

14 **Abstract**

15 Mutations in the *PINK1* and *PRKN* genes are the most common cause of early-onset familial
16 Parkinson disease. These genes code for the PINK1 and Parkin proteins, respectively, which are
17 involved in the degradation of dysfunctional mitochondria through mitophagy. An early step in
18 PINK1 – Parkin mediated mitophagy is the ubiquitination of the mitofusin proteins MFN1 and -2. The
19 ubiquitination of MFN1 and -2 in patient samples may therefore serve as a biomarker to determine
20 the functional effects of *PINK1* and *PRKN* mutations, and to screen idiopathic patients for potential
21 mitophagy defects. We aimed to characterise the expression of the PINK1 – Parkin mitophagy
22 machinery in peripheral blood mononuclear cells (PBMCs) and assess if these cells could serve as a
23 platform to evaluate mitophagy via analysis of MFN1 and -2 ubiquitination. Mitophagy was induced
24 through mitochondrial depolarisation by treatment with the protonophore CCCP and ubiquitinated
25 MFN proteins were analysed by western blotting. In addition, *PINK1* and *PRKN* mRNA and protein
26 expression levels were characterised with reverse transcriptase quantitative PCR and western
27 blotting, respectively. Whilst CCCP treatment led to MFN ubiquitination in primary fibroblasts, SH-
28 SY5Y neuroblastoma cells and Jurkat leukaemic cells, treatment of PBMCs did not induce
29 ubiquitination of MFN. *PRKN* mRNA and protein was readily detectable in PBMCs at comparable
30 levels to those observed in Jurkat and fibroblast cells. In contrast, PINK1 protein was undetectable
31 and *PINK1* mRNA levels remarkably low in control PBMCs. Our findings suggest that the PINK1 –
32 Parkin mitophagy signalling pathway is not functional in PBMCs. Therefore, PBMCs are not a suitable
33 biosample for analysis of mitophagy function in Parkinson disease patients.

34

35 Introduction

36 Parkinson disease (PD) has a heterogeneous clinical presentation. An important goal in PD
37 research is to identify if and how this heterogeneity is related to biochemical and cell biological
38 differences, which inform the pathophysiology of the disease [1]. The identification of such
39 differences is important for three interrelated reasons: (1) the elucidation of mechanistic targets for
40 drug development, (2) the selection of the most appropriate patient cohorts for clinical trials, and (3)
41 to provide functional readouts allowing the identification of target engagement and changes in
42 cellular and biochemical phenotypes.

43 Although most cases of PD are idiopathic, there are some rare early-onset familial forms,
44 which may provide insights into potential biochemical subtypes that exist within the wider,
45 idiopathic PD population. The most common forms of early-onset familial PD are caused mutations
46 in the *PINK1* and *PRKN* genes, which encode PTEN induced putative kinase 1 (PINK1) [2] and Parkin,
47 respectively [3]. Both proteins act in the same quality control pathway to sense damaged
48 mitochondria and target them for degradation through a specialised form of macro-autophagy, also
49 known as mitophagy. PINK1 is a mitochondrial kinase imported into the mitochondria via the
50 preprotein translocase complexes where it is constitutively degraded by the presenilin-associated
51 rhomboid-like protein (PARL) [4]. Loss of mitochondrial membrane potential disrupts the
52 mitochondrial import and degradation of PINK1, which subsequently accumulates on the outer
53 mitochondrial membrane (OMM) [5]. Here, PINK1 undergoes autophosphorylation [6] and
54 phosphorylates Parkin at serine 65, promoting its mitochondrial translocation and stabilisation [7].
55 Phosphorylation of ubiquitin at serine 65 by PINK1 further promotes the full activation of Parkin [8,
56 9]. Parkin, an E3 ubiquitin ligase ubiquitinates multiple OMM proteins, including mitofusin 1 (MFN1)
57 and mitofusin 2 (MFN2) [10]. Recruitment of autophagy adaptors by ubiquitin chains conjugated to
58 OMM proteins [11] leads to the engulfment of impaired mitochondria by autophagosomes, which
59 fuse with lysosomes causing the eventual degradation of defective mitochondria.

60 The clinical, molecular and functional genetics of *PINK1* and *PRKN* are complex. Disease
61 causing mutations can include missense mutations, deletions and copy number variations, which can
62 be inherited in homozygous and compound heterozygous patterns [2, 3, 12, 13]. Pathogenic *PINK1*
63 and *PRKN* mutations are generally associated with a loss of function of the respective proteins.
64 Furthermore, recent findings demonstrate that heterozygously inherited *PINK1* mutations can confer
65 increased PD risk, an effect that may be mediated at the molecular level by a dominant negative
66 mechanism during PINK1 dimerisation [14, 15]. However, the effects of these mutations on Parkin
67 and PINK1 function, and on downstream mitophagy, have not been fully characterised. For instance,
68 there are over 200 *PRKN* variants, some of which are pathological and which have differing effects

69 on Parkin function and mitophagy [16]. Moreover, mitophagy is impaired in skin fibroblasts from PD
70 patients with no known genetic cause [17, 18]. These findings suggest that defects in mitophagy may
71 contribute to idiopathic PD in some patients [19].

72 Although the role of mitophagy in the pathogenesis of PD is debated, functional readouts of
73 the PINK1 – Parkin mitophagy signalling pathway may provide patient stratification, even if they are
74 epiphenomenal to disease causing processes. Such readouts will also allow the functional
75 characterisation of novel *PINK1* and *PRKN* variants and those of unknown significance. One such
76 functional readout is the ubiquitination of the MFN proteins following mitochondrial depolarisation,
77 a lack of which distinguishes *PINK1* and *PRKN* mutant fibroblasts from those isolated from healthy
78 controls [20]. In this study we aimed to translate this readout to peripheral blood mononuclear cells
79 (PBMCs), which represent a minimally invasive source of biological tissue for biomarker analysis. We
80 have also characterised the expression of the PINK1 – Parkin signalling pathway in different cell
81 types and demonstrate that the pathway is not functional in PBMCs. This work thus adds an
82 important contribution to knowledge on the mitochondrial biology of PBMCs and their utility in PD
83 research.

84

85 **Results**

86 **Treatment of fibroblasts with CCCP induces MFN ubiquitination in a** 87 **PINK/Parkin dependent manner**

88 We began by characterising the effect of mitochondrial depolarisation upon the
89 ubiquitination of the OMM proteins MFN1 and MFN2 in primary fibroblast cultures. Fibroblasts from
90 2 healthy controls were treated with 20 μ M carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) for
91 2 hours to depolarise the mitochondria followed by western blot analysis. In untreated cells, MFN1
92 was detected as a double band, with the lower and upper bands migrating at 75 and 78 kDa,
93 respectively, and MFN2 was detected as a single band with an apparent M_r of 75 kDa (Fig 1). CCCP
94 treatment led to the appearance of an extra anti-MFN1 reactive band, which migrated with an
95 apparent M_r that was 9 kDa larger than the lower MFN1 band detected in untreated cells. In
96 addition, an extra anti-MFN1 reactive band that was 9 kDa larger than the MFN2 band in untreated
97 cells was detected (Fig 1). This size change is consistent with mono-ubiquitination and our previous
98 work [10] indicates that the extra MFN1 and MFN2 bands detected post-CCCP treatment are
99 ubiquitin positive. Next, we performed the same treatment and analysis on fibroblast cultures from
100 two early-onset PD patients, one with a homozygous nonsense mutation in *PINK1* and one with a
101 homozygous deletion in *PRKN*. Consistent with previous reports [20], CCCP treatment failed to

102 induce ubiquitination of either MFN1 or MFN2 in fibroblasts from either patient (Fig 1). These results
103 demonstrate that CCCP-induced ubiquitination of MFN1 and MFN2 in fibroblasts depends upon the
104 presence of functional PINK1 and Parkin proteins.

