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**PARK6 PINK1 mutants are defective in maintaining
mitochondrial membrane potential and inhibiting ROS
formation of substantia nigra dopaminergic neurons**

Hung-Li Wang^{1*}, An-Hsun Chou³, Ai-Shun Wu¹, Si-Ying Chen¹, Yi-

Hsin Weng⁴, Yu-Cheng Kao¹, Tu-Hsueh Yeh⁴, Po-Ju Chu² and

Chin-Song Lu⁴

Departments of ¹Physiology and Pharmacology and ²Biomedical Sciences, Chang Gung University School of Medicine; Departments of ³Anesthesiology and ⁴Neurology, Chang Gung Memorial Hospital, Kwei-San, Tao-Yuan, Taiwan, R.O.C.

*Correspondence: Hung-Li Wang, Ph.D.

Department of Physiology
Chang Gung University School of Medicine
Kwei-San, Tao-Yuan, Taiwan, R.O.C.

E-mail: hlwns@mail.cgu.edu.tw

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Abstract

Mutations in PTEN-induced kinase 1 (PINK1) gene cause recessive familial type 6 of Parkinson's disease (PARK6). PINK1 is believed to exert neuroprotective effect on SN dopaminergic cells by acting as a mitochondrial Ser/Thr protein kinase. Autosomal recessive inheritance indicates the involvement of loss of PINK1 function in PARK6 pathogenesis. In the present study, confocal imaging of cultured SN dopaminergic neurons prepared from PINK1 knockout mice was performed to investigate physiological importance of PINK1 in maintaining mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial morphology and test the hypothesis that PARK6 mutations cause the loss of PINK1 function. PINK1-deficient SN dopaminergic neurons exhibited a depolarized $\Delta\Psi_m$. In contrast to long thread-like mitochondria of wild-type neurons, fragmented mitochondria were observed from PINK1-null SN dopaminergic cells. Basal level of mitochondrial superoxide and oxidative stressor H_2O_2 -induced ROS generation were significantly increased in PINK1-deficient dopaminergic neurons. Overexpression of wild-type PINK1 restored hyperpolarized $\Delta\Psi_m$ and thread-like mitochondrial morphology and inhibited ROS formation in PINK1-null dopaminergic cells. PARK6 mutant (G309D), (E417G) or (C Δ 145) PINK1 failed to rescue mitochondrial dysfunction and inhibit oxidative stress in PINK1-deficient dopaminergic neurons. Mitochondrial toxin rotenone-induced cell death of dopaminergic neurons was augmented in PINK1-null SN neuronal culture. These results indicate that PINK1 is required for maintaining normal $\Delta\Psi_m$ and mitochondrial morphology of cultured SN dopaminergic neurons and exerts its neuroprotective effect by inhibiting ROS formation. Our study also provides the evidence that PARK6 mutant (G309D), (E417G) or (C Δ 145) PINK1 is defective in regulating mitochondrial functions and attenuating ROS production of SN dopaminergic cells.

Keywords: Parkinson's disease; PARK6; PTEN-induced kinase 1; Substantia nigra dopaminergic neurons; Mitochondrial membrane potential; Mitochondrial fragmentation; Reactive oxygen species.

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1. Introduction

Patients with familial type 6 of Parkinson's disease (PARK6) exhibit early-onset parkinsonism symptoms and autosomal recessive inheritance [1]. Subsequent molecular genetic studies demonstrated that homozygous missense or truncating mutations of PTEN-induced kinase 1 (PINK1) gene are implicated in the pathogenesis of PARK6 and that PINK1 is the second most frequent causative gene in early-onset Parkinson's disease (PD) [2-7]. PINK1 was initially discovered as the one of transcriptionally activated genes by tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) [8]. PINK1 mRNA is ubiquitously expressed with an abundant expression in brain, heart, skeletal muscle and testis [8]. Within the brain, PINK1 mRNA is mainly found in neurons with little expression in glial cells. A high level of neuronal PINK1 expression is observed in the substantia nigra, neocortex and hippocampus [9,10]. PINK1 contains 581 amino acids and consists of an N-terminal mitochondrial targeting motif, a possible transmembrane domain, a Ser/Thr kinase domain and a C-terminal autoregulatory domain [11-13]. N-terminal mitochondrial targeting sequence is sufficient for the mitochondrial import of PINK1 protein [12,14]. Within the mitochondria, PINK1 is expressed in the mitochondrial intermembrane space, inner and outer mitochondrial membranes [10,12, 15,16].

