authors thank Dr. Christian Jäger and Xiangyi Dong from the Luxembourg Centre for Systems Biomedicine Metabolomics Platform for the generation of metabolomics data. Finally, this project was supported by the Imaging Facility and the Disease Modelling and Screening Platform, Luxembourg Centre for Systems Biomedicine, University of Luxembourg, and Luxembourg Institute of Health, Luxembourg. Open access funding enabled and organized by Projekt DEAL.

## Data Availability Statement

Raw data for the midbrain tissue sample from the *PRKN* mutation carrier is available in the GEO with accession number GSE166790. Raw data for the two control samples were previously sequenced used in this study are available in the GEO with the accession number GSE157783 (data sets: C2-GSM4774937, C3-GSM4774938). Other data are available upon request.

## References

1. Panigrahi B, Martin KA, Li Y, et al. Dopamine Is Required for the Neural Representation and Control of Movement Vigor. Cell 2015; 162(6):1418–1430. https://doi.org/10.1016/j.cell.2015.08.014
2. Lücking CB, Dürr A, Bonifati V, et al. Association between early-onset Parkinson's disease and mutations in the parkin gene. N Engl J Med 2000;342(21):1560–1567. https://doi.org/10.1056/NEJM200005253422103
3. Grünewald A, Kumar KR, Sue CM. New insights into the complex role of mitochondria in Parkinson's disease. Prog Neurobiol 2019; 177:73–93. https://doi.org/10.1016/j.pneurobio.2018.09.003
4. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998; 392(6676):605–608. https://doi.org/10.1038/33416
5. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol 2008;183(5):795–803. https://doi.org/10.1083/jcb.200809125
6. Shin JH, Ko HS, Kang H, et al. PARIS (ZNF746) repression of PGC-1α contributes to neurodegeneration in Parkinson's disease. Cell 2011;144(5):689–702. https://doi.org/10.1016/j.cell.2011.02.010
7. Chocron ES, Munkácsy E, Pickering AM. Cause or casualty: The role of mitochondrial DNA in aging and age-associated disease. Biochim Biophys Acta Mol Basis Dis 2019;1865(2):285–297. https://doi.org/10.1016/j.bbadis.2018.09.035
8. West AP, Khoury-Hanold W, Staron M, et al. Mitochondrial DNA stress primes the antiviral innate immune response. Nature 2015; 520(7548):553–557. https://doi.org/10.1038/nature14156
9. Sliter DA, Martinez J, Hao L, et al. Parkin and PINK1 mitigate STING-induced inflammation. Nature 2018;561(7722):258–262. https://doi.org/10.1038/s41586-018-0448-9
10. Pickrell AM, Huang CH, Kennedy SR, et al. Endogenous Parkin Preserves Dopaminergic Substantia Nigral Neurons following Mitochondrial DNA Mutagenic Stress. Neuron 2015;87(2):371–381. https://doi.org/10.1016/j.neuron.2015.06.034
11. Seibler P, Graziotto J, Jeong H, Simunovic F, Klein C, Krainc D. Mitochondrial Parkin recruitment is impaired in neurons derived from mutant PINK1 induced pluripotent stem cells. J Neurosci Off J Soc Neurosci. 2011;31(16):5970–5976. https://doi.org/10.1523/JNEUROSCI.4441-10.2011
12. Kriks S, Shim JW, Piao J, et al. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. Nature 2011;480(7378):547–551. https://doi.org/10.1038/nature10648
13. Haenseler W, Sansom SN, Buchrieser J, et al. A Highly Efficient Human Pluripotent Stem Cell Microglia Model Displays a Neuronal-Co-culture-Specific Expression Profile and Inflammatory Response. Stem Cell Rep 2017;8(6):1727–1742. https://doi.org/10.1016/j.stemcr.2017.05.017
14. Reinhardt P, Glatza M, Hemmer K, et al. Derivation and expansion using only small molecules of human neural progenitors for neurodegenerative disease modeling. PloS One 2013;8(3):e59252. https://doi.org/10.1371/journal.pone.0059252
15. Sandor C, Robertson P, Lang C, et al. Transcriptomic profiling of purified patient-derived dopamine neurons identifies convergent perturbations and therapeutics for Parkinson's disease. Hum Mol Genet 2017;26(3):552–566. https://doi.org/10.1093/hmg/ddw412
16. Arena G, Cissé MY, Pyrdziak S, et al. Mitochondrial MDM2 Regulates Respiratory Complex I Activity Independently of p53. Mol Cell 2018;69(4):594–609.e8. https://doi.org/10.1016/j.molcel.2018.01.023
17. Vengellur A, LaPres JJ. The role of hypoxia inducible factor 1alpha in cobalt chloride induced cell death in mouse embryonic fibroblasts. Toxicol Sci Off J Soc Toxicol 2004;82(2):638–646. https://doi.org/10.1093/toxsci/kfh278
18. McCormack JG. Mitochondria. A practical approach; Edited by V M Darley-Usmar, D Rickwood and M T Wilson. pp 340. IRL Press, Oxford. 1987. £29 or £18 (pbk) ISBN 1-85221-034-6 or 033-8 (pbk). Biochem Educ 1988;16(2):118. https://doi.org/10.1016/0307-4412(88)90107-0

