

**Author contributions**

K.O., T.O., K.T. and N.M. designed the research and analysed the data. K.O., T.O., M.K., F.K., Y.F., K.S-F. and S.S. carried out immunoblotting experiments. K.O., M.I., K.I., M.K. and E.G. performed immunocytochemistry. M.F. carried out statistical analysis. H.K., N.T. and H.T. performed mass spectrometric analysis. H.S. did structural modelling. N.M. wrote the manuscript with help and supervision from M.K., N.H., K.M. and K.T.

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# VPS35 Mutation in Japanese Patients with Typical Parkinson's Disease

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**ABSTRACT:** Vacuolar protein sorting 35 (*VPS35*) was recently reported to be a pathogenic gene for late-onset autosomal dominant Parkinson's disease (PD), using exome sequencing. To date, *VPS35* mutations have been detected only in whites with PD. The aim of the present study was to determine the incidence and clinical features of Asian PD patients with *VPS35* mutations. We screened 7 reported nonsynonymous missense variants of *VPS35*, including p.D620N, known as potentially disease-associated variants of PD, in 300 Japanese index patients with autosomal dominant PD and 433 patients with sporadic PD (SPD) by direct sequencing or high-resolution melting (HRM) analysis. In addition, we screened 579 controls for the p.D620N mutation by HRM analysis. The p.D620N mutation was detected in 3 patients with autosomal dominant PD (1.0%), in 1 patient with SPD (0.23%), and in no con-

trols. None of the other reported variants of *VPS35* were detected. Haplotype analysis suggested at least 3 independent founders for Japanese patients with p.D620N mutation. Patients with the *VPS35* mutation showed typical tremor-predominant PD. We report Asian PD patients with the *VPS35* mutation. Although *VPS35* mutations are uncommon in PD, the frequency of such mutation is relatively higher in Japanese than reported in other populations. In *VPS35*, p.D620N substitution may be a mutational hot spot across different ethnic populations. Based on the clinical features, *VPS35* should be analyzed in patients with PD, especially autosomal dominant PD or tremor-predominant PD. ©2012 Movement Disorder Society

**Key Words:** Parkinson's disease; *VPS35*; autosomal dominant; hotspot; mutation.

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive motor disturbances manifested by tremor, rigidity, akinesia, and postural instability. Neuropathologically, PD is characterized by selective loss of dopaminergic neurons in the substantia nigra and the presence of cytosolic inclusions called Lewy bodies (LBs) in the remaining neurons. The pathogenesis of PD is multifactorial, including genetic-environmental interaction. PD is a common disease in the elderly, with an incidence of about 1%–2% in individuals older than 60 years.<sup>1</sup> Among PD patients, approximately 5%–10% have a positive family history of PD,<sup>2</sup> and among these, the Mendelian forms of PD can contribute to the elucidation of the molecular pathways that lead to the degeneration and death of dopaminergic neurons.

Mutations in the vacuolar protein sorting 35 (*VPS35*) gene have recently been identified in families with autosomal dominant late-onset PD (MIM 601501).<sup>3,4</sup> Patients with *VPS35* mutations present with tremor-predominant dopa-responsive parkinsonism.<sup>3,4</sup> *VPS35*, a key component of the retromer cargo-recognition complex, is thought to associate with sorting cargos into the tubular endosomal network for retrieval to the trans-Golgi network.<sup>5</sup> Therefore, pathogenic mutations of *VPS35* may cause disruption of the retrograde transport system and contribute to dopaminergic neuronal cell death in PD. One missense mutation has been reported to be pathogenic for PD.<sup>3,4</sup> Mutation of c.1858G>A (p.D620N) was identified in 3 Austrian families and 1 family each in Switzerland, the United States, Tunisia, and the United Kingdom, as well as 1 family and 1 patient with sporadic PD (SPD) among Yemenite Jews from Israel.<sup>3,4,6</sup> In addition, several variants, such as p.M57I, p.I241M, p.P316S, and p.R524W, have been reported in Europe and the United States as potentially pathogenic for PD.<sup>3,4</sup>

Although multipopulation screenings for *VPS35* mutations were performed in recent reports, there is still no report of PD patients with *VPS35* mutations of Asian ancestry.<sup>3,4,6–8</sup> In the present study, we screened Japanese patients with autosomal-dominant PD (ADPD), Japanese patients with SPD, and control subjects for mutations of *VPS35*, with a special focus on 7 reported nonsynonymous variants that were found in patients with PD, including the p.D620N. Here, we report 3 families and 1 SPD patient with the p.D620N mutation in *VPS35* and describe their clinical features.

## Patients and Methods

### Subjects

The study was approved by the ethics committee of Juntendo University, and all subjects gave written

informed consent to participate in the genetic research. The study subjects were 308 Japanese patients (300 index patients) with ADPD (age at disease onset [AAO; mean  $\pm$  SD],  $51.1 \pm 11.7$  years; range, 8–83 years; female/male [F/M] ratio, 1.35) and 433 Japanese SPD patients (AAO,  $47.2 \pm 12.9$  years; range, 5–88 years; F/M ratio, 1.09) selected from the gene bank of Juntendo University. Some of the selected subjects had been confirmed negative for *SNCA*, *PARK2*, *PINK1*, *PARK7*, *LRRK2*, and *PLA2G6* mutations.<sup>9–14</sup> From the same gene bank, we also selected 579 healthy Japanese subjects without a family history of parkinsonism (age at sampling,  $58.0 \pm 9.3$  years; range, 23–89 years; F/M ratio, 1.54). The criteria for the diagnosis of PD were adopted by the participating neurologists and were established based on the United Kingdom Parkinson's Disease Society Brain Bank.<sup>15</sup>

### Genetic Analysis

Genomic DNA was extracted from peripheral blood using a standard protocol. Patients with ADPD and SPD were examined for the following 7 variants: p.M57I (exon 3), p.I241M (exon 7), p.P316S (exon 9), p.R524W (exon 13), p.D620N (exon 15), p.A737V (exon 16), and p.L774M (exon 17) of *VPS35* (RefSeq accession number NM\_018206.4). PCR direct sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing kit and 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) or 3730 DNA Analyzer (Applied Biosystems). In addition, SPD patients and control subjects were also genotyped for c.1858G>A (p.D620N) mutation by high-resolution melting (HRM) analysis using LightScanner and LCGreen plus (Idaho Technology, Salt Lake City, UT). HRM analysis was performed using a previously described protocol<sup>16</sup> and the following primers: forward, GAGGATGGTTGGTCCTTGAA; reverse, TGCCAATGATCAAGGTGATG. All exons of *VPS35* were also analyzed in patients with the p.D620N mutation using the method described previously.<sup>3</sup>

Haplotype analysis of the *VPS35* flanking region was performed using 3130 Genetic analyzer and GeneMapper software (Applied Biosystems, Foster City, CA). To adjust the size of PCR products, we also genotyped Centre d'Étude du Polymorphisme Humain (CEPH) control samples (1331-01 and 1331-02) for comparison of haplotypes with previously reported patients carrying the p.D620N mutation. The sequences of the PCR primers were reported previously.<sup>3</sup>

## Results

### Detection of p.D620N Mutation

We detected the heterozygous missense p.D620N mutation in 3 unrelated patients with ADPD and 1

TABLE 2. Clinical features of patients with p.D620N mutation

	Patient ID			
	AII-11	BIII-8	CII-3	D
Age at disease onset (y)	62	55	34	42
Disease duration (y)	15	2	7	21
Resting tremor	+	+	+	+
Bradykinesia	+	+	+	+
Rigidity	+	+	+	+
Gait disturbance	+	—	—	+
Postural instability	+	—	—	+
Clinical response to levodopa	+	+	+	+
Wearing off	+	—	+	+
Asymmetry at onset	+	+	+	+
Orthostatic hypotension	+	—	—	—
Incontinence	+	—	—	—
Urinary urgency	—	—	—	—
Levodopa-induced dyskinesia	+	—	+	+
Sleep benefit	+	—	+	Unknown
Dystonia at onset	—	—	—	—
Hyperreflexia	—	—	—	—
Hallucination	—	—	—	—
Other psychosis	—	—	—	—
Dementia	+	—	—	—
Gaze palsy	—	—	—	—
Brain MRI	WNL	WNL	WNL	WNL
Cardiac MIBG scintigraphy	H/M ratio (E/L), 2.38/2.68; washout ratio, 4.15% <sup>a</sup>	Not performed	Not performed	Not performed

<sup>a</sup>MIBG scintigraphy was performed when AII-11 was 76 years old. WNL, within normal limit; H/M ratio, heart-to-mediastinum ratio; (E/L), early/late stage.

Clinical Presentation

Table 2 summarizes the clinical features of the 4 VPS35 mutation-positive patients. Patient AII-11 was a 77-year-old man who developed right upper limb rest tremor at age 62. At age 75, he underwent gastrostomy for progressive dysphagia, then developed cognitive dysfunction without hallucination. Single-photon emission computed tomography of cerebral blood flow showed no reduction in blood flow in the basal ganglia. His father and 4 of 8 siblings were diagnosed with PD (Fig. 1A) and presented levodopa-responsive typical parkinsonism: upper limb tremor and small-step gait. His nephew and niece were also diagnosed with PD, and the nephew developed parkinsonism in his early fifties. Patients BIII-8 and CII-3 both developed upper limb rest tremor at ages 34 and 55, respectively. The mother and aunts of patient BIII-8

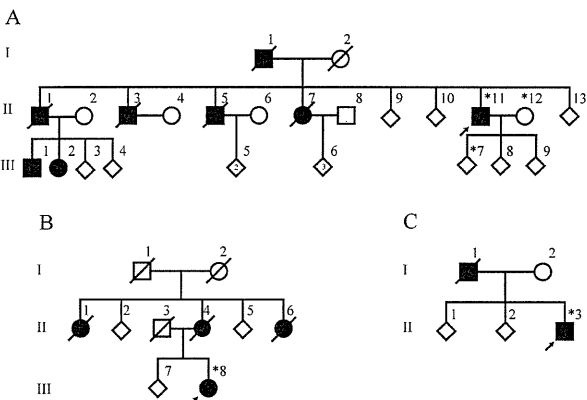


FIG. 1. Pedigrees of families with the VPS35 p.D620N mutation (open symbol, unaffected family member; closed symbol, affected member; arrow, proband; asterisk, individual analyzed for the p.D620N mutation and/or haplotype; forward slash through symbol, deceased individuals; square, male; circle, female; diamond, unspecified sex).

patient with SPD (Fig. 1). The p.D620N has been reported previously as a pathogenic mutation for familial PD.<sup>3,4,6</sup> This mutation was not found in 1158 control chromosomes. Patients carrying the p.D620N mutation did not have any other variants in all exons of VPS35. In our population, the incidence of the p.D620N mutation was 1.0% (3 of 300) in ADPD and 0.23% (1 of 433) in SPD. The remaining variants analyzed in this study were not identified in any patients.