Mitochondria play a critical role in the ATP production, calcium homeostasis, oxidative stress response and initiation of cell death process [17]. Multiple lines of evidence indicate that mitochondrial dysfunction is involved in the neurodegeneration of substantia nigra pars compacta (SNpc) dopaminergic cells in PD patients [18-20]. Impaired activity of complex I and increased levels of oxidative stress products were found in the substantia nigra of PD patients [20]. Mitochondrial complex I inhibitors, including MPTP and rotenone, caused cell death of SNpc dopaminergic neurons by activating mitochondrial

apoptotic pathway and were used to prepare PD animal model [18,20]. Homozygous mutations of PINK1 gene cause autosomal recessive PD, and the sequence of Ser/Thr kinase is the only functional domain in PINK1 protein. Therefore, PINK1 is believed to regulate mitochondrial functions of SNpc dopaminergic neurons and exert neuroprotective effect against various cellular stresses by functioning as a mitochondrial Ser/Thr protein kinase [2,7,13,16]. In accordance with this hypothesis, previous studies using cell lines, *Drosophila* or zebrafish model showed that PINK1 is involved in maintaining the normal function and structural integrity of mitochondria. Deletion of PINK1 gene in *Drosophila* muscles caused defects in mitochondrial morphology, an impaired ATP production and a reduction in mitochondrial DNA and protein levels [21,22]. PINK1 knockdown in zebrafish resulted in impaired mitochondrial functions, including depolarized mitochondrial membrane potential and increased reactive oxygen species (ROS) level, and a decrease in the number of central dopaminergic neurons [23]. Knockdown of PINK1 expression by RNA interference in HeLa cells or dopaminergic neuroblastoma cell line led to depolarization of mitochondrial membrane potential and truncated or fragmented mitochondrial morphology [24-29]. PINK1 is the first protein directly linking the mitochondrial abnormality to the neurotoxicity of SN dopaminergic cells and PD pathogenesis. Therefore, it is essential to investigate PINK1 regulation of mitochondrial function and morphology using SN dopaminergic neurons.

The autosomal recessive inheritance indicates that PARK6 mutation-induced loss of PINK1 function is involved in PARK6 pathogenesis. Wild-type PINK1 is believed to be required for maintaining normal mitochondrial functions of SN dopaminergic neurons and exert a neuroprotective effect [7,13,16]. PARK6 PINK1 mutations could impair the ability of PINK1 to maintain mitochondrial function and cause the loss of PINK1-mediated neuroprotective effect, which subsequently leads to the neurotoxicity of SN dopaminergic cells. Consistent with this hypothesis, our previous study demonstrated that when expressed

in cell lines, mitochondrial PINK1 produces a cytoprotective effect by inhibiting apoptotic stimuli-induced opening of mitochondrial permeability transition pore, cytochrome-c release and subsequent caspase-3 activation [14]. In contrast, several PARK6 PINK1 mutants expressed in cell lines failed to inhibit mitochondrial release of cytochrome-c and exert anti-apoptotic effect [2,14,30]. However, PARK6 mutations-induced loss of PINK1-mediated mitochondrial and neuroprotective functions in SN dopaminergic neurons should be further confirmed. To better understand physiological functions of PINK1 in SN dopaminergic cells and molecular pathogenesis of PARK6, in the present study cultured SN dopaminergic neurons prepared from PINK1 knockout mice were used to investigate physiological importance of PINK1 in maintaining mitochondrial function and morphology and test the hypothesis that PARK6 mutations cause the loss of PINK1 function.

2. Materials and methods

2.1. Generation of *PINK1*-deficient mice

BAC clone containing the mouse *PINK1* gene was obtained from Invitrogen. To target the exon 1 of *PINK1*, 1.1 kb and 5.9 kb DNA fragments surrounding the exon 1 were prepared by performing PCR amplification with BAC clone DNA and used as the 5' and 3' homologous sequences, respectively. The 5' homologous arm corresponds to the 5' region of exon 1, and the 3' homologous arm spans 3' terminal portion of exon 1 to the exon 3 of *PINK1* gene. Target vector of *PINK1* gene was constructed by subcloning 5' and 3' homologous DNA fragments into pBluescript vector containing the PGK-Neo selection cassette. The linearized target vector was transfected by electroporation into mouse ES cells derived from the 129/Sv strain. Neomycin-resistant colonies were obtained, and Southern blot analysis described below was performed to select ES cell lines with correct homologous recombination of target vector. Correctly targeted ES clone was injected into C57BL/6J blastocysts. The resulting chimeric mice were mated with C57BL/6J mice and bred to obtain F1 heterozygous mutant mice. *PINK1*^{-/-} mice with mixed genetic backgrounds of C57BL/6J and 129/Sv strains were obtained by the interbreeding of F1 heterozygous mice.