19. Delcambre S, Ghelfi J, Ouzren N, et al. Mitochondrial Mechanisms of LRRK2 G2019S Penetrance. Front Neurol 2020;11:881. https://doi.org/10.3389/fneur.2020.00881
20. Grünewald A, Rygiel KA, Hepplewhite PD, Morris CM, Picard M, Turnbull DM. Mitochondrial DNA Depletion in Respiratory Chain-Deficient Parkinson Disease Neurons. Ann Neurol 2016;79(3):366–378. https://doi.org/10.1002/ana.24571
21. Rygiel KA, Grady JP, Taylor RW, Tuppen HAL, Turnbull DM. Triplex real-time PCR--an improved method to detect a wide spectrum of mitochondrial DNA deletions in single cells. Sci Rep 2015;5:9906. https://doi.org/10.1038/srep09906
22. Meiser J, Delcambre S, Wegner A, et al. Loss of DJ-1 impairs antioxidant response by altered glutamine and serine metabolism. Neurobiol Dis 2016;89:112–125. https://doi.org/10.1016/j.nbd.2016.01.019
23. Smajić S, Prada-Medina CA, Landoulsi Z, et al. Single-cell sequencing of human midbrain reveals glial activation and a Parkinson-specific neuronal state. Brain J Neurol. Published online December 17 2021;awab446. https://doi.org/10.1093/brain/awab446
24. Sarraf SA, Raman M, Guarani-Pereira V, et al. Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. Nature 2013;496(7445):372–376. https://doi.org/10.1038/nature12043
25. Palikaras K, Lionaki E, Tavernarakis N. Balancing mitochondrial biogenesis and mitophagy to maintain energy metabolism homeostasis. Cell Death Differ 2015;22(9):1399–1401. https://doi.org/10.1038/cdd.2015.86
26. Kuroda Y, Mitsui T, Kunishige M, et al. Parkin enhances mitochondrial biogenesis in proliferating cells. Hum Mol Genet 2006;15(6):883–895. https://doi.org/10.1093/hmg/ddl006
27. Zheng L, Bernard-Marissal N, Moullan N, et al. Parkin functionally interacts with PGC-1α to preserve mitochondria and protect dopaminergic neurons. Hum Mol Genet 2017;26(3):582–598. https://doi.org/10.1093/hmg/ddw418
28. Cantó C, Auwerx J. PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. Curr Opin Lipidol 2009;20(2):98–105. https://doi.org/10.1097/MOL.0b013e328328d0a4
29. Amat R, Planavila A, Chen SL, Iglesias R, Giralt M, Villarroya F. SIRT1 controls the transcription of the peroxisome proliferator-activated receptor-gamma Co-activator-1alpha (PGC-1alpha) gene in skeletal muscle through the PGC-1alpha autoregulatory loop and interaction with MyoD. J Biol Chem 2009;284(33):21872–21880. https://doi.org/10.1074/jbc.M109.022749
30. Williamson DH, Lund P, Krebs HA. The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. Biochem J 1967;103(2):514–527. https://doi.org/10.1042/bj1030514
31. Gleyzer N, Vercauteren K, Scarpulla RC. Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators. Mol Cell Biol 2005;25(4):1354–1366. https://doi.org/10.1128/MCB.25.4.1354-1366.2005
32. Jornayvaz FR, Shulman GI. Regulation of mitochondrial biogenesis. Essays Biochem 2010;47:69–84. https://doi.org/10.1042/bse0470069
33. Suen DF, Narendra DP, Tanaka A, Manfredi G, Youle RJ. Parkin overexpression selects against a deleterious mtDNA mutation in heteroplasmic cybrid cells. Proc Natl Acad Sci U S A 2010;107(26):11835–11840. https://doi.org/10.1073/pnas.0914569107
34. Borsche M, König IR, Delcambre S, et al. Mitochondrial damage-associated inflammation highlights biomarkers in PRKN/PINK1 parkinsonism. Brain J Neurol 2020;143(10):3041–3051. https://doi.org/10.1093/brain/awaa246
35. Mertens J, Paquola ACM, Ku M, et al. Directly Reprogrammed Human Neurons Retain Aging-Associated Transcriptomic Signatures and Reveal Age-Related Nucleocytoplasmic Defects. Cell Stem Cell 2015;17(6):705–718. https://doi.org/10.1016/j.stem.2015.09.001
36. Kano M, Takanashi M, Oyama G, et al. Reduced astrocytic reactivity in human brains and midbrain organoids with PRKN mutations. NPJ Park Dis 2020;6(1):33. https://doi.org/10.1038/s41531-020-00137-8
37. Okarmus J, Havelund JF, Ryding M, et al. Identification of bioactive metabolites in human iPSC-derived dopaminergic neurons with PARK2 mutation: Altered mitochondrial and energy metabolism. Stem Cell Rep. 2021;16(6):1510–1526. https://doi.org/10.1016/j.stemcr.2021.04.022
38. Rothfuss O, Fischer H, Hasegawa T, et al. Parkin protects mitochondrial genome integrity and supports mitochondrial DNA repair. Hum Mol Genet 2009;18(20):3832–3850. https://doi.org/10.1093/hmg/ddp327
39. Gensler S, Weber K, Schmitt WE, et al. Mechanism of mammalian mitochondrial DNA replication: import of mitochondrial transcription factor A into isolated mitochondria stimulates 7S DNA synthesis. Nucleic Acids Res 2001;29(17):3657–3663. https://doi.org/10.1093/nar/29.17.3657
40. Zanon A, Kalvakuri S, Rakovic A, et al. SLP-2 interacts with Parkin in mitochondria and prevents mitochondrial dysfunction in Parkin-deficient human iPSC-derived neurons and Drosophila. Hum Mol Genet 2017;26(13):2412–2425. https://doi.org/10.1093/hmg/ddx132
41. LaVoie MJ, Hastings TG. Dopamine quinone formation and protein modification associated with the striatal neurotoxicity of methamphetamine: evidence against a role for extracellular dopamine. J Neurosci Off J Soc Neurosci 1999;19(4):1484–1491.
42. Graves SM, Xie Z, Stout KA, et al. Dopamine metabolism by a monoamine oxidase mitochondrial shuttle activates the electron transport chain. Nat Neurosci 2020;23(1):15–20. https://doi.org/10.1038/s41593-019-0556-3
43. Reeve A, Simcox E, Turnbull D. Ageing and Parkinson's disease: why is advancing age the biggest risk factor? Ageing Res Rev 2014;14:19–30. https://doi.org/10.1016/j.arr.2014.01.004
44. Agarwal E, Goldman AR, Tang HY, et al. A cancer ubiquitome landscape identifies metabolic reprogramming as target of Parkin tumor suppression. Sci Adv 2021;7(35):eabg7287. https://doi.org/10.1126/sciadv.abg7287


## Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

SGML and CITI Use Only
DO NOT PRINT

## Author Roles

(1) Research Project: A. Conception, B. Organization, C. Execution; (2) Statistical Analysis: A. Design, B. Execution, C. Review and Critique; (3) Manuscript: A. Writing of the First Draft, B. Review and Critique.

K.W: 1C, 2C, 3A, 3B
S.S.: 1C, 2C, 3A, 3B
J.G.: 1C, 3B
S.D.: 1C, 3B
C.A.P.-M.: 1C, 2C, 3A, 3B
E.K.: 1C, 3B
G. Arena: 1C, 3B
p.M.: 1C, 3B
G. Agyeah: 1C, 3B
A.R.: 1C, 3B
I.B.: 1C, 3B
K.B.: 1C, 3B
J.O.: 1C, 3B
J.-J.G.: 1C, 3B
M.T.: 1C, 3B
J.T.: 1C, 3B
M.M.: 1C, 3B
N.H.: 1C, 3B
C.K.: 1C, 3B
P.A.: 1C, 2C, 3A, 3B
P.S.: 1C, 3B
M.S.: 1C, 2C, 3A, 3B
S.L.P.: 1C, 2C, 3A, 3B
A.G.: 1A, 1B, 3B

## Full Financial Disclosures for Previous 12 Months

Kobi Wasner, Semra Smajic, Jenny Ghelfi, Sylvie Delcambre, Giuseppe Arena, Patrycja Mulica, Gideon Agyeah, Ibrahim Boussaad, Katja Badanjak, and Paul Antony report grants from the Luxembourg National Research Fund (FNR) and employment with the University of Luxembourg. Cesar A. Prada-Medina reports grants from the German Research Foundation (DFG), Max Planck Foundation, and Deutsches Zentrum für Luft-und Raumfahrt (DLR) and employment with the Max Planck Institute for Molecular Genetics Berlin and University of Oxford. Evelyn Knappe reports employment with the University of Lübeck. Aleksandar Rakovic and Philip Seibler report grants from the DFG and employment with the University of Lübeck. Jochen Ohnmacht reports grants from the FNR and Pelican Foundation and employment with the University of Luxembourg. Jean-Jacques Gérardy reports grants from the FNR and employment with Laboratoire National de Santé Luxembourg. Masashi Takanashi reports employment with Juntendo University. Joanne Trinh reports grants from the Peter and Traudl Engelhorn Fellowship, Canadian Institutes of Health Research (CIHR) Fellowship, Joachim Herz Stiftung Add-on Fellowship, and the DFG and employment with the University of Luebeck. Michel Mittelbronn reports employment with the University of Luxembourg, Laboratoire National de Santé Luxembourg, Luxembourg Center of Neuropathology, Luxembourg Institute of Health, and University of Luxembourg. Nobutaka Hattori reports grants from the Japan Society for Promotion of Science, Japan Agency for Medical Research and Development, and Japan Science and Technology Agency; a Health Labour Sciences Research Grant; and employment with Juntendo University. Christine Klein reports consultancies as medical advisor to Centogene and Biogen; honoraria from the Wellcome Trust Review Board; member of the scientific advisory board of the Else Kroener Fresenius Foundation; grants from the Movement Disorder Society, Hermann