Haplotype analysis demonstrated that the Japanese patients with the p.D620N mutation had different genotypes from those of white patients with the same mutation.<sup>3</sup> One disease allele was detected by analyzing patient AII-11 and his relatives. Patients AII-11 and BIII-8 in this study carried at least the same single allele of microsatellites in the flanking region of VPS35 (Table 1). On the other hand, patients CII-3 and D had a different genotype of D16S3105, with a locus mapped very close to VPS35, compared with the disease allele of AII-11 (Table 1, boldface).

TABLE 1. Haplotype analysis of VPS35 p.D620N mutation carriers

Microsatellite	Patient ID			
	AII-11	BIII-8	CII-3	D
D16S401	170	166/170	166/172	166/170
D16S3068	143	141/145	145/147	145/145
D16S753	272	272/268	268/276	264/268
VPS35 p.D620N	A	A/G	A/G	A/G
Chr16_45.333M	294	294/298	294/300	294/304
D16S3105	191	191/189	189/193	187/187
Chr16_45.615M	147	147/147	147/145	147/145
Chr16_45.806M	246	246/238	246/244	246/244
Chr16_45.835M	237	237/237	237/237	237/237
Chr16_45.855M	212	212/210	210/210	210/216
D16S3044	195	195/195	195/197	197/197

Both alleles are shown when markers of phase could not be determined.

and the father of patient CII-3 also developed PD (Fig. 1B, C). Patient D, who developed upper limb rest tremor at age 42, had no family history of PD. She underwent subthalamic nucleus deep brain stimulation (STN-DBS) at age 60 because of disabling motor fluctuation and dyskinesia refractory to pharmacological treatment. All affected patients were born to nonconsanguineous parents.

## Discussion

*VPS35* has been reported as the pathogenic gene for ADPD, and only 1 mutation, p.D620N, has been reported in several unrelated white families. To our knowledge, there have been no reports of Asian PD patients with *VPS35* mutations.<sup>3,8</sup> Based on this background, we set out in this study to determine the incidence of *VPS35* mutations in Japanese patients with PD. We detected the heterozygous p.D620N mutation of *VPS35* in 3 ADPD families and 1 SPD patient with East Asian ancestry. On the other hand, we could not conclude the pathogenicity of 6 other variants that had been reported as potentially pathogenic for PD because none of the variants was detected in our patients with PD.

The frequency of the p.D620N mutation in Japanese patients was 1.0% in ADPD and 0.23% in SPD. Although the exact frequency among whites is undetermined, the frequency is relatively higher in Japanese patients compared with that reported in previous studies (0%–1.22%).<sup>3,4,6,7,17</sup> Moreover, the frequency in Japanese patients also differs greatly from those of other Asian populations such as Taiwanese patients and mainland Chinese patients (0%).<sup>3,8</sup> Although the mutation frequency was expected to be lower than that of other pathogenic genes for ADPD, such as multiplication of *SNCA*<sup>9,18</sup> and point mutation of *LRRK2*,<sup>19–21</sup> *VPS35* may be one of the most important genes in Japanese PD. Because we screened for only 7 reported variants, we cannot determine the exact frequency of *VPS35* mutations in ADPD; we would need to analyze all 17 exons of *VPS35* in ADPD patients to screen for other variants and to assess the incidence of all disease-associated *VPS35* mutations.<sup>3,4</sup> Furthermore, we would need to perform mutational analysis for SPD patients, in addition to ADPD, to identify Asian population-specific variants, such as *LRRK2* p.G2385R, associated with susceptibility for PD.<sup>19</sup>

Based on haplotype analysis reported in previous studies, the substitution of *VPS35* c.1858G>A (p.D620N) occurs from independent mutational events.<sup>3</sup> We were able to determine the chromosomal phase only in patient AII-11 (family A). The p.D620N mutation possibly shared a common founder between Japanese ADPD families A and B; however, it was inconclusive because the phase of patient BIII-8 was

undetermined. On the other hand, the same p.D620N mutation probably occurred independently in patient CII-3 (family C) and patient D. By genotyping of D16S3105, which is located approximately 1.5 kb centromeric of *VPS35*, there were at least 3 different haplotypes in Japanese because families A and C and patient D (SPD) did not have the same alleles for this microsatellite. To determine the chromosomal phase of families B and C, detailed genetic analyses of other family members are needed in future studies. These results suggest the existence of 3 or more founders in Japanese patients, in addition to the reported white patients with the p.D620N mutation or de novo mutations, indicating that the p.D620N mutation site is a mutational hot spot in *VPS35* across different ethnic populations.

According to previous reports, the average AAO of patients with the *VPS35* mutation was 50–60 years ( $50.6 \pm 7.3$  years),<sup>3</sup> with a distinctive feature of a slightly younger AAO compared with patients with idiopathic PD. In our study, the AAO was nonspecific with a wide range between 30–70 years. Because the family history of patient D was unknown, she was categorized as SPD. With regard to *VPS35* mutation penetrance, it is incomplete from the results of a previous report.<sup>3</sup> Therefore, although the frequency is low, patients with p.D620N mutation could be found among SPD patients.

The clinical symptoms of our patients with *VPS35* mutation closely resembled the idiopathic PD form, with tremor-dominant dopa-responsive parkinsonism. Psychiatric problems were inconspicuous; however, dementia may occur in patients with a long disease course, similar to patient AII-11, who had PD for 15 years. Our patients with *VPS35* mutations had normal brain MRI and cardiac MIBG scintigraphy. There have been no definite pathological mutations of *VPS35* in the spectrum of LB disorders. On the basis of these results, patients with *VPS35* mutation could show comparatively benign disease course without widespread LB pathology.<sup>22,23</sup>

*VPS35* assembles into the retromer cargo-recognition complex that associates with the cytosolic face of the endosomes. The retromer mediates the retrograde transport of transmembrane cargo from the endosomes to the trans-Golgi network.<sup>5</sup> The p.D620N mutation of *VPS35* might cause impairment of interaction with other components of the retromer complex and impaired retrograde trafficking of recycling proteins,<sup>4</sup> similar to  $\alpha$ -synuclein and *LRRK2*, which are involved in vesicle trafficking.<sup>24,25</sup> Mutations in familial PD genes, including *VPS35*, may cause disruption of intracellular trafficking and lead to neurodegeneration. These findings suggest that impairment of intracellular trafficking systems is associated with the pathogenesis of PD. Although the association between the p.D620N mutation of *VPS35* and PD remains unknown, further functional studies might shed light on the pathogenesis

of VPS35 mutation and the effects of interaction with other known pathogenic gene products on PD.

In conclusion, we have reported Asian PD patients with the VPS35 p.D620N mutation. The p.D620N substitution may be a mutational hot spot across different ethnic populations. The frequency of VPS35 mutation was low in ADPD; however, it is relatively high in Japanese patients compared with that reported in other populations.<sup>3,4,6–8</sup> Based on the clinical features of patients with VPS35 mutation, VPS35 should be analyzed in patients with PD, especially ADPD or tremor-predominant PD. ■

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RESEARCH

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# Mitochondrial dysfunction associated with increased oxidative stress and $\alpha$ -synuclein accumulation in PARK2 iPSC-derived neurons and postmortem brain tissue

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## Abstract

**Background:** Parkinson's disease (PD) is a neurodegenerative disease characterized by selective degeneration of dopaminergic neurons in the substantia nigra (SN). The familial form of PD, PARK2, is caused by mutations in the *parkin* gene. *parkin*-knockout mouse models show some abnormalities, but they do not fully recapitulate the pathophysiology of human PARK2.

**Results:** Here, we generated induced pluripotent stem cells (iPSCs) from two PARK2 patients. PARK2 iPSC-derived neurons showed increased oxidative stress and enhanced activity of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. iPSC-derived neurons, but not fibroblasts or iPSCs, exhibited abnormal mitochondrial morphology and impaired mitochondrial homeostasis. Although PARK2 patients rarely exhibit Lewy body (LB) formation with an accumulation of  $\alpha$ -synuclein,  $\alpha$ -synuclein accumulation was observed in the postmortem brain of one of the donor patients. This accumulation was also seen in the iPSC-derived neurons in the same patient.

**Conclusions:** Thus, pathogenic changes in the brain of a PARK2 patient were recapitulated using iPSC technology. These novel findings reveal mechanistic insights into the onset of PARK2 and identify novel targets for drug screening and potential modified therapies for PD.