2.2. Southern blot analysis

Genomic DNA was digested with XbaI, electrophoresed in 0.8 % agarose gel, and transferred to Hybond N⁺ filter. The blot was hybridized with 3' external DNA probe (~600 bp) radiolabeled by random priming at 65 °C for 16-18 hours. Then, the blot was washed (0.5 × SSC with 0.1 % SDS) at 65 °C and exposed to X-ray film. The expected sizes of XbaI fragment in wild-type allele and targeted allele are 16 kb and 9 kb, respectively.

2.3. Northern blot analysis and RT-PCR assay of *PINK1* mRNA expression

Total RNA was prepared from the brain of wild-type, PINK1^{+/-} or PINK1^{-/-} mouse by using Trizol Reagent (Invitrogen), electrophoresed in 1 % formaldehyde agarose gel, and transferred to Hybond N⁺ filter. The blot was hybridized with radiolabeled DNA probe (~600 bp) corresponding to C-terminal fragment of human PINK1 cDNA at 65 °C for 16-18 hours. Then, the blot was washed at 65 °C and exposed to X-ray film. The expected size of PINK1 mRNA transcript is 2.3 kb. As a control, the blot was also hybridized with DNA probe specific for mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

For RT-PCR analysis, the first strand cDNA was synthesized in a reaction volume of 30 µl containing total RNA (5 µg), 1 × the first-strand buffer, 10 mM dithiothreitol, 1 mM of each dNTP, 10 ng/µl random hexamers, 20 U of RNase inhibitor (Promega) and 100 U of SuperScript III RT (Invitrogen) for 1 hour at 50 °C. Subsequently, the reaction mixture was heated at 70 °C for 15 minutes, chilled on the ice, and used as DNA template for PCR amplification. PCR amplification of PINK1 cDNA was performed with forward primer (5'TGATGTGGAATATCTCGGCAGGTTC3') and reverse primer (5'ACACTCCAGGT TAGCCAGAAACAGC3'). The expected size of PINK1 cDNA fragment is 1050 bp. PCR amplification of GAPDH mRNA was used as the control.

2.4. Preparation and immunofluorescence staining of cultured substantia nigra neurons

Cultured substantia nigra (SN) neurons were prepared as described previously [31]. Briefly, substantia nigra was dissected from postnatal 3- or 4-day-old wild-type or PINK1^{-/-} mice and incubated with DMEM/F12 medium containing pronase (0.5 mg/ml, Roche) and DNase I (0.3 mg/ml, Roche) for 50 minutes at 37 °C. Tissue fragments were subsequently triturated, and dissociated cells were plated onto poly-L-lysine- and collagen-coated dishes. SN neurons were cultured in DMEN/F12 medium supplemented with 5 % fetal bovine serum, 3 % horse serum and GDNF (glial cell line-derived neurotrophic growth factor; 25

ng/ml). From the second day in culture, proliferation of glial cells was prevented by adding 5'-fluoro-2'-deoxyuridine and uridine into culture medium.

Cultured SN neurons were fixed with 4 % paraformaldehyde, permeabilized in 0.1 % Triton x-100, and then incubated at 4 °C overnight with diluted monoclonal anti-tyrosine hydroxylase antibody (Chemicon). Following the wash, neurons were incubated with FITC-conjugated horse anti-mouse IgG (Vector). Then, dopaminergic neurons were viewed and photographed under a Leica DM LFS A upright microscope equipped with Epifluorescence system.

2.5. Confocal imaging of mitochondrial membrane potential and reactive oxygen species (ROS) formation

For the determination of mitochondrial membrane potential ($\Delta\Psi_m$), cultured SN dopaminergic neurons of wild-type or PINK1^{-/-} mice were loaded with a potential sensitive dye TMRM (tetramethylrhodamine methyl ester; 100 nM; Molecular Probes) in HEPES buffered saline (NaCl 140 mM, KCl 5 mM, MgCl₂ 1 mM, CaCl₂ 2mM, glucose 10 mM, HEPES 5 mM, pH 7.3) for 10 minutes at the room temperature. SN neurons were then washed with HEPES buffered saline, and transferred to the recording chamber mounted on a Leica DM6000 microscope equipped with Leica TCS SP5 confocal spectral scanning system and a 100 × oil immersion objective. TMRM was excited at 543 nm with a HeNe green laser, and the emitted fluorescent signal (emission, 560–620 nm) was collected. Z-stacks of 15 confocal TMRM fluorescence images (with an acquisition interval along the z-axis of 0.3 μm) were processed and analyzed by LAS AF software (Leica).

Confocal MitoSOX Red staining was performed to visualize mitochondrial level of superoxide anion in wild-type or PINK1-deficient SN dopaminergic neurons. MitoSOX Red selectively targeted to the mitochondria is oxidized by superoxide and exhibits red

fluorescence. Cultured SN neurons were incubated with 5 μ M MitoSOX Red (Molecular Probes) for 10 minutes at 37 °C. After washout, MitoSOX Red was excited at 514 nm with an Ar-blue laser, and fluorescence signal was detected (emission, 540–620 nm).