105

106 **Treatment of Jurkat cells with CCCP induces MFN ubiquitination**

107 We next asked whether CCCP-induced MFN ubiquitination was a shared feature across
108 different cell types. We first opted to analyse the immortalised T-lymphocyte cell line Jurkat as a
109 surrogate model for primary leucocyte cells. Similar to that observed in control fibroblasts, CCCP
110 treatment produced extra anti-MFN1 and anti-MFN2-2 reactive bands, whose migration was
111 consistent with mono-ubiquitination of the respective proteins (Fig 2A). In addition, we observed
112 faint anti-MFN1 and anti-MFN2-2 reactive bands suggestive for polyubiquitination. Time course
113 analysis demonstrated that CCCP-induced ubiquitination of MFN1 and MFN2 occurred as early as 30
114 minutes post-treatment and persisted up to 24 hours post-treatment (Fig 2B). The maximum
115 ubiquitinated MFN signal was detected at 2 hours post-treatment, for both MFN1 and MFN2 (S1
116 Fig). Thus, Jurkat cells respond in the same way as fibroblasts to CCCP treatment in terms of MFN
117 ubiquitination.

118

119 **Treatment of PBMCs from healthy controls with CCCP does not** 120 **induce MFN ubiquitination**

121 Following these findings, we aimed to compare the CCCP-induced MFN ubiquitination
122 response across 4 different cell types: the immortalised neuroblastoma SH-SY5Y cell line, the
123 immortalised Jurkat T-lymphocyte cell line, primary cultured fibroblasts and PBMCs derived from
124 healthy controls. Similarly to the results obtained with fibroblasts and Jurkat cells, and consistent
125 with our previous report [10], treatment of SH-SY5Y cells with CCCP produced an anti-MFN1 and
126 anti-MFN2 reactive profile consistent with ubiquitination (Fig 3). As demonstrated in Figs 1 and 2,
127 anti-MFN1 and anti-MFN2 reactive bands consistent with mono-ubiquitination were also detected in
128 CCCP-treated Jurkat cells and fibroblasts; however, we did not detect anti-MFN1 and anti-MFN2
129 reactive bands consistent with MFN ubiquitination in CCCP-treated PBMC samples (Fig 3). Long
130 exposure images also did not reveal any signal consistent with ubiquitination of either MFN1 or -2.
131 These results demonstrate that under the conditions tested, CCCP treatment does not induce
132 ubiquitination of MFN1 or -2 in PBMCs.

133

134 **Parkin is expressed in PBMCs from healthy controls**

135 In order to shed light on lack of CCCP-induced MFN ubiquitination in PBMCs, we next
136 determined the relative expression of PINK1 and Parkin. We began by assessing the relative
137 abundance of *PRKN* transcripts in Jurkat, fibroblast, SH-SY5Y and PBMC samples. RNA was isolated
138 from the cells and reverse transcribed to create cDNA libraries from which *PRKN* transcripts were
139 amplified by real-time quantitative PCR (qPCR). *PRKN* mRNA was readily amplified from all cell types,
140 and when normalised, fibroblasts (2 independent cultures) and SH-SY5Y cells exhibited 11.8, 10.2
141 and 12.6 times the *PRKN* levels detected in Jurkat cells (Fig 4A). *PRKN* transcripts were also amplified
142 from 5 independent control PBMC cultures. Levels in these cultures were similar to those detected
143 in Jurkat cells (Fig 4A).

144 By western blot analysis, Parkin protein was readily detected in 3 independent control
145 fibroblast cultures as well as in Jurkat cells and SH-SY5Y cells as a single band migrating at ~48 kDa
146 (Fig 4B). SH-SY5Y cells demonstrated the highest Parkin signal (~23 fold more than Jurkat and
147 fibroblast cells, Fig 4D). A ~48-kDa band was also detected in PBMC samples from 6 healthy controls
148 (Fig 4C). This protein co-migrated with that detected in SH-SY5Y cells and Jurkat cells, and
149 importantly, was absent from HeLa cells, which have been demonstrated to not express Parkin
150 protein [21]. Therefore, we assume that the ~48-kDa band detected in PBMCs represents Parkin.
151 Quantitatively, the Parkin signal from PBMCs was not significantly different from that detected in
152 either Jurkat or fibroblast cells (Fig 4D). Collectively, these findings demonstrate that Parkin is
153 expressed in PBMCs and, consequently, that the lack of CCCP-induced MFN ubiquitination in these
154 cells is not due to a lack of Parkin expression.

155

156 **PINK1 is undetectable in PBMCs from healthy controls**

157 In order to investigate the expression levels of *PINK1* mRNA in PBMCs, RNA was isolated
158 from Jurkat, fibroblast, SH-SY5Y and PBMC samples and followed by reverse transcriptase qPCR.
159 *PINK1* transcript levels in fibroblasts exceeded those detected in Jurkat cells by 33.8 and 32.7 times
160 (for two independent control cultures), whilst levels in SH-SY5Y cells were similar to those in Jurkat
161 cells (Fig 5A). Levels of *PINK1* mRNA in PBMCs from healthy controls were 20–100 times lower than
162 those detected in Jurkat cells and >3000 times lower than those detected in fibroblast cultures (Fig
163 5A).

164 *PINK1* protein is constitutively degraded by PARL in the mitochondria and, as a result,
165 undetectable under basal conditions. In order to detect *PINK1*, the mitochondrial membrane
166 potential must be dissipated, for example with prolonged CCCP treatment. Therefore, control
167 fibroblasts, SH-SY5Y cells, Jurkat cells and PBMCs were treated with 20 μ M CCCP for 24 hours,
168 followed by western blot analysis for *PINK1*. *PINK1* antibodies have been reported to give a panoply

169 of non-specific bands when used in western blotting, including at the predicted M_r of PINK1, as
170 determined in knockout cells [22]. Such observations may confound the interpretation of results and
171 render the use of biological controls as critical when studying PINK1 protein. Indeed, even in
172 untreated cells we detected numerous cross reactive bands in fibroblast, SH-SY5Y and Jurkat
173 samples with a PINK1 antibody from Novus Biologicals (Fig 5B). However, when these cells were
174 treated with CCCP, an additional band appeared at ~60 kDa, the predicted molecular weight of
175 mature PINK1 (Fig 5B). This band was absent in untreated cells and is, thus, consistent with PINK1.
176 The levels of PINK1 protein were significantly higher in Jurkat cells than either fibroblast or SH-SY5Y
177 cells. Fibroblast cells expressed the lowest levels of PINK1 after CCCP treatment, 10 times lower than
178 detected in Jurkat cells (Fig 5C). However, in PBMCs the pattern of PINK1 cross reactive proteins was
179 unchanged between treated and untreated cells (Fig 5B). Specifically we did not detect an additional
180 band at 60 kDa when cells were treated with CCCP, thus suggesting that PINK1 protein is not
181 detectable in PBMCs. With an alternative PINK1 antibody from Cell Signaling Technologies, we also
182 failed to detect PINK1 in CCCP-treated PBMCs, despite adequate detection in the other cell types (S2
183 Fig). The lack of PINK1 detection in PBMCs may explain the absence of CCCP-induced MFN
184 ubiquitination in these cells.

185 During mitophagy, mitochondrial proteins are degraded in the lysosome. Accordingly,
186 mitophagy can be monitored by assessing the levels of mitochondrial proteins post mitophagic
187 induction. Indeed, in Jurkat, fibroblast and SH-SY5Y cells, 24 hour CCCP treatment led to significant
188 reductions in subunit A of the mitochondrial succinate dehydrogenase complex (SDHA) (Fig 5D). In
189 PBMCs, conversely, treatment with CCCP did not significantly affect SDHA levels (Fig 5D). These
190 findings suggest differences in CCCP-induced mitophagy between cultured SH-SY5Y, fibroblast and
191 Jurkat cells on the one hand, and PBMCs on the other.