**Keywords:** Induced pluripotent stem cells, Parkinson's disease, Parkin, Oxidative stress, Mitochondria,  $\alpha$ -synuclein

## Background

*Parkin* is a causative gene of autosomal recessive juvenile Parkinson's disease (PARK2). It encodes a component of an E3 ubiquitin ligase involved in mitochondrial homeostasis [1-5]. *Parkin* deficiency is thought to result in aberrant ubiquitination and compromised mitochondrial integrity, leading to neuronal dysfunction and degeneration. Several PARK2 mouse models exist, but they do

not replicate all of the pathogenic changes seen in human PARK2 neurons; thus, these models do not fully account for the molecular mechanisms of PD [6-9]. A recent report demonstrated that there is a defect in dopamine (DA) utilization in PARK2 induced pluripotent stem cell (iPSC)-derived neurons [10]. However, it is not known whether neuronal homeostasis is disrupted in PARK2 patients. Furthermore, studies have yet to demonstrate whether the phenotype of PD-specific iPSC-derived neurons recapitulates the *in vivo* phenotype of the corresponding cell donor. To address these questions, we generated iPSCs from two PARK2 patients

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(PA and PB) [11]. In PARK2 iPSC-derived neurons, but not PARK2 fibroblasts or iPSCs, abnormal mitochondrial morphology and aberrant tubulovesicular structures adjacent to the Golgi were observed, as was increased oxidative stress. Although  $\alpha$ -synuclein accumulation and Lewy body (LB) formation are very rare in PARK2 patients [1,12,13], we observed pathological changes and prominent LB formation, including the accumulation of  $\alpha$ -synuclein, in postmortem brain tissue from one of the donor patients (PA). However, we obtained autopsied brain tissue from the father of donor PB, who carried the same *parkin* deletion as PB, and observed no evidence of LB formation or  $\alpha$ -synuclein-positive cells. Consistent with these observations in postmortem brain tissue, increased  $\alpha$ -synuclein accumulation was clearly observed in PA iPSC-derived neurons *in vitro*, but not in PB iPSC-derived neurons. These results are the first demonstration of pathogenic changes in the brain of a PARK2 patient that were recapitulated using iPSC technology. Our findings also provide mechanistic insights into PARK2 pathophysiology.

**Results & discussion**

**Generation of PARK2 iPSCs**

iPSCs were generated from dermal fibroblasts isolated from two PARK2 patients carrying *parkin* mutations and two control subjects using retroviruses carrying *Oct4*, *Sox2*, *Klf4*, and *c-Myc* to reprogram the cells as previously described [14,15]. The PARK2 patients were a 71-year-old female (PA) with a homozygous deletion of *parkin* exons 2–4 and a 50-year-old male (PB) with a homozygous deletion of exons 6 and 7 (Table 1 and Additional file 1A and B). Patient PA died 1 year after enrollment in the study at the age of 72. A previously-established human iPSC clone from control subject A, 201B7 (B7), was also used [15]. In addition, the following human embryonic stem cell (hESC)-like iPSC clones were selected for detailed analysis: three controls (B7 and YA9 from control A, and WD39 from control B), three from patient PA (PA1, PA9 and PA22), and four from patient PB (PB1, PB2, PB18 and PB20) (Figure 1A and Additional file 2A and B).

The PARK2 iPSCs expressed pluripotent hESC markers (Figure 1A and Additional file 2A-C) and formed teratomas containing all three germ layers (Additional file 2D).

All of the retroviral transgenes were silenced in each clone (Additional file 2E). The iPSCs derived from PA and PB retained the corresponding homozygous *parkin* deletions and exhibited genomic stability (Figure 1B-D; Additional file 3A and B; and Table 1). All of the clones differentiated into neurons, including tyrosine hydroxylase (TH)-positive neurons, through a process of embryoid body and neurosphere formation (Figure 1A). Thus, all of the lines were successfully reprogrammed into a pluripotent state and were suitable for further analysis.

**Increased oxidative stress accompanied by activation of the Nrf2 pathway in PARK2 iPSC-derived neurons**

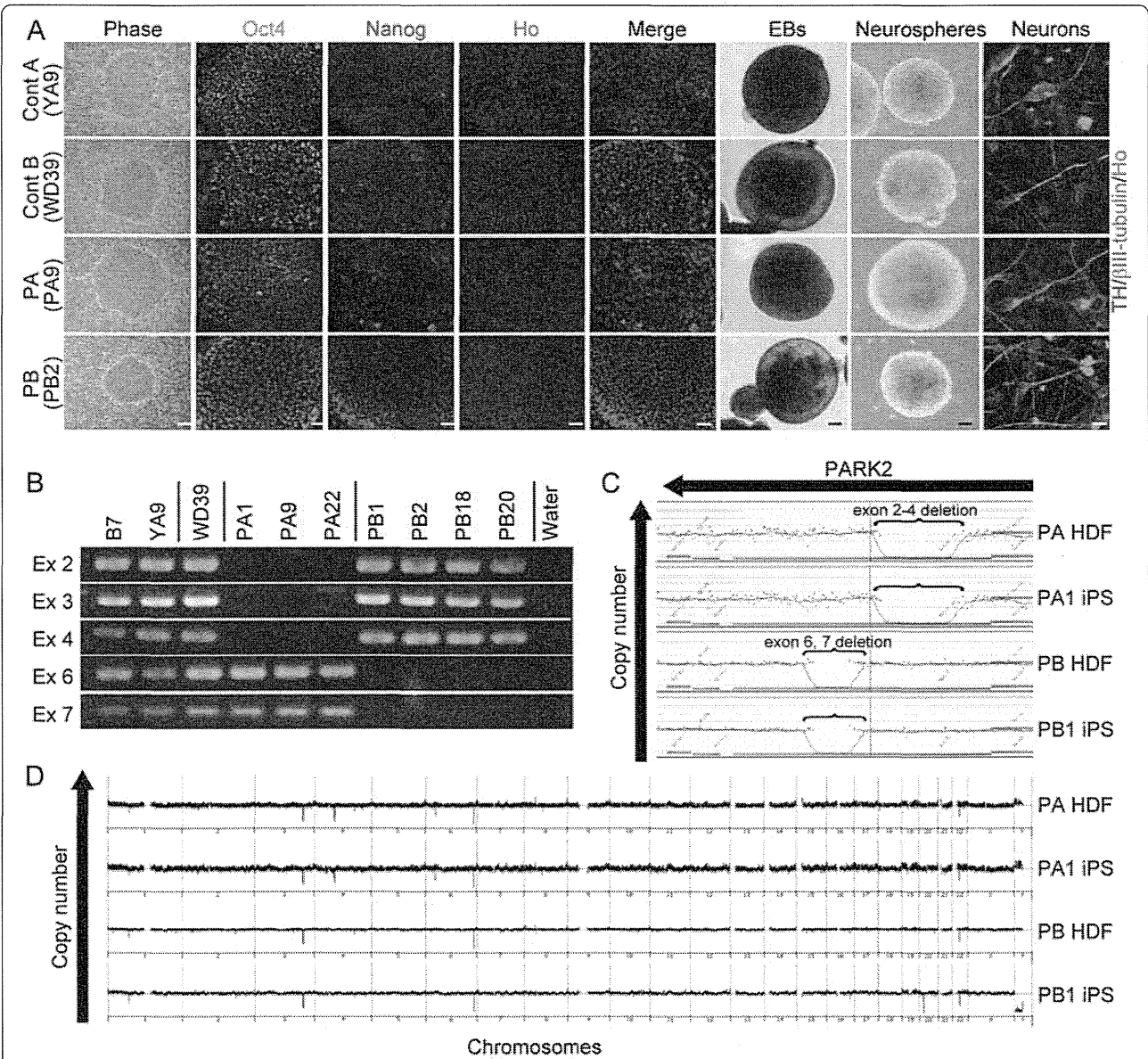
Because increased levels of oxidative stress have been documented in other PD models [7,10,16,17], we examined oxidative metabolism in the iPSC clones by measuring the cellular levels of reduced glutathione (GSH). GSH reacts with reactive oxygen species (ROS) and is catalyzed by glutathione S-transferase[18]. Consistent with previous results from patient-derived cells [16], the levels of GSH in PARK2 iPSC-derived neurospheres were significantly lower than those in control iPSC-derived neurospheres (Figure 2A). We also examined ROS production using 2', 7'-dichlorodihydrofluorescein (DCF) fluorescence to measure the levels of intracellular oxidants. The DCF fluorescence intensity in the PARK2 iPSC-derived neurons was significantly higher than that in control iPSC-derived neurons (Figure 2B and C), which indicated an increased level of oxidative stress. A recent study showed that, in PARK2 iPSC-derived neurons, monoamine oxidase (MAO)-A and -B levels and oxidative stress levels are increased, as is spontaneous DA release [10]. Here, we found no significant differences in MAO-A and -B expression levels between PARK2 and control neurons (Additional file 4A and B).

The Nrf2 pathway plays a cytoprotective role under conditions of ROS accumulation. Recent studies show that activation of the Nrf2 pathway reduces oxidative stress and provides partial protection from MPTP-mediated neurotoxicity[19]. Elevated Nrf2 expression was observed in the postmortem brain of a PD patient [20]. These data suggest a putative link between the Nrf2 pathway and PD, and prompted a closer investigation of this signaling pathway in control and PARK2 iPSC-derived neurons [19-21]. The expression of Nrf2 pathway proteins, such as Nrf2 and NADH quinone oxidoreductase (NQO1), was significantly increased in PARK2 iPSC-derived neurons (Figure 2D and E). These data are in line with previous reports [19-21], and suggest that the Nrf2 cytoprotective pathway may be activated in PARK2 iPSC-derived neurons to prevent further damage from oxidative stress. Taken together, these data demonstrated an increased level of

**Table 1 PA and PB patient information**

	PA patient	PB patient
Race	Japanese	Japanese
Age	72 y/o	50 y/o
Sex	Female	Male
Age of onset	62 y/o	28 y/o
Mutation of <i>parkin</i>	Exon 2–4 homozygous deletions	Exon 6, 7 homozygous deletions