To visualize oxidative stressor H₂O₂-induced formation of intracellular reactive oxygen species (ROS), wild-type or PINK1^{-/-} SN dopaminergic neurons were loaded with 1 μ M CM-H₂DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; Molecular Probes), which becomes intensely fluorescent after being oxidized by intracellular ROS, for 40 minutes at room temperature. Following the washout, CM-H₂DCFDA signal was detected by 488 nm excitation using Ar-blue laser, and the emitted fluorescence (emission, 510–560 nm) was collected. MitoSOX Red or CM-H₂DCFDA fluorescence images were analyzed by Leica LAS AF software.

2.6. Confocal imaging of mitochondrial morphology

To visualize mitochondrial morphology, cultured wild-type or PINK1-deficient SN dopaminergic neurons were incubated with Mitotracker Green (150 nM; Molecular Probes) for 15 minutes at the room temperature. Mitotracker Green is a cell-permeable mitochondrial-specific dye and becomes fluorescent when associated with lipids within the mitochondria. After being loaded with Mitotracker Green, SN neurons were washed with HEPES buffered saline and transferred to the recording chamber for confocal microscopy observation. Mitotracker Green was excited at 488 nm with an Ar-blue laser, and fluorescent image was detected. Confocal Mitotracker Green images were analyzed by LAS AF software (Leica).

2.7. Preparation of recombinant adenoviruses containing cDNA of wild-type or PARK6 mutant PINK1

Influenza hemagglutinin epitope (HA, YPYDVPDYA) was added to the C-terminus of human wild-type PINK1 by performing PCR amplification. Oligonucleotide-directed

mutagenesis using PCR amplification was performed to prepare cDNA encoding PARK6 mutant PINK1 [14]. According to our previous studies [31,32], AdEasy Adenoviral Vector System (Stratagene) was used to prepare recombinant adenoviruses containing cDNA of wild-type or PARK6 mutant PINK1 by homologous recombination. Briefly, cDNA of HA-tagged wild-type or mutant PINK1 was subcloned into pShuttle vector provided in the kit. The resulting plasmid was cotransformed into *E. coli* BJ5183 cells with adenoviral backbone plasmid pAdEasy-1. Recombinant adenoviral plasmid was then transfected into HEK 293 cells, which provide E1 gene required for the production of infectious viral particles. Following the amplification in HEK 293 cells, viral stocks were purified by CsCl gradient ultracentrifugation and titered using Adeno-X rapid titer kit (Clontech). One day after plating, cultured SN neurons were infected with recombinant adenovirus at multiplicity of infection (MOI) of 20-30 pfu/cell.

2.8. Western blot analysis of HA-tagged PINK1 expressed in cultured SN neurons

Following the infection of adenoviruses containing cDNA of wild-type or PARK6 mutant PINK1, mitochondrial and cytosolic fractions of cultured SN neurons were prepared as described previously [14]. Briefly, SN neurons were lysed in ice-cold buffer containing 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH = 7.3, 1 mM EGTA, 1 mM DTT, 5 µg/ml pepstain, 5 µg/ml leupeptin, 5 µg/ml aprotinin, and 0.3 mM PMSF. Cells were homogenized using a Dounce tissue grinder on ice, and cell lysate was centrifuged at 500 g for 10 min at 4 °C. The supernatant was centrifuged at 9,500 g for 9 min at 4 °C to pellet the mitochondrial fraction. The resulting supernatant was further centrifuged at 16,000 g for 20 min at 4 °C, and final supernatant was used as cytosolic fraction. Protein samples were fractionated on 10 % SDS-polyacrylamide and transferred to polyvinylidene difluoride (PVDF) membrane. Subsequently, the membrane was incubated with diluted polyclonal anti-HA antiserum (Santa Cruz) overnight at 4 °C. Then, the membrane was incubated with

donkey anti-rabbit horseradish peroxidase-linked secondary antibody (Amersham). Subsequently, immunoreactive proteins on the membrane were visualized by using enhanced chemiluminescence protocol (ECL Kit, Amersham). To verify the purity of mitochondrial fraction, membrane was stripped and reblotted with monoclonal anti-cytochrome c oxidase subunit IV (COX-IV), a mitochondrial marker (Molecular Probes).

2.9. Analysis of neuronal death

Two days after treating cultured SN neurons with rotenone, neuronal death was analyzed by performing MTT assay. Briefly, SN neurons were incubated with 0.2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 hours at 37 °C. Subsequently, neurons were washed with PBS, and formazan released was quantified at 560 nm by using an ELISA plate reader.