192

193 **The PINK1– Parkin mitophagy signalling pathway is not expressed** 194 **in activated T cells and lymphoblastoid cell lines**

195 PBMCs cultured under normal conditions are non-proliferative, whereas Jurkat cells are
196 robustly proliferating T lymphocyte-like cells. For that reason, we investigated whether inducing the
197 proliferation of T cells in the PBMC samples would shift their response to CCCP treatment.
198 Moreover, we analysed how Epstein-Barr virus (EBV) immortalised lymphoblastoid cell lines (LCLs)
199 from healthy controls responded to CCCP treatment. The T-cell fraction of PBMCs were induced to
200 proliferate by incubation with T-activator CD3/CD28 Dynabeads. This treatment led to the
201 appearance of cell clusters, which were visually similar to cell clusters observed in both Jurkat and
202 LCL cultures (Fig 6A). Despite these morphological similarities, neither activated PBMCs nor LCLs

203 responded to 2-hour CCCP treatment with ubiquitination of MFN1 or MFN2 (Fig 6B). Furthermore,
204 PINK1 protein did not accumulate in either cell type following prolonged CCCP treatment (Fig 6C).
205 *PINK1* mRNA levels in LCLs were also 10–25 times lower than those detected in fibroblasts (S3 Fig).
206 As shown in Fig 4, western blot analysis detected Parkin in Jurkat cells and non-proliferating PBMCs;
207 however, Parkin was undetectable in PBMCs activated with CD23/CD8 Dynabeads and in 4
208 independent LCLs (Fig 6D). Collectively, these findings demonstrate that whilst Jurkat cells exhibit
209 CCCP-induced PINK1 – Parkin signalling, this pathway is absent from activated PBMCs and LCLs.
210

211 Discussion

212 In this study we aimed to assess the utility of PBMCs as a platform for studying mitophagy
213 defects in PD patients. Our analysis demonstrated that PBMCs are unsuitable for this purpose as
214 they do not functionally express the PINK1 – Parkin mitophagy signalling pathway. The
215 ubiquitination of MFN proteins following CCCP treatment can be used as a marker of early
216 mitophagy in fibroblasts, and distinguishes control fibroblasts from those with *PINK1* or *PRKN*
217 mutations [20]. Our findings demonstrate that this process is not conserved in all cell types.
218 Specifically, PBMCs and lymphocytes immortalised by EBV transduction lack the necessary
219 machinery to transduce loss of mitochondrial membrane potential into MFN ubiquitination.

220 In agreement with previous work, we detected *PRKN* mRNA and Parkin protein in PBMCs
221 from healthy controls [23, 24]. Parkin is a multi-functional protein with roles in diverse cellular
222 processes, including those related to immunity [25]. Its expression in these cells is thus expected.
223 However, we were unable to detect PINK1 protein in PBMCs, and levels of *PINK1* mRNA in these cells
224 were remarkably low, up to 3000 times lower than those detected in fibroblast cells. The low levels
225 of PINK1 expression in PBMCs likely explains their lack of CCCP-induced MFN ubiquitination, as
226 PINK1 accumulation is required to activate Parkin on the mitochondrial surface [8]. PINK1 protein
227 was also undetectable in LCLs and mRNA levels were 10–25 times lower than those detected in
228 fibroblasts.

229 Jurkat cells underwent CCCP-induced MFN ubiquitination and time dependent reduction in
230 SDHA levels, indicating functional mitophagy similar to that observed in fibroblasts and SH-SY5Y
231 cells. Moreover, Jurkat cells expressed readily detectable levels of *PINK1* and *PRKN* mRNA and the
232 respective proteins. Jurkat cells are an EBV-negative T-cell line derived from an acute lymphoblastic
233 leukaemia biopsy, and proliferate rapidly in culture [26, 27]. We thus considered that it may have
234 been the quiescence of primary PBMCs, which rendered them insensitive to CCCP-induced
235 mitophagy. However, stimulation of PBMCs with CD3/CD28 antibodies, whilst leading to robust
236 proliferation [28], did not result in CCCP-induced MFN ubiquitination. In fact, stimulated PBMCs

237 massively downregulated Parkin expression. PBMCs isolated from patients are a heterogeneous
238 cellular sample [29], and this reduction in Parkin expression post-stimulation may have been due to
239 the clonal expansion of PBMC sub-populations lacking Parkin expression. In favour of this
240 hypothesis, we noted that EBV-transformed lymphocytes from four independent controls did not
241 express Parkin protein at detectable levels. It is tempting to speculate that the lack of Parkin in these
242 immunoactive cells may reflect a specific function. For instance, the downregulation of Parkin in
243 blood cells may promote viral clearance through promoting mitochondrial ROS production [30].

244 **Conclusions**

245 Overall, our findings demonstrate PBMCs from control subjects do not undergo MFN
246 ubiquitination after acute induction of mitophagy with CCCP. PBMCs express extraordinarily low
247 *PINK1* mRNA levels and PINK1 protein is undetectable. The physiology of these cells thus precludes
248 their use as a platform in studying the PINK1 – Parkin mitophagy signalling pathway in PD research.

249

250 **Methods**

251 **Attainment of samples**

252 Primary human dermal fibroblast cultures were established from skin explants of two early-
253 onset PD patients, one female patient with a homozygous p.R246X nonsense mutation in *PINK1* and
254 a heterozygous deletion of exons 4–6 in *PRKN*, and one female patient with a homozygous deletion
255 of exons 4–5 in *PRKN*. In addition, skin biopsies were obtained from age-matched healthy control
256 subjects. Fibroblast cultures were established according to standard procedures [31]. Ethical
257 approval for this work was obtained from the Royal Free Hospital and Medical School Research
258 Ethics Committee (REC 07/H0720/161). Ethical approval for PBMC work was obtained from Camden
259 and King's Cross Research Ethics Committee (REC 17/LO/1166). All donors provided prior informed
260 written consent and all work was performed in compliance with national legislation and the
261 Declaration of Helsinki.

262

263 **PBMC isolation, culture and treatment**

264 PBMCs were isolated using standard Ficoll gradient separation protocols. Blood collected in
265 EDTA-coated vacutainer blood tubes was mixed with an equal volume of phosphate-buffered saline
266 (PBS). Diluted blood (20 ml) was layered on to 15 ml of Lymphoprep (Stemcell Technologies) in 50-ml
267 Falcon tubes and centrifuged at 400× *g* for 30 min without deceleration. The resultant PBMC layer
268 was then aspirated into a new 15-ml Falcon tube and washed twice in PBS, centrifuging at 300× *g* to

269 reduce platelet contamination. Fresh PBMCs were then re-suspended in RPMI medium (Thermo
270 Fisher Scientific, 61870) and counted using a flow cytometer (Moxi GO™, Orflo Technologies).

271 For routine analysis, PBMCs were cultured at 1×10^6 cells/ml in RPMI supplemented with 10%
272 foetal bovine serum (FBS) and 1 mM sodium pyruvate at 37°C, 5% CO₂. After 24 h, cells were treated
273 with 20 μM CCCP and harvested at the indicated time points. For harvesting, 2 volumes of PBS were
274 directly added to the suspended cells, followed by centrifugation at 500× *g* for 10 min. The pellet
275 was resuspended in 1 ml of PBS and centrifuged at 17,000× *g* for 10 min at 4°C. The supernatant was
276 aspirated and the pellet was stored at -80°C until further analysis. The age and sex of the donors of
277 the PBMC samples used in this study are shown in S4 Table.