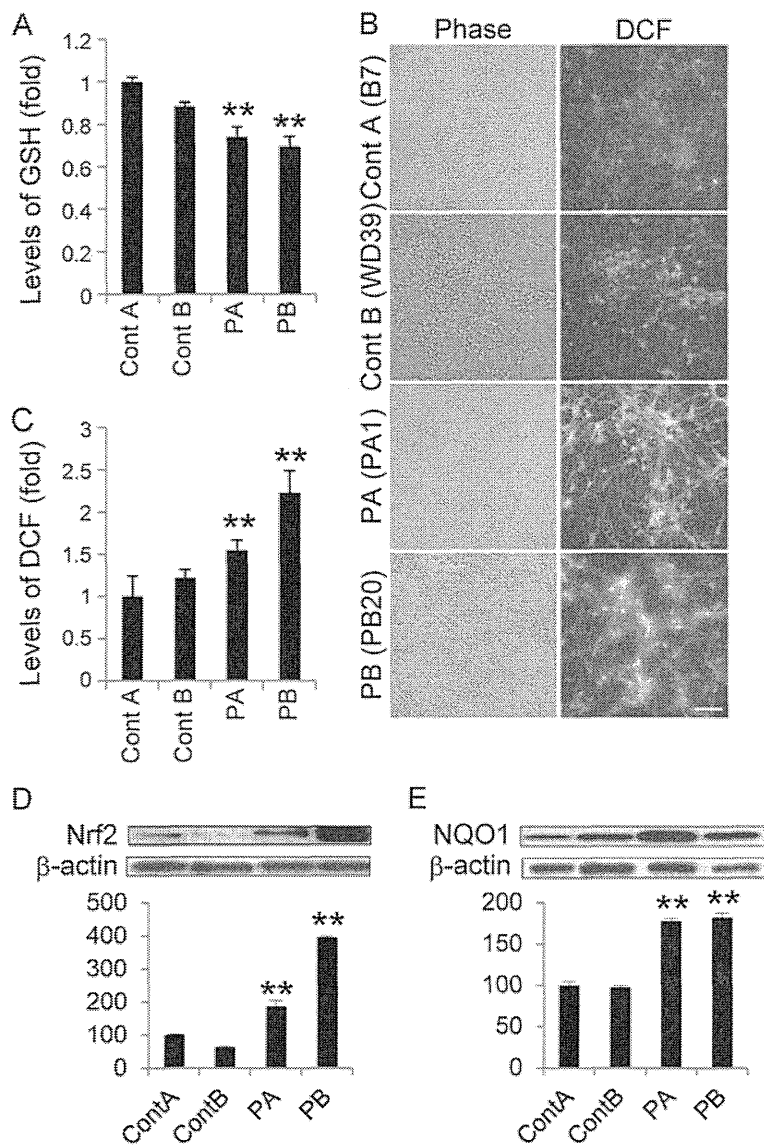


**Figure 1 Generation of PARK2 iPSCs.** (A) iPSCs derived from control and PARK2 subjects, embryoid bodies (EBs), neurospheres (NSs), and neurons. *Left three rows:* iPSCs from Control A (YA9), Control B (WD39), PA (PA9), and PB (PB2) were immunopositive for the pluripotency markers Oct4 (green) and Nanog (red). *Right three rows:* Differentiation of iPSCs into tyrosine hydroxylase (TH)-positive (red) neurons via EB and NS formation. Scale bars: Phase contrast, 400  $\mu$ m; Nanog and Oct4 immunostaining, 100  $\mu$ m; EBs, 25  $\mu$ m; NSs, 50  $\mu$ m; neurons, 10  $\mu$ m. (B) Deletion of exons 2–4 in clones PA1, 9 and 22; and of exons 6 and 7 in clones PB1, 2, 18, and 20 was confirmed. (C) Exons 2–4 were deleted in human dermal fibroblasts (HDFs) from PA and in PA1 iPSC lines. Exons 6 and 7 were deleted in HDFs from PB and PB1 iPSC lines. (D) Copy number profiles of whole chromosomes in PARK2 HDFs and iPSCs were assessed by comparative genomic hybridization (CGH) microarray analysis; there was no evidence that genomic aberrations were introduced during the process of establishing PARK2 iPSCs.

oxidative stress accompanied by activation of the Nrf2 pathway in PARK2 neurons.

**Abnormal mitochondrial morphology and impaired mitochondrial turnover in PARK2 iPSC-derived neurons**  
Increased oxidative stress (which affects anti-oxidant defense systems) and mitochondrial dysfunction are implicated in the pathogenesis of PD [1,13,21-23]. Furthermore,

ROS accumulation causes both oxidative damage and mitochondrial dysfunction in the substantia nigra (SN) of *parkin*-deficient mice [7]. However, the exact mechanism of mitochondrial pathogenesis associated with PARK2 is controversial. For example, while *Drosophila parkin* mutants show abnormal mitochondrial morphology, *parkin*-knockout mice do not [7,24]. In addition, while a greater degree of mitochondrial branching is observed in



**Figure 2 Increased oxidative stress accompanied by activation of the Nrf2 pathway in PARK2 iPSC-derived neurons.** (A) GSH levels were significantly reduced in PARK2 (PA1, 9 and 22, and PB2, 18 and 20) iPSC-derived neurospheres compared with those in control A (YA9) and B (WD39) neurospheres. (B, C) DCF fluorescence intensity in PARK2 (PA1, 9 and 22, and PB2 and 20) iPSC-derived neurons was significantly higher than that in control A (B7) and B (WD39) neurons. (D, E) Immunoblot analysis of Nrf2 and NQO1 levels in iPSC-derived neurons from PA and PB. Expression of Nrf2 and NQO1 in PARK2 (PA9 and PB2) iPSC-derived neurons was significantly higher than that in control A (YA9) and B (WD39) neurons. Relative protein abundance was normalized to  $\beta$ -actin. \*\* indicates  $P < 0.01$  (Mann-Whitney  $U$ -test). Data represent the mean and SEM of at least three experiments for each group.

fibroblasts derived from PARK2 patients, the detailed morphology of the mitochondria in these cells has not been characterized [25]. To investigate these mitochondrial abnormalities in more depth, we performed a detailed morphological analysis of mitochondria in PARK2 iPSC-derived neurons using electron microscopy. Mitochondria in PARK2 neurons from both patients showed a highly electron-dense matrix and swollen mitochondrial cristae within the inner mitochondrial membrane (IMM) (Figure 3A, black arrowheads). The perikaryal volume

density of the abnormal mitochondria was significantly increased in PA and PB iPSC-derived neurons relative to control clones (Figure 3B). Furthermore, the density of normal mitochondria decreased (Figure 3B). Importantly, both abnormal and normal mitochondria were observed in PARK2 neurons (Figure 3A, white arrowheads). Abnormal mitochondria were observed in 87.8% of iPSC-derived neurons from PA, and 79.5% of iPSC-derived neurons from PB. These data indicated that abnormal mitochondrial morphology was a feature of most PARK2 iPSC-derived

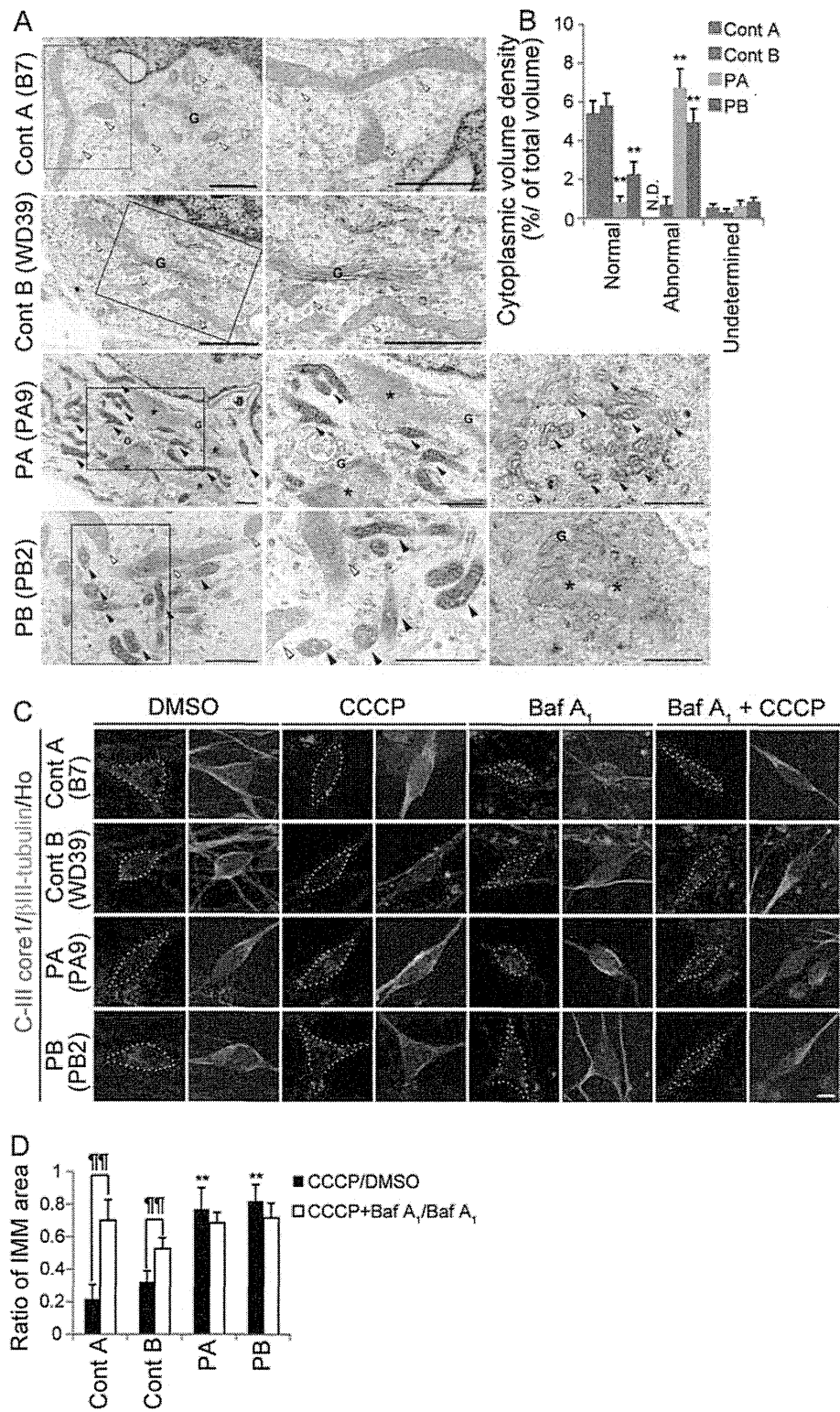


Figure 3 (See legend on next page.)