2.10. Statistics

All results are expressed as the mean \pm S. E. value of n experiments. Statistical significance among multiple experimental groups was determined by one way ANOVA followed by Dunnett's test. Unpaired Student's *t*-test (two-tailed) was used to determine the significant difference between two groups of data. A *p* value < 0.05 was considered significant.

3. Results

3.1. Generation of *PINK1*^{-/-} mice

Mouse *PINK1* gene contains eight exons. To generate homozygous mice lacking the expression of *PINK1* mRNA (*PINK1*^{-/-} mice), homologous recombination was targeted on the exon 1, which contains the start codon and initial 387 nucleotides of *PINK1* coding sequence (Fig. 1A). The targeted deletion mediated by homologous recombination removes the start codon and the majority of exon 1 (326 nucleotides of coding sequence), which is expected to result in the absence of *PINK1* mRNA expression. Linearized knockout targeting vector containing PGK-Neo selection cassette (Fig. 1A) was transfected into 129/Sv mouse ES cells. Southern blot analysis was performed to select correctly targeted ES cell lines. The expected sizes of DNA fragment in wild-type allele and targeted allele were 16 kb and 9 kb, respectively. We have obtained two clones of ES cells with corrected homologous recombination, and these cell lines were injected into C57BL/6J blastocysts and used for the generation of chimeric mice, which were then mated with C57BL/6J mice and bred to obtain F1 heterozygous mutant mice. Subsequently, F2 homozygous *PINK1* knockout mice were obtained by the interbreeding of F1 heterozygous mice. Southern blot analysis (Fig. 1B) and PCR genotyping were performed to confirm the germline transmission of targeted allele in heterozygous *PINK1*^{+/-} and homozygous *PINK1*^{-/-} mice.

The successful generation of homozygous *PINK1*^{-/-} mice was further confirmed by Northern blot analysis. In contrast to wild-type or heterozygous *PINK1*^{+/-} mouse, an expected 2.3 kb *PINK1* mRNA was absent in the brain of homozygous *PINK1*^{-/-} mouse (Fig. 1C). RT-PCR analysis also demonstrated that in contrast to wild-type mouse, *PINK1* mRNA was absent in the brain or heart of *PINK1*^{-/-} mouse (data not shown).

3.2. *PINK1* is required for maintaining normal mitochondrial membrane potential ($\Delta\Psi_m$) of SN dopaminergic neurons, and *PARK6* *PINK1* mutants are defective in

maintaining $\Delta\Psi_m$

Hyperpolarized mitochondrial membrane potential, which is the driving force behind oxidative phosphorylation and ion transportation, plays an important role in the normal function of mitochondria [17,33]. We hypothesized that PINK1 is required for maintaining mitochondrial membrane potential of SN dopaminergic neurons and that PARK6 PINK1 mutations impair the ability of PINK1 to maintain mitochondrial potential. This hypothesis was tested by performing confocal imaging of mitochondrial membrane potential using wild-type or PINK1^{-/-} dopaminergic neurons.

Cultured SN dopaminergic neurons are the ideal preparation for performing the confocal imaging of mitochondria function and morphology and investigating functional consequence of PARK6 PINK1 mutation following recombinant adenoviruses-mediated overexpression of wild-type or mutant PINK1 protein. In the present study, primary neuronal culture of SN was prepared from wild-type or PINK1^{-/-} mice. RT-PCR analysis showed that in contrast to SN neuronal culture of wild-type mouse, PINK1 mRNA was absent in cultured SN neurons of PINK1^{-/-} mouse (Fig. 2A). In accordance with our previous study [31], two subpopulations of neurons, large multipolar or oval-shaped tyrosine hydroxylase (TH)-positive dopaminergic cells (diameter = 25-30 μm) (Fig. 2B) and small non-dopaminergic cells (diameter = 15-20 μm), were found in SN neuronal culture prepared from wild-type or PINK1-deficient mouse. Cultured SN neurons prepared from wild-type and PINK1-null mice contained $37 \pm 4\%$ (n = 7 experiments) and $36 \pm 3\%$ (n = 7) of TH-positive dopaminergic cells, respectively. The number of TH-positive dopaminergic neurons in 6 to 7-day-old SN culture of PINK1^{-/-} mouse was similar to that in SN neuronal culture of wild-type mouse (n = 7 experiments).

Confocal imaging of TMRM fluorescence was performed to visualize hyperpolarized mitochondrial membrane potential ($\Delta\Psi_m$) of wild-type or PINK1-deficient SN

dopaminergic neurons at 6-7 days in culture, which were easily identified by their large diameter ($> 25 \mu\text{m}$), thick proximal dendrite and multipolar or oval-shaped cell body (Fig. 3) [31]. The selective accumulation of cationic dye TMRM in the mitochondria of health cell is driven by hyperpolarized mitochondrial membrane potential. Therefore, TMRM fluorescence intensity is an indicator of hyperpolarized $\Delta\Psi_{\text{m}}$. Compared to wild-type SN dopaminergic cells, TMRM fluorescent intensity was significantly reduced in PINK1^{-/-} SN dopaminergic neurons, indicating that PINK1 deficiency results in a depolarized mitochondrial membrane potential (Fig. 3).