278

279 **Culturing of fibroblasts, SH-SY5Y, Jurkat and LCLs**

280 Primary human skin fibroblast cultures were grown in DMEM (Thermo Fisher Scientific,
281 61965-059) supplemented with 10% FBS, 1 mM sodium pyruvate, and 50 units/ml of penicillin and
282 50 μg/ml of streptomycin at 37°C, 5% CO₂. SH-SY5Y cells were cultured in DMEM: F12 (1:1) (Thermo
283 Fisher Scientific, 31331-028) supplemented with 10% FBS, 1× non-essential amino acids and
284 penicillin/streptomycin at 37°C, 5% CO₂. When confluent, cultures were passaged using
285 trypsinisation. Jurkat cells and LCLs were cultured in suspension in RPMI medium 1640 (Thermo
286 Fisher Scientific, 61870-127) supplemented with 10% FBS and 1 mM sodium pyruvate at 37°C, 5%
287 CO₂. Cells were maintained at 5×10^5 – 3×10^6 cells/ml.

288

289 **PBMC activation with CD3/CD28 Dynabeads**

290 PBMCs were incubated with Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher
291 Scientific, 11132D) at a ratio of 1 : 1 and cultured in OpTmizer T-cell expansion SFM medium
292 supplemented with 10 mM L-glutamine, penicillin/streptomycin, serum substitute (Thermo Fisher
293 Scientific, A1048501) and 30 U/ml of interleukin-2 (IL-2; Thermo Fisher Scientific, PHC0026) at 37°C,
294 5% CO₂. Cultures were maintained at 1×10^6 – 3×10^6 cells/ml. Cells were activated for 7 days prior to
295 analysis.

296

297 **Reverse-transcriptase real-time quantitative PCR**

298 RNA was isolated from cell pellets with the RNeasy kit (Qiagen, 74104) and quantified with a
299 Nanodrop spectrophotometer (Thermo Fisher Scientific). RNA (500 ng) was reverse transcribed to
300 produce cDNA libraries using the QuantiTect Reverse Transcriptase Kit from Qiagen (205311). cDNA
301 was quantified by Qubit analysis with the 1× dsDNA HS Assay Kit (Thermo Fisher Scientific, Q33230).

302 For qPCR, 1 ng of cDNA was mixed with forward and reverse primers (final concentration 10 μ M),
303 PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, A25780) and water to a final volume of
304 20 μ l. Primers were purchased from Qiagen (*PRKN*: QT00023401; *PINK1*: QT00056630).

305 Following initial denaturation at 50°C for 2 min and activation at 95°C for 2 min,
306 thermocycling was performed with 50 cycles of denaturation (94°C, 15 sec), annealing (55°C, 30 sec)
307 and extension (72°C, 30 sec), using an Applied Biosystems StepOne real-time PCR system. Samples
308 were analysed as technical triplicates. Fluorescence was read during the extension step. Melting
309 temperature analysis was performed on the amplified products to ensure consistency within and
310 between runs. Amplicon specific C_t thresholds were applied consistently between runs, and relative
311 transcript levels were calculated by transforming the C_t values using the expression 2^{-C_t} , followed by
312 normalisation to cDNA quantity.

313

314 **Cell lysis and western blotting**

315 Cell pellets were lysed in 0.1% Triton X-100 in PBS containing a Halt Protease Inhibitor
316 Cocktail (Thermo Fisher Scientific; 78430). Lysates were vortexed and incubated for 15 min on ice,
317 followed by clarification at 17,000 $\times g$ for ten min at 4°C. Following determination of the protein
318 concentration in the supernatants (Pierce™ BCA Protein Assay Kit; Thermo Fisher Scientific, 23250),
319 samples were prepared for denaturing gel electrophoresis by addition of Laemmli Sample Buffer
320 (BioRad, 161-0747), Sample Reducing Agent (Thermo Fisher Scientific, NP0009) and water to
321 consistent protein concentrations.

322 Samples were resolved on Mini-Protean® TGX 4–20% gels or TGX 7.5% gels (BioRad
323 Laboratories, 4568095 and 4568025). Separated proteins were transferred to Trans-Blot Turbo 0.2-
324 μ m PVDF membranes (BioRad, 170-4157), using the BioRad Trans-Blot Turbo Transfer System.
325 Membranes were blocked in 10% non-fat dry milk powder in PBS prior to incubation with primary
326 antibodies overnight at 4°C in 5% milk, 0.15% Tween-20, PBS. Following washing with 0.15% Tween-
327 20, PBS, membranes were incubated with secondary antibodies conjugated to HRP enzymes (Dako,
328 P0447 and P0448) and washed again. Blots were developed with Clarity Western ECL Substrate
329 (BioRad, 170-5060). Capturing of the chemiluminescent signals was performed with the BioRad
330 Chemidoc™ MP Imaging System. Signals were quantified with BioRad Image Lab 6.0.1 software. The
331 primary antibodies are specified in S5 Table.

332

333 **Statistical analyses**

334 Graphs and statistical analyses were executed with GraphPad Prism® version 6.01 software.
335 Data are presented as mean ± standard error of the mean. Student's t test was used to examine
336 statistical significance and significance levels were set to p<0.05.