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**Figure 3 Dysregulation of mitochondrial homeostasis in PARK2 iPSC-derived neurons.** (A) Electron micrographs of control A (B7), control B (WD39) and PARK2 (PA9 and PB2) iPSC-derived neurons. Boxed areas are shown in the enlarged images to the right. Control mitochondria showed a characteristically long, cylindrical profile with well-organized cristae, and the electron density of the matrix was relatively low (white arrowheads). By contrast, increased electron density of the matrix was evident in PARK2 mitochondria (black arrowheads), and the cristae often appeared swollen. As shown in PB2, some of the neurons contained both morphologically intact (white arrowheads) and abnormal (black arrowheads) mitochondria. Furthermore, abnormal tubulovesicular structures (asterisks) were observed adjacent to the Golgi cisternae (G). (B) The relative perikaryal volume of the abnormal mitochondria was significantly increased, and that of the normal mitochondria was decreased, in PARK2 neurons compared with control neurons. (C) Double labeling for the IMM marker, ComplexIII coreI (CIII-Core I; magenta) and  $\beta$ III-tubulin (green) of control A (B7), control B (WD39) and PARK2 (PA9 and PB2) iPSC-derived neurons. The volume of the IMM area was reduced in control neurons treated with CCCP, but not in PARK2 neurons treated with CCCP. Administration of Baf A<sub>1</sub> rescued the CCCP-induced phenotype in control neurons. (D) The CCCP/DMSO ratio in control A (B7 and YA9) and B (WD39) neurons was reduced after CCCP treatment. This reduction was not observed in PARK2 (PA1, 9 and 22, and PB2 and 20) iPSC-derived neurons (black bars indicate CCCP/DMSO ratio; white bars indicate Baf A<sub>1</sub>+CCCP/Baf A<sub>1</sub> ratio). \*\* indicates  $P < 0.01$  compared with the control; ¶¶ indicates  $P < 0.01$  when comparing the black and white bars (Mann-Whitney U-test). At least three experiments were performed for each group, with 5–36 cells quantified per experiment. Scale bars: a, 1  $\mu$ m; c, 10  $\mu$ m. Error bars represent the SEM. N.D., not detected.

neurons from these patients. In addition, abnormal tubulovesicular structures were observed adjacent to the Golgi cisternae in PARK2 iPSC-derived neurons (Figure 3A). These abnormal mitochondrial and tubulovesicular structures were not observed in PARK2 fibroblasts or in undifferentiated iPSCs (Additional file 5A and B). These histological abnormalities represent novel PARK2-related neuronal pathologies.

PARKIN is involved in the mitochondrial fission/fusion system and is recruited to depolarized mitochondria to promote mitophagy [5,26–29]. In iPSC-derived neurons containing a mutation in PINK1 (a protein kinase upstream of PARKIN), PARKIN is not recruited appropriately to mitochondria [30]. We hypothesized that PARKIN-deficient human neurons would show aberrant removal of depolarized mitochondria. To examine the turnover of damaged mitochondria, we treated iPSC-derived neurons with carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), which triggers the loss of mitochondrial membrane potential and results in the removal of damaged mitochondria. The intensity of TMRE, a mitochondrial membrane potential-dependent dye, clearly decreased in both control and PARK2 iPSC-derived neurons treated with CCCP, which indicated a reduced mitochondrial membrane potential in both sets of neurons (Additional file 6). To determine the extent to which the damaged mitochondria were eliminated, we measured the area of the IMM after CCCP treatment. Compared with untreated cells, there was a dramatic loss of IMM area in the treated control neurons, but not in the treated PARK2 neurons (Figure 3C, left four columns; Figure 3D, black bars). To assess whether lysosomes were involved in the CCCP-induced elimination of mitochondria, we treated cells with Bafilomycin (Baf) A<sub>1</sub>, an inhibitor of the vacuolar type H(+)-ATPase. Baf A<sub>1</sub> attenuated the CCCP-dependent reduction in the IMM area in control neurons (Figure 3C, right four columns; Figure 3D, white bars). To confirm that the abnormal turnover of damaged mitochondria was characteristic of neuronal cells, PARK2 fibroblasts and undifferentiated

iPSCs were treated with CCCP. CCCP-treated PARK2 fibroblasts and undifferentiated iPSCs exhibited the same mitochondrial dynamics as CCCP-treated control cells (Additional file 5C–E). Together, these data indicated aberrant degradation of mitochondria damaged by CCCP treatment in PARK2 iPSC-derived neurons.

These results support a recently proposed working model for PD, in which damaged mitochondria accumulate due to a disruption in PARKIN-mediated mitochondrial quality control [28]. The electron microscopy data, which showed a mixture of abnormal and normal mitochondria, indicated that PARKIN-mediated mitochondrial quality control is compromised, even in young PARK2 iPSC-derived neurons. In these cells, residual normal mitochondria may have compensated for the damaged ones. Thus, while our findings suggest that the PARKIN-dependent mechanisms that regulate mitochondrial homeostasis are disrupted in PARK2 cells, further detailed analyses are required to fully understand the mechanism underlying this disruption and the implications for PD.

#### Patient-specific accumulation of $\alpha$ -synuclein in PARK2 iPSC-derived neurons and its correlation with LB formation

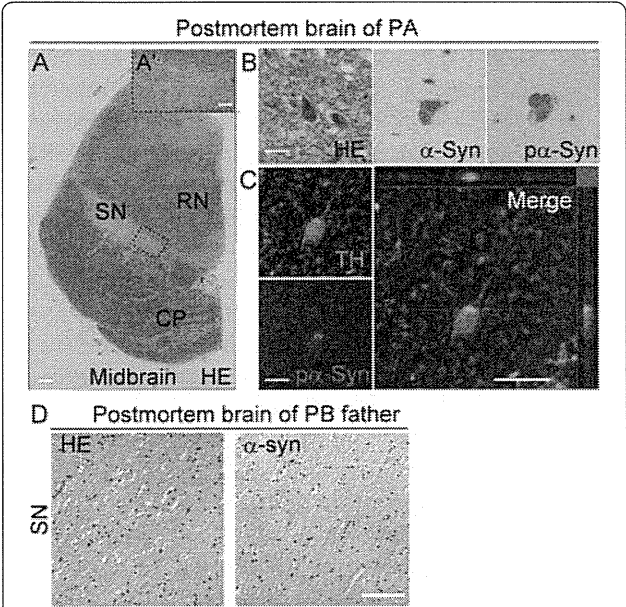
LBs are pathological neuronal inclusions composed principally of  $\alpha$ -synuclein. They are typically associated with PD and certain forms of dementia [1,13,31]. Although LBs are generally thought to be absent from PARK2 patients [1,13,31], rare cases of LB formation in the brains of PARK2 patients have been reported recently [12,32,33]. The PARKIN protein co-localizes with LBs in some patients with sporadic PD [34], and a functional interaction between PARKIN and  $\alpha$ -synuclein is indicated by both *in vitro* and *in vivo* findings [35–37]. These results suggest that PARKIN-pathway may contribute to LB formation in PD patients.

We were able to conduct a histopathological analysis of postmortem brain tissue from patient PA. Hematoxylin and eosin staining of the SN revealed low levels of brown-black

melanin pigment compared with healthy SN tissue (Figure 4A and A'). Surprisingly, LBs accumulated in the SN and other areas of the brain in patient PA (Figure 4B and Table 2). Furthermore,  $\alpha$ -synuclein and p $\alpha$ -synuclein immunoreactive puncta and neurites were observed in the areas where LBs were present (Figure 4B). TH/p $\alpha$ -synuclein double-positive neurons were also detected in the SN (Figure 4C). Of note,  $\alpha$ -synuclein-positive/TH-negative or p $\alpha$ -synuclein-positive/TH-negative neurons in the SN and other areas of the brain tissue from patient PA's brain were observed (Table 2). These data suggested that  $\alpha$ -synuclein accumulated not only in TH+ neurons, but also in other types of neurons. Postmortem tissue from the brain of the father of patient PB was also examined. The father carried a homozygous deletion of exons 6 and 7 of the *parkin* gene (Figure 1B, Additional file 1B and 7A), similar to patient PB. There was no evidence of LBs or  $\alpha$ -synuclein-positive neurons in the autopsied brain tissue of the father (Figure 4D). Thus, since the genetic background of patient PB and his father are likely to be very close (Additional file

1B and 7A), these results are probably reflective of a specific phenotype of patient PB, which was different from that in patient PA (Figure 4A-D).