To further confirm the essential role of PINK1 in maintaining $\Delta\Psi_{\text{m}}$ of SN dopaminergic neurons and test the hypothesis that PARK6 mutations cause the loss-of-function of PINK1 in regulating $\Delta\Psi_{\text{m}}$, HA-tagged human wild-type and PARK6 PINK1 mutants, (G309D) PINK1, (E417G) PINK1 and C-terminal truncated (C Δ 145) PINK1 (the number represents amino acid residues deleted from the C-terminal end) [2,4], were overexpressed in the mitochondria of cultured PINK1^{-/-} SN dopaminergic neurons with the aid of recombinant adenoviruses [29,30]. Consistent with our previous study [14], subcellular fractionation and subsequent Western blot study using anti-HA antiserum indicated that three days after the infection of recombinant adenoviruses containing cDNA of wild-type or mutant PINK1, HA-tagged wild-type PINK1, PARK6 mutant (G309D) or (E417G) PINK1 (M. W. = ~65 kDa) was mainly expressed in the mitochondrial fraction of PINK1-null SN dopaminergic neurons (Fig. 4) with a minimal expression in the cytosol. When cultured SN neurons were infected with adenoviruses containing the cDNA of (C Δ 145) PINK1, molecular weight of C-terminal truncated (C Δ 145) PINK1 expressed in the mitochondria was reduced to ~48-kDa (Fig. 4).

When overexpressed in 6 to 7-day-old SN neuronal culture of PINK1^{-/-} mice, wild-type

PINK1 increased TMRM fluorescence intensity and restored normal $\Delta\Psi_m$ of PINK1-deficient SN dopaminergic neurons (Fig. 5). As a control, infection of adenoviruses containing cDNA of GFP or wild-type ataxin-3-Q22 did not affect TMRM signal and mitochondrial membrane potential of PINK1-null SN dopaminergic cells (data not shown; n = 4 cells). In contrast to wild-type PINK1, PARK6 mutant (G309D), (E417G) or (CΔ145) PINK1 was defective in increasing TMRM fluorescence signal and reversing depolarized $\Delta\Psi_m$ of PINK1-deficient SN dopaminergic cells (Fig. 5). These results indicate that PARK6 mutations cause the impairment of PINK1 in maintaining the mitochondrial membrane potential.

3.3. PINK1 deficiency leads to mitochondrial fragmentation in SN dopaminergic neurons, which is rescued by wild-type PINK1 but not by PARK6 mutant PINK1

To investigate PINK1 regulation of mitochondrial morphology, confocal Mitotracker Green staining was performed to visualize the mitochondria of 6 to 7-day-old cultured wild-type or PINK1^{-/-} SN dopaminergic neurons. In the majority (~95 %) of wild-type SN dopaminergic neurons, mitochondria displayed a normal long thread-like tubular structure (Fig. 6A and 6B). Fragmented mitochondria, which are truncated and shortened (<1.5 μm), were only found in about 5 % of wild-type dopaminergic cells (Fig. 6B). On the other hand, the percentage of dopaminergic neurons (42 ± 4 %; n = 5 experiments) with fragmented mitochondria was greatly increased in PINK1-deficient SN neuronal culture (Fig. 6A and 6B). This finding suggests that PINK1 is required for maintaining the normal morphology and structural integrity of mitochondria in SN dopaminergic neurons.

Following the infection of recombinant adenoviruses containing the cDNA of wild-type PINK1, long thread-like morphology of mitochondria was restored in PINK1-deficient dopaminergic neurons, and the number of PINK1-null dopaminergic cells with mitochondrial fragmentation was significantly decreased in PINK1^{-/-} SN neuronal culture

(Fig. 6A and 6B). In contrast, overexpression of PARK6 mutant (G309D), (E417G) and (CΔ145) PINK1 failed to significantly decrease the percentage of PINK1-deficient dopaminergic cells with fragmented mitochondria (Fig. 6A and 6B). Therefore, PARK6 mutations cause the loss of PINK1 function in maintaining the normal mitochondrial morphology of SN dopaminergic neurons.