337

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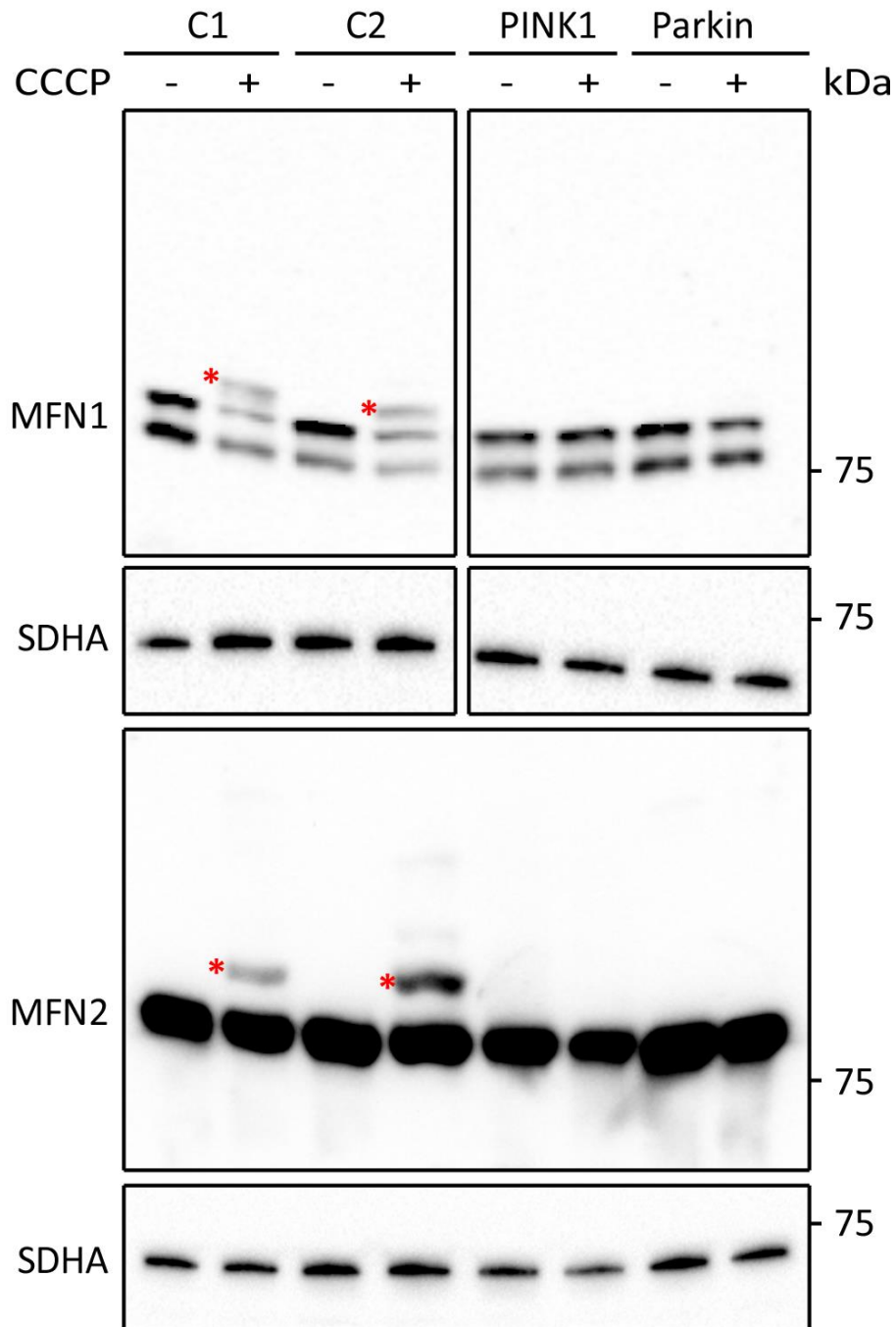
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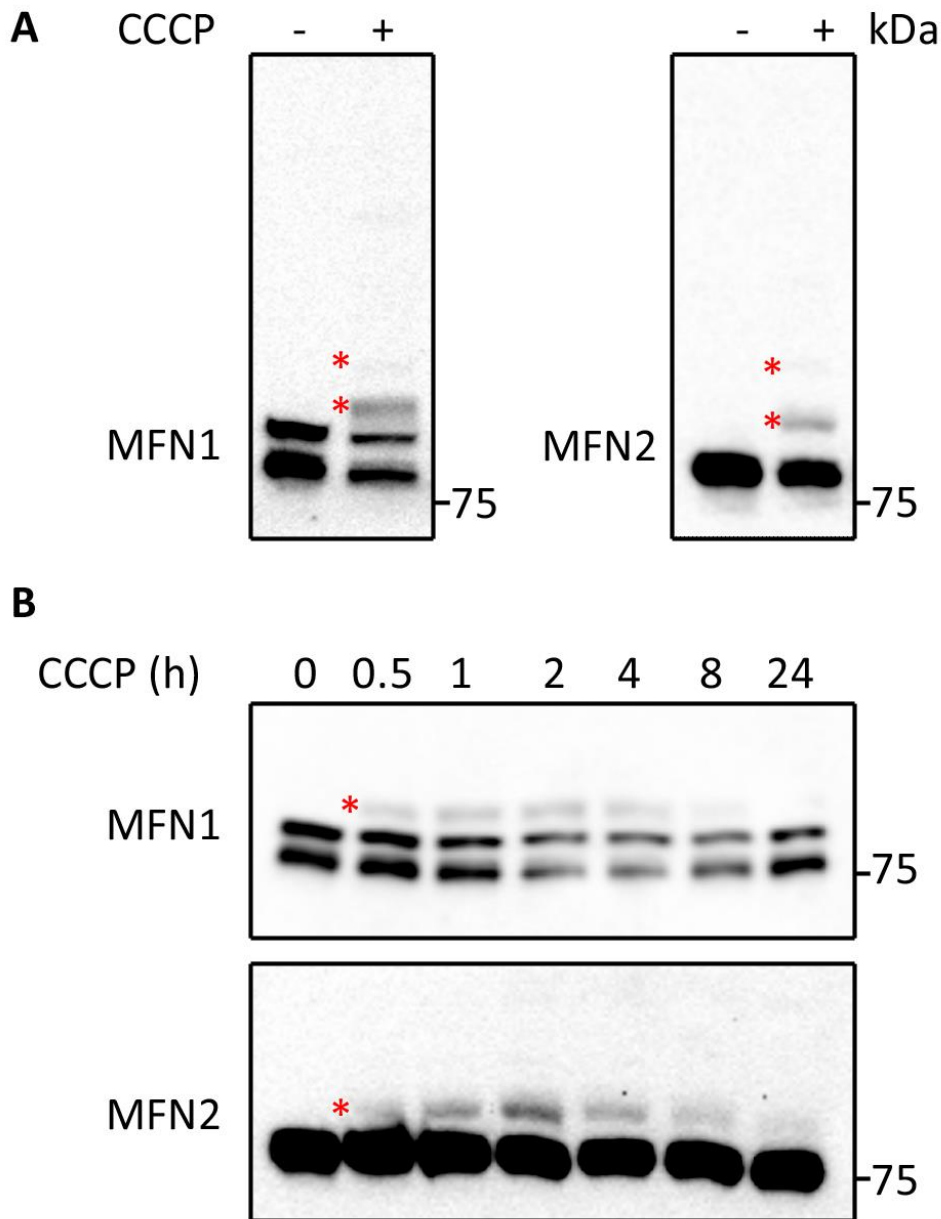
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417 **Fig 1. Analysis of CCCP-induced MFN ubiquitination in cultured fibroblast cells.** Western blot
418 analysis of fibroblasts from healthy controls (C1 and C2) and two early-onset PD patients with
419 mutations in *PINK1* or *PRKN*. Cells were left untreated or were treated for 2 hours with 20 μ M CCCP.
420 Blots were developed with antibodies against MFN1 and MFN2. Antibodies against SDHA were used
421 as loading control. Asterisks indicate bands representing ubiquitinated MFN proteins.

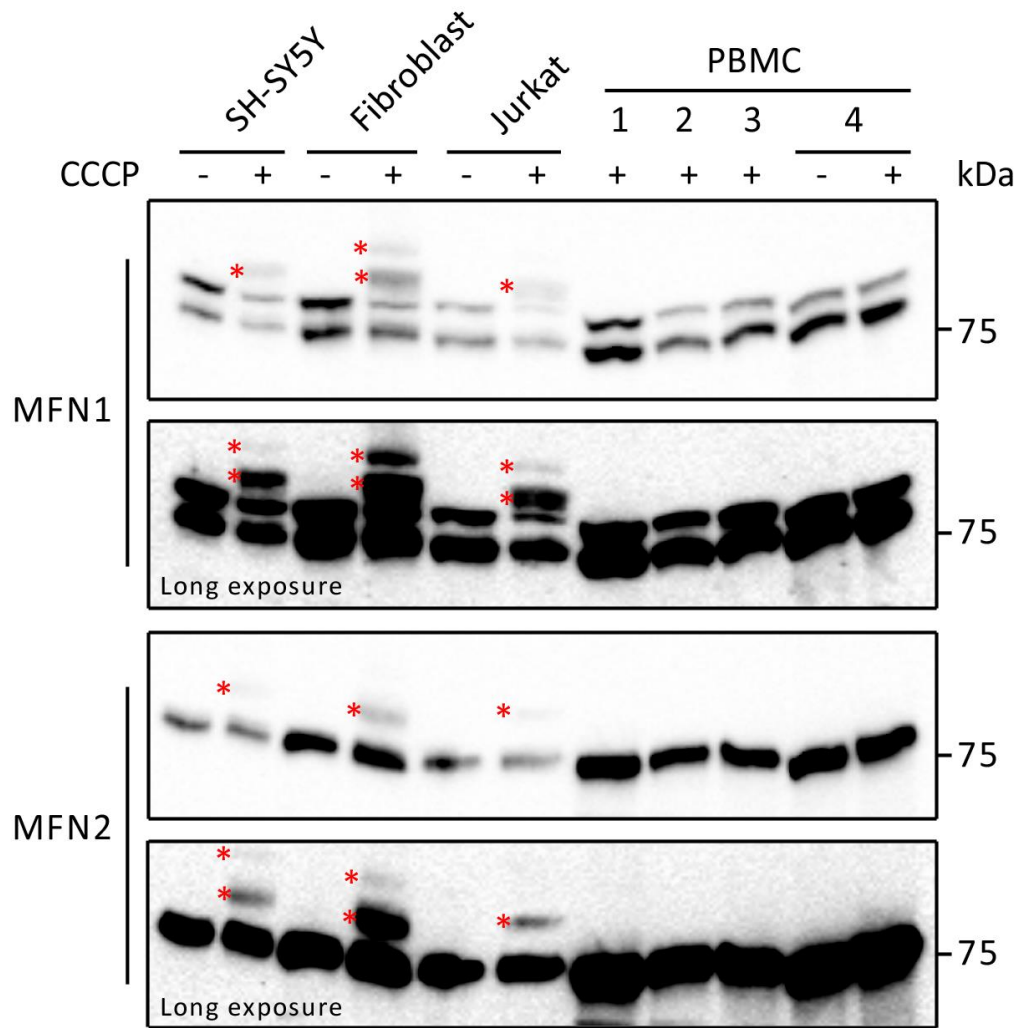


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423 **Fig 2. Analysis of CCCP-induced MFN ubiquitination in Jurkat cells.** (A) Jurkat cells left untreated or
424 treated for 2 hours with 20 μ M CCCP were analysed by western blotting with antibodies against
425 MFN1 and MFN2. (B) Western blot analysis of Jurkat cells treated with CCCP for increasing periods of
426 time (0.5–24 hours), developed with antibodies against MFN1 and MFN2. Asterisks indicate bands
427 representing ubiquitinated MFN proteins.