To determine whether iPSC-derived neurons recapitulated the *in vivo* phenotypes of the corresponding cell donors, we next examined  $\alpha$ -synuclein accumulation in PARK2 iPSC-derived neurons. To rule out the possibility that  $\alpha$ -synuclein expression in undifferentiated PARK2 iPSCs was increased by multiplication of the *SNCA* gene, genomic aberrations acquired during the process of iPSC establishment, or by repeated passage of the cells, the *SNCA* gene copy number in iPSCs was quantified by genomic qPCR. A comparison with control iPSCs showed that iPSCs from both PA and PB carried the normal number of *SNCA* gene copies (Additional file 8A). Moreover, immunostaining for  $\alpha$ -synuclein did not reveal any increase or decrease in  $\alpha$ -synuclein protein levels in PARK2 iPSCs (Additional file 8B). As a control for LB formation, we generated iPSC-derived neurons from a 106-year-old woman (designated Cent1-8), since previous work suggested that aging is a predisposing factor for LB formation in PD patients [31,38]. Since  $\alpha$ -synuclein was also expressed in non-neural cells, triple labeling for  $\alpha$ -synuclein,  $\beta$ III-tubulin, and TH was performed to ensure that only neurons were examined (Figure 5A, asterisks). The proportion of  $\alpha$ -synuclein-positive iPSC-derived neurons that were also positive for  $\beta$ III-tubulin from PB was similar to that in the controls (including Cent1-8); however, the proportion was significantly higher in PA. These results were consistent with the *in vivo* phenotypes of the cell donors based on analysis of postmortem brain tissue (1629, 357, 805, 3747, and 4330 iPSC-derived  $\beta$ III-tubulin+ neurons in control A, control B, Cent1-8, PA and PB respectively; Figure 5A-C, arrows and arrowheads). Thus, the increase in  $\alpha$ -synuclein expression levels seen in PARK2 iPSC-derived neurons



**Figure 4 Accumulation of LBs in the postmortem brain of patient PA. (A–C)** Immunohistochemical staining of postmortem brain tissue from patient PA. (A) Low magnification image of a midbrain section stained with hematoxylin and eosin (H&E). (A') High magnification image of the boxed area. Melanin levels were reduced in most of the substantia nigra (SN). (B) (Left) High magnification image of a midbrain section stained with H&E showing the presence of Lewy bodies (LBs) in the SN. (Middle and Right)  $\alpha$ -synuclein-positive and p $\alpha$ -synuclein-positive cells in the SN. (C) Confocal microscopy image of a TH (green) and p $\alpha$ -synuclein (red) double-positive SN neuron and a projected merged image: p $\alpha$ -synuclein accumulated in the TH-positive neuron. (D) Melanin levels were reduced in most of the SN. No LBs or  $\alpha$ -synuclein-positive neurons were observed in postmortem brain tissue from the father of patient PB. Scale bars: A, 1000  $\mu$ m; A', 350  $\mu$ m; B, C, 50  $\mu$ m; D, 100  $\mu$ m.

**Table 2 LB type pathology in PA patient's postmortem brain**

Brain area		LB type pathology
Brainstem lesion	IX-X	+++
	LC	+++
	SN	++
Basal forebrain/Limbic	nbM	++
	Amy	++
	Ent	+
	Cing	+
Neocortical	T	-
	F	-
	P	-

IX-X, motor cranial nerves IX-X; LC, Locus Coeruleus; SN, Substantia Nigra; nbM, nucleus basal of Meynert; Amy, Amygdala; Ent, Entorhinal cortex; T, Temporal lobe; F, Frontal lobe; P, Parietal lobe.

cannot be attributed solely to the effects of aging, but associated with the disease phenotype.

The obvious LB-formation was observed in the postmortem brain of PA patient, who showed a late onset at 61 years, corresponding to the enhanced  $\alpha$ -synuclein accumulation in the iPSC-derived neurons from the same patient. Thus, it is likely that early-stage LB formation was recapitulated *in vitro* in iPSC-derived neurons. Furthermore, the present findings are consistent with recent work by several groups, which suggest that the age of onset of PARK2 in patients with LB formation (41 on average) is later than in patients without LB formation (below 40) [12,32,33]. The earlier onset in patient PB (at 28 years) than in PA (at 61 years) would be consistent with the finding of lower  $\alpha$ -synuclein accumulation in PB iPSC-derived neurons compared with PA iPSC-derived neurons. On the other hand, and in contrast to the observations of brain tissue from PA, analysis of brain tissue from the father of patient PB, in whom the onset of PD was 39 years of age, revealed no evidence of LB formation (Figure 4D). Importantly, PA iPSC-derived neurons showed significantly more  $\alpha$ -synuclein accumulation than PB iPSC-derived neurons (Figure 5A and C). These results suggest that the extent of  $\alpha$ -synuclein accumulation is an important factor in LB formation. Then, how can we explain the difference of  $\alpha$ -synuclein accumulation between PA and PB patients-derived neuronal cells? It is possible that PA is a rare example of PARK2 complicated by sporadic PD. Although both PA and PB iPSCs showed a normal SNCA gene copy number, it is possible that PA-derived cells acquired an unknown gene mutation relating to LB formation. Thus, we cannot rule out the possibility that other factors may affect LB formation in PARK2 patients. Further analyses will be required to identify these putative factors. Although iPSC clones from sporadic and familial PD patients were recently established [17,30,39-42], this report is the first to demonstrate that the phenotype of PD-specific iPSC-derived neurons replicates the *in vivo* phenotype seen in postmortem brain tissue from the corresponding cell donor.

## Conclusions

In summary, dysfunctional neuronal homeostasis (characterized by increased oxidative stress and activation of the Nrf2 pathway), impaired mitochondrial function, and increased  $\alpha$ -synuclein accumulation were observed in PARK2 iPSC-derived neurons. These results indicate that PARK2-associated phenotypes may appear soon after, or possibly even before, the onset of PARK2. Detailed analyses of PARK2 iPSC-derived neurons, particularly mature neurons, to determine the time course of LB accumulation and synaptic dysfunction will be of great interest. Such analyses will further our understanding of the pathogenesis of PARK2 as well as sporadic PD. The ultimate goal is the

development and application of novel preventative therapies for PD.

## Materials & methods

### Isolation of human skin fibroblasts and generation of iPSCs

For control A, human dermal fibroblasts (HDFs) from the facial dermis of a 36-year-old Caucasian female (Cell Applications Inc.) were used to establish iPSCs (201B7; Passage 20-29, YA9; Passage 15-24). The 201B7 iPSCs were kindly provided by Dr. Yamanaka [15]. A skin-punch biopsy from a healthy 16-year-old Japanese female obtained after written informed consent (Keio University School of Medicine) was used to generate the control B iPSCs (WD39; Passage 8-17). PA iPSCs (PA1, 9, and 22; Passage 10-19) and PB iPSCs (PB1, 2, 18, and 20; Passage 8-17) were generated from a 71-year-old Japanese female patient and a 50-year-old Japanese male patient, respectively, using the same methods used to generate control B iPSCs. The maintenance of HDFs, lentiviral production, retroviral production, infection, stem cell culture and characterization, and teratoma formation were performed as described previously [14,15]. All of the experimental procedures for skin biopsy and iPS production were approved by the Keio University School of Medicine Ethics committee (Approval Number: 20-16-18) and Juntendo University School of Medicine Ethics committee (Approval Number: 2012068). hESCs (KhES-1; Passage 29-38 (kindly provided by Dr. Norio Nakatsuji) were cultured on feeder cells in iPS culture media [43].

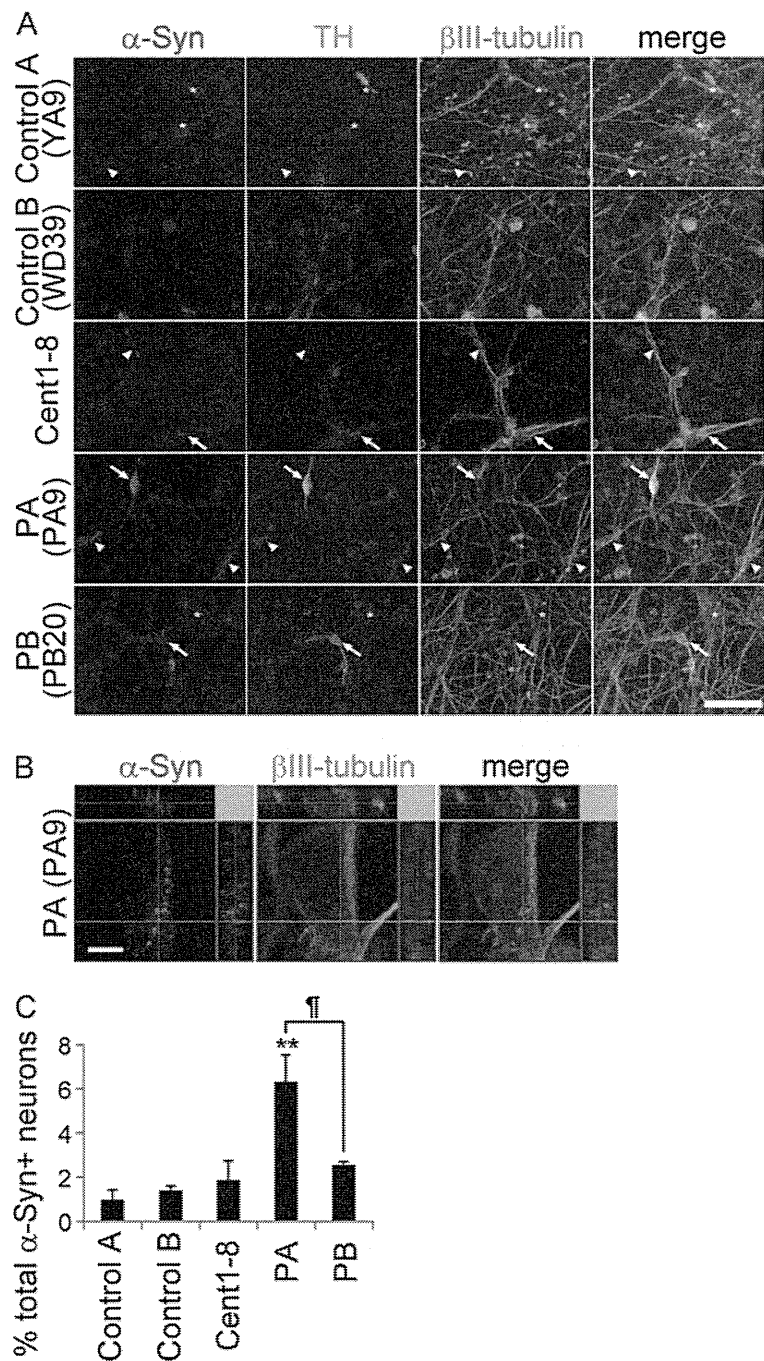
### *In vitro* differentiation of human iPSCs

Neural differentiation of iPSCs was performed as previously described [44] with slight modifications (Okada et al., manuscript in preparation). Briefly, iPSC colonies were detached from feeder layers and cultured in suspension as EBs for about 30 days in bacteriological dishes. EBs were then enzymatically dissociated into single cells and the dissociated cells cultured in suspension in serum-free media (MHM) [44] for 10 to 14 days to allow the formation of neurospheres. Neurospheres were passaged repeatedly by dissociation into single cells followed by culture in the same manner. Typically, neurospheres between passages 3 and 8 were used for analysis. For terminal differentiation, dissociated or undissociated neurospheres were allowed to adhere to poly-L-ornithine- and fibronectin-coated coverslips and cultured for 10 days.