3.4. PINK1 deficiency enhances ROS generation in SN dopaminergic neurons, which is reversed by wild-type PINK1 but not by PARK6 mutant PINK1

Mitochondrial electron transport chain is the major intracellular source of reactive oxygen species (ROS), including superoxide anion, hydroxyl radical, various peroxides and hydroperoxides [34]. An excessive ROS formation and resulting oxidative stress is believed to be involved in PD pathogenesis [18-20]. It is very likely that PINK1 protects SN dopaminergic neurons from oxidative stress-induced neurotoxicity by inhibiting ROS formation. According to this hypothesis, mitochondrial ROS generation and oxidative stress inducer-evoked intracellular ROS production are expected to be augmented in PINK1^{-/-} SN dopaminergic cells.

In the present study, confocal MitoSOX Red staining was performed to visualize mitochondrial level of superoxide anion, a major ROS produced by the mitochondria, in 6 to 7-day-old cultured wild-type or PINK1-deficient SN dopaminergic neurons. MitoSOX Red is selectively targeted to the mitochondria and exhibits red fluorescence following the oxidation by superoxide anion. Compared to wild-type SN dopaminergic cells, MitoSOX Red fluorescence intensity, an indicator of mitochondrial superoxide level, was significantly increased in PINK1^{-/-} dopaminergic neurons (Fig. 7). Following the overexpression of wild-type PINK1, fluorescence signal of MitoSOX Red and mitochondrial superoxide generation was greatly reduced in PINK1-deficient SN dopaminergic cells (Fig. 7). In contrast, infecting PINK1-null SN dopaminergic neurons with recombinant adenoviruses containing the cDNA of PARK6 mutant (G309D), (E417G) or (CΔ145) PINK1 was defective in

decreasing mitochondrial level of superoxide (Fig. 7).

Confocal CM-H₂DCFDA staining was performed to visualize oxidative stressor H₂O₂-induced intracellular ROS generation in wild-type or PINK1-deficient SN dopaminergic neurons. CM-H₂DCFDA, a cell-permeant ROS indicator, is nonfluorescent and then converted to highly fluorescent 2',7'-dichlorofluorescein (DCF) following the removal of the acetate groups by intracellular esterases and ROS-induced oxidation (Fig. 8A and 8B). Compared to wild-type SN dopaminergic cells, H₂O₂ (1mM)-induced DCF fluorescence and ROS formation was greatly augmented in PINK1^{-/-} dopaminergic neurons (Fig. 8A and 8B). After the infection of adenoviruses containing the cDNA of wild-type PINK1, oxidative stressor H₂O₂ (1mM)-evoked DCF fluorescence signal and ROS production was significantly attenuated in PINK1-deficient SN dopaminergic cells (Fig. 8B and 8C). In contrast to wild-type PINK1, overexpression of PARK6 mutant (G309D), (E417G) or (CA145) PINK1 failed to significantly inhibit H₂O₂-induced DCF fluorescence and ROS generation in PINK1-null dopaminergic neurons (Fig. 8B and 8C). These findings suggest that lack of functional PINK1 expression caused by PARK6 mutations could augment the vulnerability of dopaminergic neurons to oxidative stress-induced neurotoxicity.

3.5. PINK1 deficiency enhances rotenone-induced cell death in SN neuronal culture

The pesticide rotenone is an inhibitor of mitochondrial complex I and induces cell death of SN dopaminergic neurons by causing oxidative stress. Rotenone has been proposed to act as an environmental mitochondrial toxin involved in PD pathogenesis and used to prepare PD animal model [18,20]. Our results demonstrate that PINK1 deficiency in SN dopaminergic cells leads to depolarization of mitochondrial membrane potential and elevated mitochondrial ROS generation, which is likely to enhance the susceptibility of dopaminergic neurons to mitochondrial toxin rotenone. In accordance with this hypothesis, MTT assays indicated that compared to 6 to 7-day-old wild-type neuronal culture of SN, rotenone-induced cell death of dopaminergic neurons was significantly enhanced in PINK1-

deficient SN neuronal culture (Fig. 9A). Following the overexpression of wild-type PINK1, augmentation of rotenone (150 nM)-induced cell death was significantly prevented in PINK1^{-/-} SN neuronal culture (Fig. 9B). In contrast, infecting PINK1-null SN neurons with recombinant adenoviruses containing the cDNA of mutant (G309D), (E417G) or (CΔ145) PINK1 did not inhibit enhancement of rotenone-induced neuronal death (Fig. 9B).

4. Discussion

Missense or truncating mutations of PINK1 gene cause recessive familial type 6 of Parkinson's disease (PARK6) [2-6]. PINK1 is believed to function as a mitochondrial Ser/Thr protein kinase and protect SN dopaminergic neurons against various cellular stresses [2,7,13,16]. Autosomal recessive inheritance mode indicates that PARK6 mutation-induced loss of PINK1 function is involved in the pathogenesis of PARK6. This hypothesis is supported by previous studies showing that when expressed in cell lines, PARK6 PINK1 mutants lose the ability to inhibit mitochondrial release of cytochrome-c and exert anti-apoptotic effect [2,14,30]. PINK1 is the first protein directly linking the mitochondrial abnormality to the neurotoxicity of SN dopaminergic cells and PD pathogenesis. Therefore, it is essential to study PINK1 regulation of mitochondrial functions and PARK6 mutation-induced loss of PINK1 function in SN dopaminergic neurons.