and Lilly Schilling Foundation, DFG, the German Federal Ministry of Education and Research (BMBF), and European Community; intramural funds from the University of Luebeck; and employment with the University of Luebeck; and royalties from Oxford University Press. Malte Spielmann reports grants from the DFG, Max Planck Foundation, and DLR and employment with the University of Lübeck. Sandro L. Pereira reports grants from the FNR and Le Foyer Assurances Luxembourg and employment with the University of Luxembourg and Luxembourg Institute of Health. Anne Grünewald reports grants from the FNR, DFG, The Michael J. Fox Foundation, and Le Foyer Assurances Luxembourg and employment with the University of Luxembourg.

## Funding Agencies

K.W., J.G., G. Arena, S.L.P., and A.G. were supported by the Luxembourg National Research Fund (FNR) within the ATTRACT program (Model-IPD, FNR9631103). A.G. and S.L.P were generously supported by a donation from Le Foyer Assurances Luxembourg and obtained funding from the FNR within the framework of PARK-QC DTU (PRIDE17/12244779/PARK-QC). S.L.P. was further supported by the FNR INTER Mobility scheme (INTER/MOBILITY/19/13512561). In addition, A.G. and G. Arena are supported by an FNR CORE grant (MiRisk-PD, C17/BM/11676395). A.G., J.G., and S.D. received funding through an FNR INTER grant (ProtectMove, FNR11250962 and INTER/DFG/19/14429377) and via a grant from The Michael J. Fox Foundation within the framework of the Mitochondrial Biomarkers Spring 2018 program. In addition, P.M. and S.S. were supported by the FNR via the PARK-QC DTU (PRIDE17/12244779/PARK-QC) and G. Agyeah obtained funding from the FNR within the framework of the $i_2$TRON DTU (PRIDE19/14254520/i2TRON). K.B. received funding from the FNR within the framework of the PRIDE program (CriTiCS, FNR10907093). The work of I.B., J.O., and P.A. was supported by an Excellence Grant of the FNR within the PEARL program (FNR/P13/6682797 awarded to Rejko Krüger). M.M. and J.-J.G. were supported by an FNR PEARL Excellence Grant (P16/BM/11192868). Moreover, I.B., P.A., and A.G. received funding from the FNR as part of the National Centre of Excellence in Research on Parkinson's disease initiative. J.O. is supported by a Postdoctoral Grant of the Pelican Foundation, Luxembourg. A.G. (GR 3731/5-1, GR 3731/5-2), A.R., C.K., E.K., P.S., and J.T. are supported by the German Research Foundation, DFG (Research Unit ProtectMove, FOR 2488). M.S. and C.A.P.-M. are supported by grants from the DFG (SP1532/3-1, SP1532/4-1 and SP1532/5-1), the Max Planck Foundation and the Deutsches Zentrum für Luft-und Raumfahrt (DLR 01GM1925). N.H. is supported by the Japan Society for Promotion of Science (Grant-in-Aid for Scientific Research [A]; 18H04043, 21H04820), the Japan Agency for Medical Research and Development (Grant-in-Aid for Challenging Research [Exploratory]; 19K22603), the Health Labour Sciences Research Grant (JP20dm0307101, JP20dm0207070, JP20ek0109358, JP19ek0109393, JP19gm0710011, JP19km0405206), and the Japan Science and Technology Agency (20FC1049, H29-FC1-062, H29-FC1-033). The Newcastle Brain Tissue Resource is supported by the UK MRC (MR/L016451/1), the Alzheimer's Society, and Alzheimer's Research Trust through the Brains for Dementia Research Initiative and receives funding from the National Institutes for Health Research Biomedical Research Centre Newcastle.