### Immunocytochemical analysis of iPSCs and neurons

For immunocytochemical analysis, cells were fixed with phosphate buffered saline (PBS) containing 4% paraformaldehyde (PFA) for 30 min at room temperature (RT). The cells were analyzed by immunofluorescence staining using antibodies to the following proteins:  $\beta$ -III-tubulin (1:1000,





**Figure 5 α-synuclein accumulation in PARK2 iPSC-derived neurons. (A–C)** Triple labeling for α-synuclein (red), tyrosine hydroxylase (TH; cyan), and βIII-tubulin (green) along with Hoechst (blue) staining of control A (B7), control B (WD39), Cent1-8, and PARK2 (PA9 and PB20) iPSC-derived neurons. **(A)** Arrows indicate α-synuclein+/TH+/βIII-tubulin+ neurons; arrowheads indicate α-synuclein+/TH-/βIII-tubulin+ neurons. Note the presence of α-synuclein+/TH-/βIII-tubulin- non-neural cells (asterisks). **(B)** High magnification confocal projection image of an α-synuclein (magenta)/βIII-tubulin (green) double-positive PA9 iPSC-derived neuron. **(C)** The proportion of α-synuclein+/βIII-tubulin+ neurons relative to βIII-tubulin-positive neurons was significantly higher in PA (PA1, 9 and 22) iPSC-derived neurons than in control A (B7 and YA9), control B (WD39) and Cent1-8 iPSC-derived neurons. Scale bars: A, 50 μm; C, 5 μm. \*\* indicates  $P < 0.01$ ; \* and ¶ indicate  $P < 0.05$  (Mann–Whitney U-test). Data represent the mean and SEM of at least three experiments for each group.

Sigma), NANOG (1:100, ReproCELL), OCT3/4 (1:200, Santa Cruz Biotechnology), SSEA-4 (1:200, Millipore), TRA-1-60 (1:200, Millipore), TH (1:100, Millipore),  $\alpha$ -synuclein (1:500, Invitrogen), p $\alpha$ -synuclein (1:1000, Wako), cleaved-Caspase3 (1:500, Cell Signaling) and ComplexIII (C-III)-core I (1:200, Invitrogen). Cells were washed with PBS after incubation with the primary antibody, followed by incubation with an Alexa Fluor 488-, Alexa Fluor 555-, or Alexa Fluor 647-conjugated secondary antibody (1:500, Invitrogen). Images were obtained using Apotome (Zeiss) or LSM-710 confocal (Zeiss) microscopes.

### PCR amplification of genomic DNA

Genomic DNA was purified from HDFs and iPSCs using a DNeasy kit (Qiagen). The PCR conditions used have been previously described [2,42].

### Reverse transcription (RT)-PCR

RNA isolation and reverse transcription (RT)-PCR were performed as previously described [44]. The amount of cDNA was normalized to  $\beta$ -actin mRNA. Real-time RT-PCR was performed on a ABI PRISM Sequence detection System 7900HT (Applied BioSystems) using SYBR premix ExTaq (Takara). Primers for the detection of Oct4, the transgenes *Oct4-tg*, *Sox2-tg*, *Klf4-tg* and *c-Myc-tg*, and MAO-A, and -B have been previously described [10,15].

### Teratoma assay

To assess teratoma formation, iPSCs were injected into the testis of 8-week-old NOD/SCID mice (OYG International) as previously described [14]. Eight weeks after transplantation, tumors were dissected and fixed with 4% PFA in PBS. Paraffin-embedded tissue was sectioned and stained with H&E. Images were obtained using a BZ-9000 (Keyence) microscope.

### CGH array

Genomic DNA was restricted, labeled, and purified using the Agilent Oligo CGH Microarray Kit (Agilent Technologies) according to the manufacturer's protocol. Labeled genomic DNA was processed for hybridization on a 4x 180K microarray (Agilent Technologies). Processing was performed as instructed by the manufacturer. The genomic analysis was performed using Agilent Genomic Workbench ver. 6.0 software (Agilent Technologies).

### Metabolism assays

Reduced GSH levels were measured according to the kit manufacturer's protocol (GSH-Glo Glutathione Assay; Promega). Chymotrypsin-like proteasome activity was measured using a Cell-Based Proteasome-Glo Assay according to the manufacturer's instructions (Promega). Briefly, neural cells ( $1.0 \times 10^4$ ) derived from neurospheres were seeded in triplicate into a white 96-well plate (Nunc).

Prepared reagent (100  $\mu$ l) was added to each well. After incubation for 10 min at RT, luminescence intensity was recorded. ROS levels were determined by measuring DCFH-DA fluorescence (Invitrogen). Briefly, neurons were incubated with 5  $\mu$ M DCFH-DA and Hoechst (1:2000) for 30 min at 37°C, after which they were washed with PBS and then incubated in differentiation media. Fluorescence was measured by an In Cell Analyzer 2000 system (GE Healthcare Biosciences).

### Protein analysis

Differentiated neurons were harvested in MAPK lysis buffer containing proteinase inhibitor, and protein concentrations were measured by BCA assay (Thermo Scientific). Samples were diluted to yield equivalent protein concentrations and then 4  $\mu$ g was denatured by the addition of 4X sample buffer (Invitrogen) supplemented with  $\beta$ -mercaptoethanol followed by boiling. Samples (7  $\mu$ l/lane) were loaded onto a 4–20% SDS-polyacrylamide gradient gel. Membranes were incubated in blocking solution with the indicated primary antibodies at 4°C overnight. Immunoreactive proteins were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and then visualized by chemiluminescence (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Quantification of band intensities was performed using an RAS4000 system. The primary antibodies used were anti-NQO1 (1:1000, Abcam), anti-NRF2 (1:1000, Santa Cruz Biotechnology) and  $\beta$ -actin (1:5000, Cell Signaling).

### CCCP and Baf A<sub>1</sub> treatments

Neurons were cultured with 30  $\mu$ M CCCP (Sigma-Aldrich) or DMSO, with or without 5  $\mu$ M Baf A<sub>1</sub> (Sigma-Aldrich), for 48 h. The cells were then fixed and stained for  $\beta$ III-tubulin and C-III Core I, and counterstained with Hoechst. To quantify the IMM area of the neurons, the cytoplasmic area was extracted as shown in Figure 3C. The C-III Core I-positive signals within the extracted area were then converted to gray-scale and digitized. The IMM area was quantified from the digitized values using Image J software.

### Tetramethylrhodamine ethyl ester (TMRE) staining

iPSC-derived neurons were incubated with 1nM TMRE (Invitrogen) for 15 min at 37°C and then observed under an Olympus IX81 microscope.

### Electron microscopy

Cells were fixed with 2% glutaraldehyde/2% PFA in 0.1 M phosphate buffer (PB) (pH7.2), post-fixed with 1% OsO<sub>4</sub> in 0.1 M PB (pH 7.2), blocked and stained with a 2% aqueous solution of uranyl acetate, dehydrated with a graded series of ethanol, and then embedded in Epon 812 (TAAB). Coverslips were detached and the embedded samples were placed under a stereomicroscope to identify the cells of



interest. Ultrathin sections were cut with a Leica UC6 or UC7 ultramicrotome (Leica Microsystems) and then stained with uranyl acetate and lead citrate. Samples were observed with a Hitachi H7100 or HT7700 electron microscope.

### Morphometry

Morphometric analysis was used to measure the volume density of mitochondria in the neuronal perikarya as previously described [45]. Briefly, electron micrographs of neurons ( $n = 20, 23, 41$ , and  $44$  for control A (B7), control B (WD39), PA9 and PB2, respectively) were obtained at a magnification of  $\times 7000$ . After enlarging to three times the original magnification, point-counting was carried out to determine the volume density using a double-lattice test system with  $1.5\text{ cm}$  spacing. Mitochondria were classified as normal, abnormal, or undetermined. The abnormal mitochondria were defined as those with irregularly arranged cristae, or with a high electron-dense matrix. The volume density (Vv) of each type of mitochondrion was expressed as percent volume according to the following formula:  $Vv = (Pi/Pt) \times 100\%$ , where Pi is the number of points falling on each mitochondrial structure and Pt is the number of points falling on the neuronal perikarya.

### Immunohistochemical analysis of autopsied brain tissue

The ethical committee of the Kitasato University School of Medicine and Juntendo University School of Medicine reviewed and approved the protocol for analysis of autopsied brain tissue. Patients and control subjects were informed of the study and gave written informed consent. Brain tissue from patient PA was obtained following her death at age 72; brain tissue from the father of patient PB was obtained when he died at age 70 [46]. Tissue was fixed with 10% formalin and then embedded in paraffin. Mid-brain sections ( $6\text{ }\mu\text{m}$  thick) were cut, deparaffinized with xylene, and then rehydrated in ethanol. After being boiled and treated with  $\text{H}_2\text{O}_2$ , sections were subjected to immunofluorescence staining with antibodies to the following proteins:  $\alpha$ -synuclein (1:500, Invitrogen),  $p\alpha$ -synuclein (1:1000, Wako), and TH (1:1000, Calbiochem). After washing with PBS, sections were incubated with a biotinylated secondary antibody (1:500; Vector Laboratories Inc.) at RT for 1 hr followed by incubation with an avidin-biotin peroxidase complex (Vector Laboratories Inc.) for 1 hr. Immunoreactive proteins were visualized using 3,3'-diaminobenzidine (DAB; Wako Pure Chemical Industries) and nuclear fast red staining. For immunofluorescence, FITC-conjugated and Cy3-conjugated secondary antibodies (1:500; Jackson ImmunoResearch Laboratories) were used. Images were obtained using a BIOREVO (Keyence) and a confocal laser-scanning LSM710 (Zeiss) microscope.