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431 **Fig 3. Analysis of CCCP-induced MFN ubiquitination in different cells types.** SH-SY5Y, fibroblast,

432 Jurkat and PBMC samples were left untreated or were treated with 20 μ M CCCP for 2 hours and

433 analysed by western blotting with antibodies against MFN1 and MFN2. Asterisks indicate bands

434 representing ubiquitinated MFN proteins.

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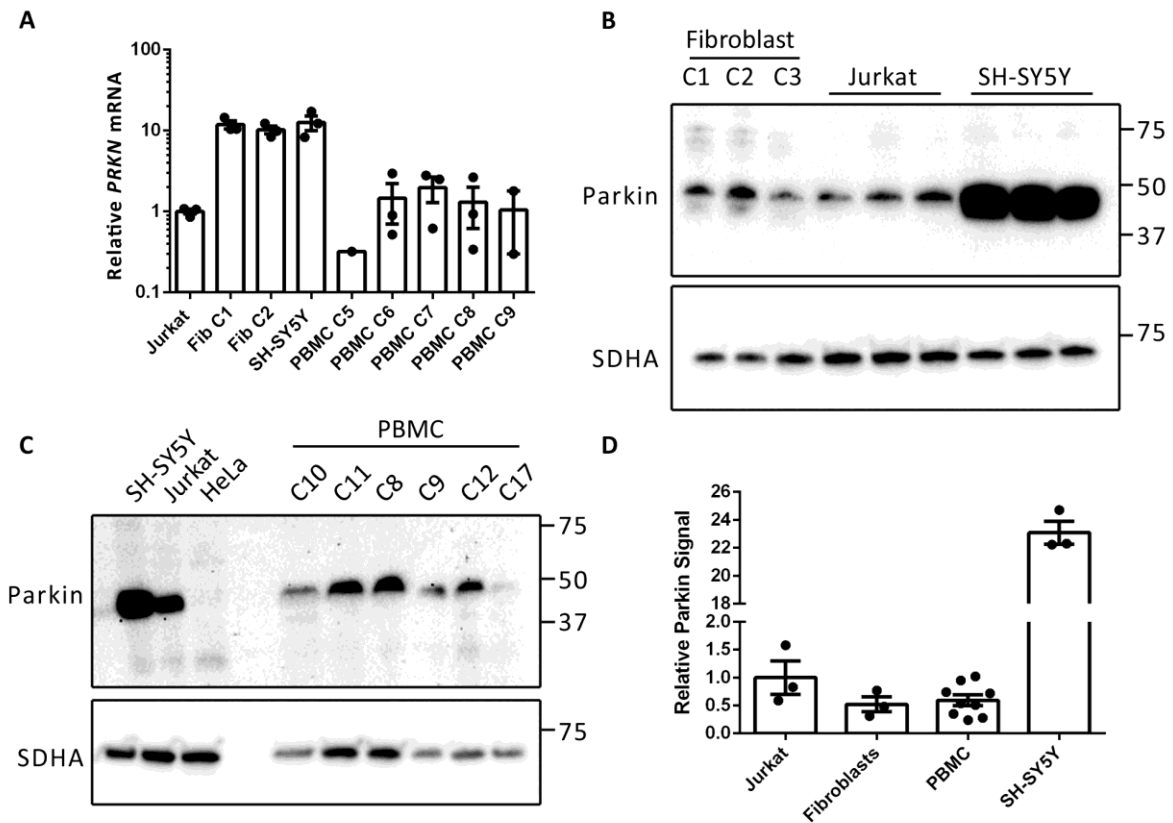
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443 **Fig 4. Analysis of Parkin expression in different cell types.** (A) Reverse transcriptase qPCR analysis of
444 *PRKN* mRNA levels in Jurkat, control fibroblast (Fib), SH-SY5Y and PBMC samples. qPCR was
445 performed on equal amounts of cDNA from each cell type and C_t values were expressed relative to
446 the Jurkat sample. (B, C) Western blot analysis of fibroblast, SH-SY5Y, Jurkat and PBMC cultures with
447 an antibody against Parkin. (D) Quantification of Parkin signal in the different cell types. Signal is
448 expressed relative to SDHA and normalised to that detected in Jurkat cells. Graphs display mean \pm
449 SEM.

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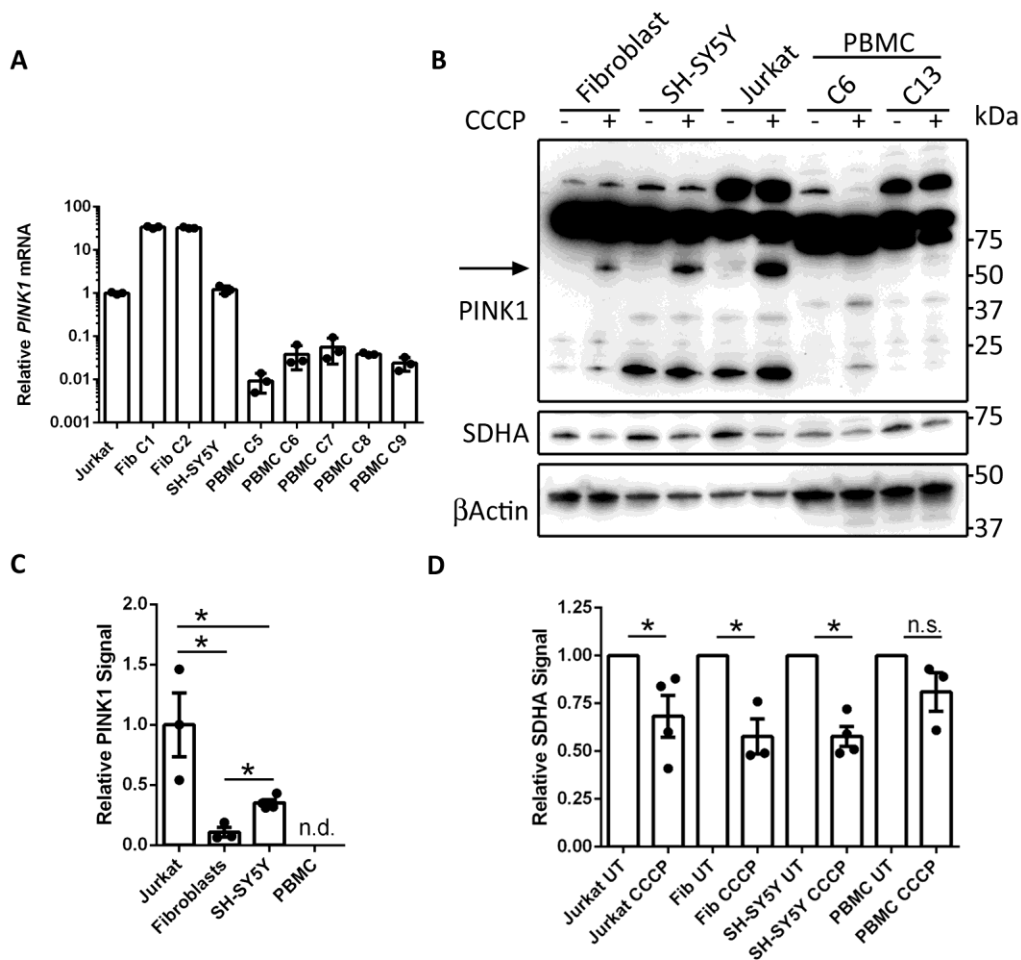
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457 **Fig 5. Analysis of PINK1 expression in different cell types.** (A) Reverse transcriptase qPCR analysis of