### Statistical analysis

Values represent the mean  $\pm$  SEM. The Mann–Whitney  $U$ -test was used to evaluate differences between groups. A  $P$  value of  $< 0.05$  was considered significant.

### Additional files

**Additional file 1: Genetic studies of family.** (A) An arrow indicates PA patient. (B) An arrow indicates PB patient. Filled circles and squares, women and men with PARK2 mutation; Open circles and squares, normal women and men; Diamond shapes, family members whose DNA samples were not analyzed. Symbols with lines through them represent the deceased.

**Additional file 2: Characterization of control and PARK2 iPSCs.** (A) Control A (YA9), Control B (WD39), PA (PA9), and PB (PB2) iPSCs expressed the pluripotency markers SSEA4 (red) and TRA1-60 (green). Scale bar,  $100\text{ }\mu\text{m}$ . (B) iPSCs established from patients PA (PA1, PA22) and PB (PB1, PB18, and PB20) were positive for the pluripotency markers Nanog (red), Oct4 (green), SSEA4 (red), and TRA1-60 (green). Scale bars: phase images,  $200\text{ }\mu\text{m}$ ; immunofluorescence images,  $100\text{ }\mu\text{m}$ . (C) Levels of endogenous Oct4 mRNA in the generated iPSCs were similar to those in KhES1 cells, a human embryonic stem cell (hESC) line [42]. Expression levels were normalized to that of KhES1 (set as 1). (D) Cont A (YA9), Cont B (WD39), PA (PA1, 9 and 22), and PB (PB1, 2, 18 and 20) iPSCs gave rise to teratomas with all three germ layers, confirming pluripotency. Scale bar,  $100\text{ }\mu\text{m}$ . (E) Silencing of transgenes in control and PARK2 iPSC clones. Expression levels were normalized to the positive control of fibroblasts in cultures assayed 6 days after retroviral infection ( $= 100$ ). Cont A, Control A; Cont B, Control B.

**Additional file 3: Confirmation of parkin deletions and genomic stability of PARK2 iPSCs using comparative genomic hybridization (CGH) microarray analysis.** (A) Exons 2–4 were deleted in the PA9 and PA22 iPSC lines. Exons 6 and 7 were deleted in the PB2, 18, and 20 iPSC lines. (B) Copy number profiles of whole chromosomes in PARK2 iPSCs assessed by CGH microarray analysis revealed that no genomic aberrations were introduced during the process of establishing PARK2 iPSCs.

**Additional file 4: Expression level of MAO-A and -B showed no difference among Control and PARK2 iPSC-derived neurons.** (A,B) qRT-PCR measurement of MAO-A and -B transcripts in PARK2 (PA (1, 9 and 22) and PB (1, 2 and 20)) iPSC-derived neurons showed no difference compared to those in Cont A (B7 and YA9). ContA; Control A, ContB; Control B.

**Additional file 5: Healthy mitochondria in PARK2 fibroblasts and iPSCs.** (A, B) Electron micrographs of fibroblasts (upper panels) and iPSCs (lower panels) from Control (Cont A and Cont B) and PARK2 patients (PA and PB). Mitochondria in the fibroblasts and iPSCs from both groups showed long, cylindrical profiles with well-organized cristae, and the electron density of the matrix was relatively low (asterisks). Scale bar,  $0.25\text{ }\mu\text{m}$ . Cont A, Control A; Cont B, Control B. (C) Fibroblasts were treated with  $30\text{ }\mu\text{M}$  CCCP or DMSO for 48 h, followed by staining for CIII core (magenta) to label the internal mitochondrial membrane (IMM) and counterstaining with Hoechst (Ho, blue). Mitochondrial size decreased after CCCP treatment in both Control (Cont A and Cont B) and PARK2 (PA and PB) fibroblasts. Scale bar,  $20\text{ }\mu\text{m}$ . (D) iPSCs were treated with  $30\text{ }\mu\text{M}$  CCCP or DMSO for 48 h and then stained for CIII core (magenta) to label IMM, Oct4 (blue) to label iPSCs, and Hoechst (Ho, white). Mitochondrial size in Control (Cont A (B7), Cont B (WD39)), and PARK2 (PA9 and PB2) iPSCs decreased after CCCP treatment. Scale bar,  $20\text{ }\mu\text{m}$ . (E) CCCP/DMSO ratios in Control (Cont A (B7, YA9), Cont B (WD39)), and PARK2 (PA9 and 22 and PB2 and 20) iPSCs (Mann–Whitney  $U$ -test). Data represent the mean and SEM ( $n > 3$  for each group).

**Additional file 6: Mitochondrial membrane potential after CCCP treatment in control and PARK2 iPSC-derived neurons.** (A) iPSC-derived neurons were treated with  $30\text{ }\mu\text{M}$  CCCP or DMSO for 48 h, after which they were stained for the mitochondrial membrane potential marker, TMRE. The intensity of TMRE (yellow) was clearly reduced in

control (Cont A (B7), Cont B (WD39)), and PARK2 (PA9 and PB2) iPSC-derived neurons. Scale bar, 50  $\mu$ m.

**Additional file 7: Confirmation of parkin deletions carried by the father of patient PB.** (A) Deletion of exons 6 and 7 was confirmed in blood samples from PB and the father of PB by PCR.

**Additional file 8:  $\alpha$ -Synuclein signals are not seen in PARK2 iPSCs.** (A) Quantitative genomic PCR analysis for SNCA exons 1 and 4 demonstrated a normal copy number in PARK2 (PA1, 9 and 22, and PB1, 2, 18 and 20) iPSCs. The copy number was the same as that observed for Cont A (B7 and YA9) and Cont B (WD39). The SNCA gene copy number was normalized to  $\beta$ -globin (*HBB*) and  $\beta$ 2-microglobulin (*B2MG*). (B) iPSCs were stained for  $\alpha$ -synuclein (red), Oct4 (green; to label iPSCs) and Hoechst (blue). No  $\alpha$ -synuclein signals were observed in Cont A (B7 and YA9), Cont B (WD39), or PARK2 (PA9 and 22, PB2 and 20) iPSCs. Scale bar, 50  $\mu$ m.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

YI, YO, WA, and HO conceived and designed the experiments. YI performed most of the experiments, analyzed data, and wrote the manuscript. YO, and HO edited the manuscript. YO developed the quality control system, neural differentiation method for the iPSCs and performed CGH microarray data analysis. WA generated the WD39 iPSCs. NK, KH, MS and AN performed western blotting analysis. TN performed some parts of *in vitro* culture assay. SS, MF, YM, HM and NH examined and recruited PARK2 patients. TK, MO, and MA performed biopsies and established the skin fibroblasts. AH, TS, TH and MS performed preliminary experiments for the metabolome analysis. TY, DI, AK and NS provided cent8-1 iPSCs. YI and NM designed the CCCP treatment experiment. MK and YU performed the electron microscopic analysis. HH, MT, HM and NH performed the histopathological studies of the postmortem brain of PA. All authors read and approved the final manuscript.

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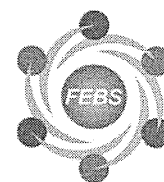
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## ATP13A2 deficiency induces a decrease in cathepsin D activity, fingerprint-like inclusion body formation, and selective degeneration of dopaminergic neurons

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### ABSTRACT

Kufor-Rakeb syndrome (KRS) was originally described as an autosomal recessive form of early-onset parkinsonism with pyramidal degeneration and dementia. *ATP13A2* was identified as the causative gene in KRS. *ATP13A2* encodes the ATP13A2 protein, which is a lysosomal type5 P-type ATPase, and *ATP13A2* mutations are linked to autosomal recessive familial parkinsonism.

Here, we report that normal *ATP13A2* localizes in the lysosome, whereas disease-associated variants remain in the endoplasmic reticulum. Cathepsin D activity was decreased in *ATP13A2*-knockdown cells that displayed lysosome-like bodies characterized by fingerprint-like structures. Furthermore, an *atp13a2* mutation in medaka fish resulted in dopaminergic neuronal death, decreased cathepsin D activity, and fingerprint-like structures in the brain. Based on these results, lysosome abnormality is very likely to be the primary cause of KRS/PARK9.

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### 1. Introduction

Parkinson's disease (PD) is one of the most common movement disorders, and it is caused by loss of dopaminergic neurons. The molecular mechanisms underlying neuronal degeneration in PD remain unknown; however, it is now clear that genetic factors

heavily contribute to the pathogenesis of this disease [1]. In approximately 10% of patients with clinical features of PD, the disease state has a strict familial etiology.

PARK9-linked PD is an autosomal recessive early-onset disorder that is characterized by levodopa-responsive parkinsonism, supranuclear gaze palsy, pyramidal signs, and dementia; this condition is also called Kufor-Rakeb syndrome (KRS), being named for a consanguineous Jordanian family containing four members with this disorder [2]. Recently, *ATP13A2* was identified as the causative gene for KRS/PARK9. The *ATP13A2* gene comprises 29 exons that encode a lysosomal type 5 P-type ATPase with 10 transmembrane domains [3]. Thus far, eight mutations have been reported in just five families and in two additional unrelated patients, and no neuropathological examination of an autopsy case has been documented [3–8]. The function of the *ATP13A2* protein remains largely unknown, but it is supposed that *ATP13A2* might participate in autophagic protein degradation via the lysosomal pathway [9].

Here, we established and analyzed a cell culture model of *ATP13A2* knockdown and *Atp13a2* mutant medaka fish to elucidate the mechanisms underlying PARK9-associated pathology.

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