458 *PINK1* mRNA levels in Jurkat, control fibroblast (Fib), SH-SY5Y and PBMC samples. qPCR was

459 performed on equal amounts of cDNA from each cell type and C_t values were expressed relative to

460 the Jurkat sample. (B) Western blot analysis of fibroblasts, SH-SY5Y, Jurkat and PBMC cultures left

461 untreated and treated with 20 μ M CCCP for 24 hour with an antibody against PINK1 (Novus

462 Biologicals, BC100-494). The band corresponding to PINK1 is indicated by the arrow. (C)

463 Quantification of PINK1 signal from CCCP-treated cells. Signal is expressed relative to β -actin and

464 normalised to levels detected in Jurkat cells. (D) Quantification of SDHA signal from untreated and

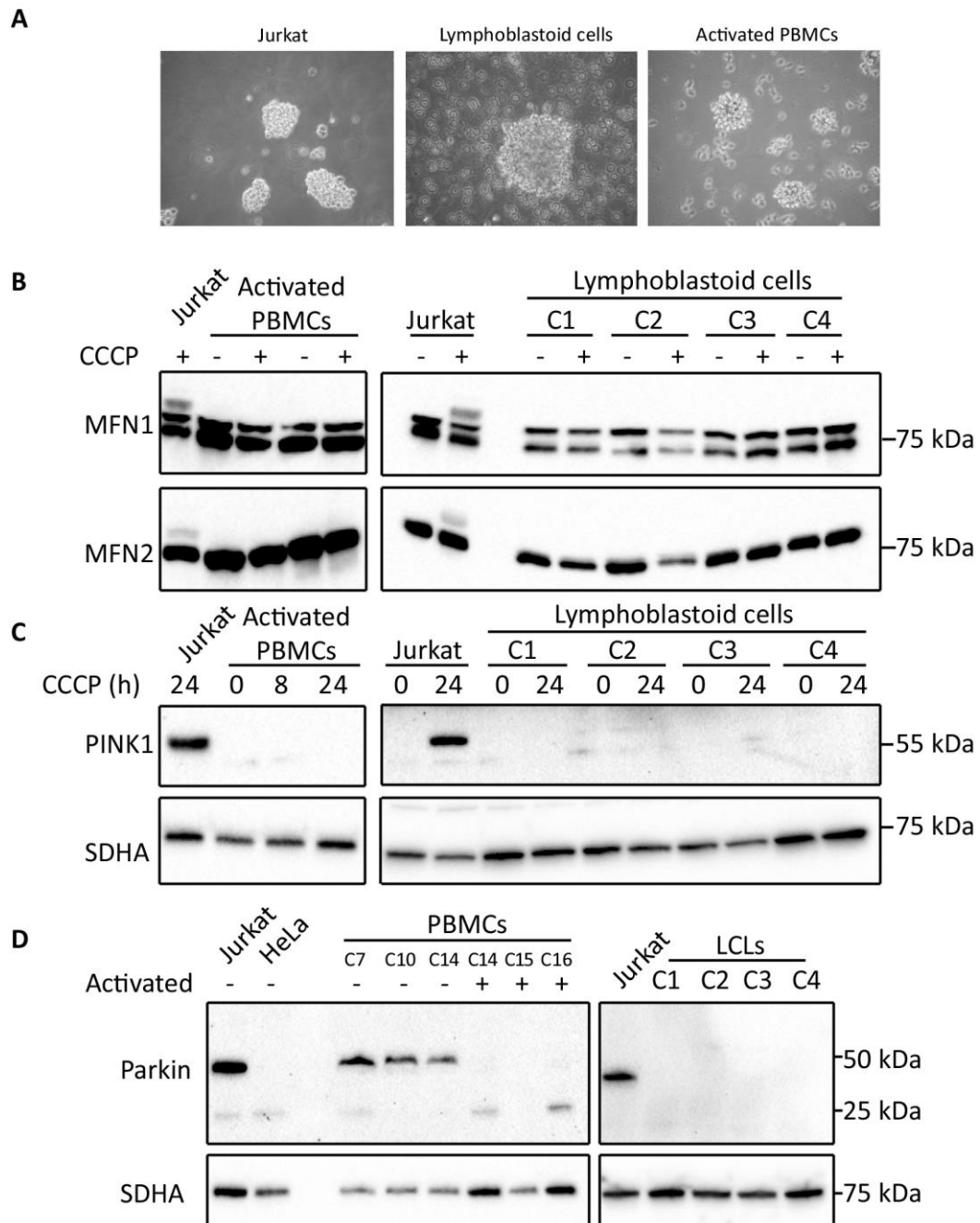
465 24-hour CCCP-treated Jurkat, fibroblast, SH-SY5Y and PBMC samples. Signal is expressed relative to

466 β -actin and normalised to the untreated condition. Graphs display mean \pm SEM; *= P <0.05, Student's

467 t-test; n.d. = not detected; n.s. = not significant.

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471 **Fig 6. Analysis of PINK1 – Parkin signalling pathway in lymphoblastoid cell lines and CD3/CD28**

472 **activated PBMCs.** (A) Phase contrast microscopy images of Jurkat cells, lymphoblastoid cell lines

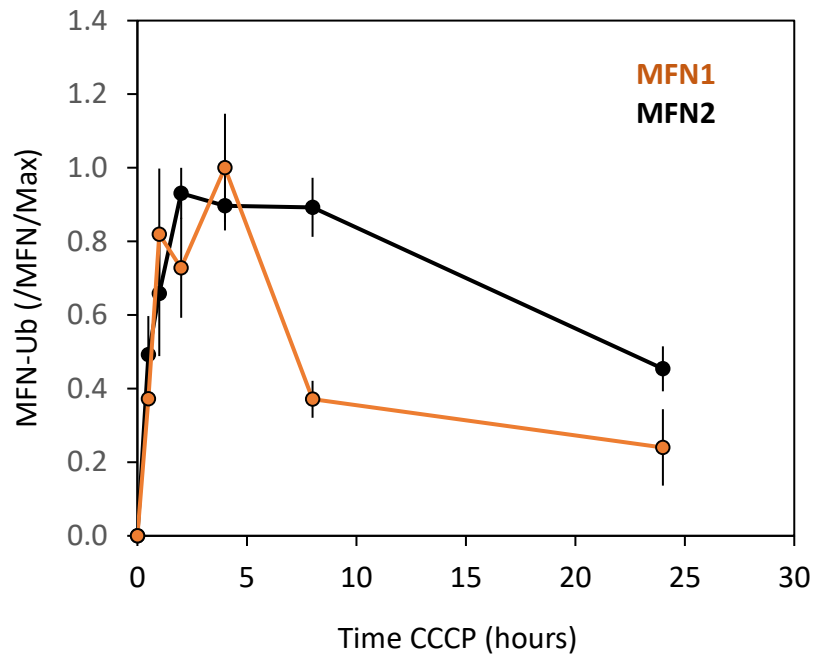
473 (LCLs) and CD3/CD28 Dynabead activated PBMCs. (B) Western blot analysis of MFN1 and -2

474 ubiquitination following CCCP treatment of activated PBMCs and LCLs. (C) Western blot analysis of

475 PINK1 expression in activated PBMCs and LCLs following prolonged CCCP treatment. (D) Western

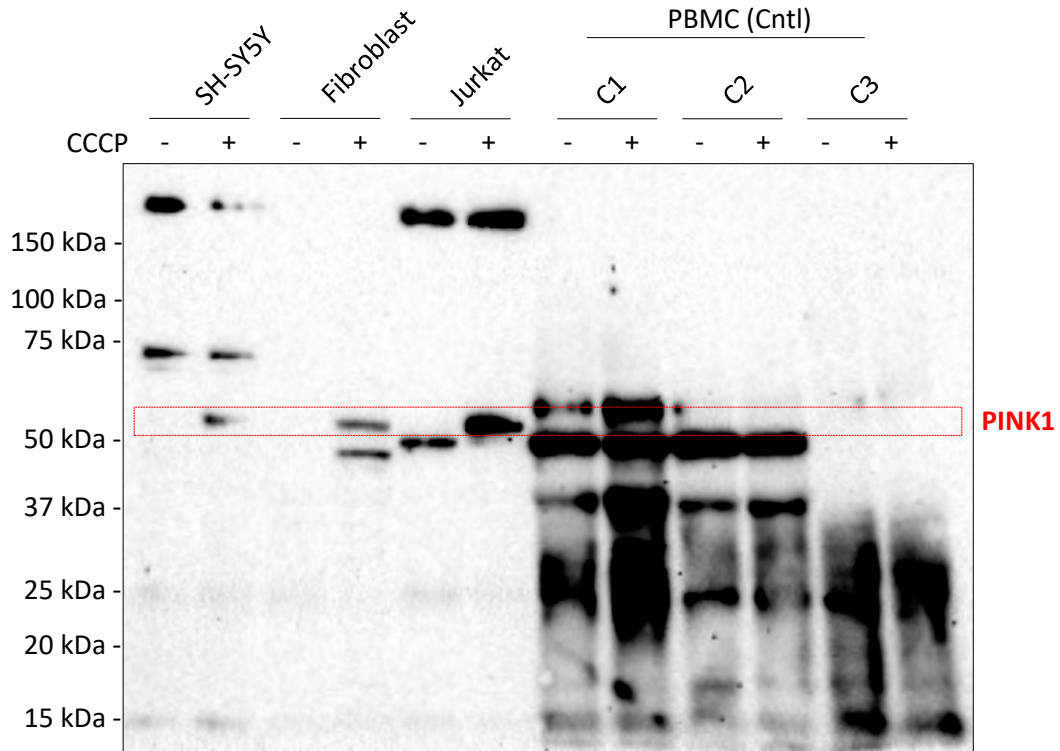
476 blot analysis of Parkin expression in resting and CD3/CD28 Dynabead activated PBMCs, and LCLs.

477 **Supporting information**



478 **S1 Fig. Time course analysis of MFN ubiquitination in Jurkat cells.** Jurkat cells were treated with 20
479 μ M CCCP for increasing periods of time and the ubiquitination of MFN1/MFN2 was analysed by
480 immunoblotting. The levels of ubiquitinated MFN1 and MFN2 were quantified relative to the
481 respective non-ubiquitinated protein. Graph represents mean \pm SEM; MFN1, n=2; MFN2, n=3.

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498 **S2 Fig. Analysis of PINK1 expression in different cell types.** SH-SY5Y, fibroblast, Jurkat and PBMC
499 cultures were left untreated or treated with 20 μ M CCCP for 24 hours. Samples were analysed by
500 western blotting with the PINK1 antibody clone D8G3 (Cell Signaling Technology). PINK1 protein is
501 indicated by the red box.

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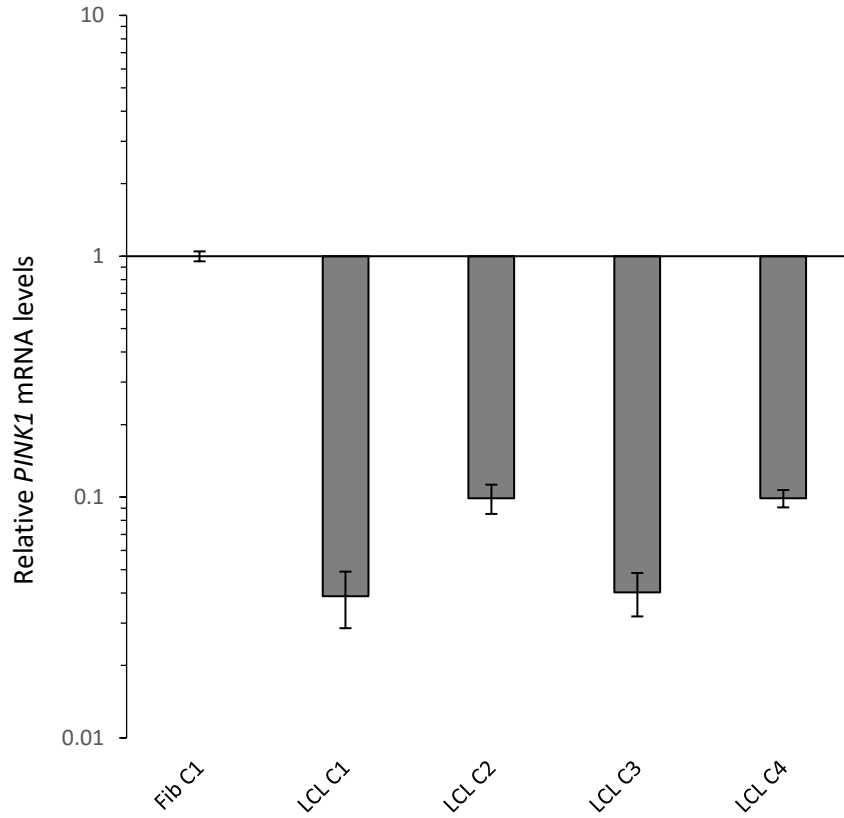
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518 **S3 Fig. Reverse transcriptase qPCR analysis of *PINK1* in LCLs.** qPCR was performed on equal
519 amounts of cDNA from each cell sample, and C_t values were expressed relative to the fibroblast (Fib)
520 sample.

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536 **S4 Table.** Age and sex of PBMC donors.

PBMC	Age	M/F
C1	31.2	F
C2	26.4	M
C3	25.3	M
C4	28.6	M
C5	60.1	M
C6	64.2	M
C7	66.9	M
C8	74.2	F
C9	27.7	M
C10	58.8	F
C11	31.4	F
C12	50.4	M
C13	34.1	F
C14	58.8	F
C15	72.8	M
C16	64.6	M

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556 **S5 Table.** Characteristics of antibodies used in this study.

Target	Host	Cat. No. (Supplier)	Immunogen	Clone	Dilution
MFN1	Ms mAB	ab57602 (Abcam)	Human Mitofusin 1 aa 1-741.	[3C9]	1:1000
MFN2	Ms mAB	ab56889 (Abcam)	Human Mitofusin 2 aa 661-757	[6A8]	1:1000
SDHA	Ms mAB	ab14715 (Abcam)	Full length native protein	[2E3GC12FB2AE2]	1:1000
bActin	Ms mAB	ab3280 (Abcam)	Chicken Actin aa 50-70	[C4]	1:1000
Parkin	Rb mAB	702785 (Thermo Fisher Scientific)	human Parkin aa 1 465	[21H24L9]	1:100
Parkin	Ms mAB	4211 (Cell Signaling Technology)	human recombinant parkin	[Prk8]	1:500
PINK1	Rb pAB	BC100-494 (Novus Biological)	Human PINK1 protein aa 175-250		1:1000
PINK1	Rb mAB	6946 (Cell Signaling Technology)	residues surrounding aa 140 of human PINK1 protein	[D8G3]	1:1000

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