In the present study, we prepared PINK1 knockout mouse by deleting the exon 1 of PINK1 gene with the aid of homologous recombination. Previously, homologous recombination-mediated targeting exons 4-7, exons 3-5 or exons 4-5 has also been successfully performed to prepare PINK1-deficient mice [35-37]. Cultured SN dopaminergic neurons are the ideal preparation for conducting confocal imaging of mitochondria function and morphology and studying functional consequence of PARK6 mutation following recombinant adenoviruses-mediated overexpression of wild-type or PARK6 mutant PINK1. Thus, cultured PINK1-deficient SN dopaminergic cells were used to investigate physiological importance of PINK1 in maintaining mitochondrial function and morphology and test the hypothesis that PARK6 mutations cause the loss of PINK1 function in SN dopaminergic neurons.

Confocal imaging of potential sensitive dye TMRM indicated that a depolarized mitochondrial membrane potential ($\Delta\Psi_m$) was observed from PINK1^{-/-} SN dopaminergic

neurons. Overexpression of wild-type PINK1 restored mitochondrial membrane potential of PINK1-null SN dopaminergic cells. In contrast, PARK6 mutant (G309D), (E417G) or C-terminal truncated (C Δ 145) PINK1 failed to significantly reverse depolarized $\Delta\Psi_m$ observed in PINK1-deficient SN dopaminergic neurons. These findings suggest that PINK1 is required for maintaining normal mitochondrial membrane potential of SN dopaminergic cells and that PARK6 mutations-induced loss of PINK1 function could cause mitochondrial dysfunction of SN dopaminergic neurons by depolarizing mitochondrial membrane potential.

The number of dopaminergic cells with truncated and shortened mitochondria was greatly increased in PINK1-null SN neuronal culture. Following an overexpression of wild-type PINK1, long thread-like morphology of mitochondria was restored in PINK1-deficient dopaminergic cells. On the other hand, PARK6 mutant (G309D), (E417G) or (C Δ 145) PINK1 was defective in rescuing mitochondrial fragmentation in PINK1^{-/-} dopaminergic neurons. Our results suggest that PINK1 is required for maintaining the normal morphology and structural integrity of mitochondria in SN dopaminergic neurons and that PARK6 mutations cause the loss of PINK1 function in controlling mitochondrial morphology.

Mitochondria form dynamic tubular networks that undergo frequent morphological changes through fission and fusion, and dysregulated process of mitochondrial fission/fusion is believed to affect neuronal survival and function [38,39]. Three mitochondrial proteins, mitofusin 1 (MFN1), mitofusin 2 (MFN2) and optic atrophy 1 (OPA1), are required for mitochondrial fusion in mammals. Mitochondrial fission in mammalian cells is mediated by dynamin-related protein 1 (Drp1) [38,39]. Cycles of mitochondrial fission and fusion allow metabolite and mitochondrial DNA mixing and are required for maintaining shape, number and bioenergetic functionality of mitochondria [38, 40]. PINK1 deficiency-induced mitochondrial fragmentation in SN dopaminergic neurons

may result from an excessive mitochondrial fission or a defective mitochondrial fusion. Recent studies showed that knockdown of Drp1, dominant negative Drp1 and overexpression of MFN2 or OPA1 prevented PINK1 deficiency-induced mitochondrial fragmentation in SH-SY5Y neuroblastoma cells [27,41]. Furthermore, PINK1 knockdown fails to cause mitochondrial fragmentation in Drp1-deficient SH-SY5Y cells [26]. These findings suggest that PINK1 regulates mitochondrial fission process by modulating Drp1 function and that mitochondrial fragmentation observed in PINK1-deficient cells results from an augmented mitochondrial fission. Mitochondrial fragmentation caused by persistent fission has been proposed to promote apoptotic neuronal death [38,39]. Thus, it is possible that PINK1 deficiency or PARK6 mutation-induced loss of PINK1 function could cause neurotoxicity of SN dopaminergic cells by augmenting mitochondrial fission. Further study is required to investigate the exact molecular mechanism by which PINK1 regulates mitochondrial fission/fusion of SN dopaminergic neurons.

Recent studies using cell lines reported that in addition to regulating mitochondrial membrane potential and morphology, PINK1 is also required for the clearance of damaged mitochondria via autophagy-a process known as mitophagy [42]. PINK1 kinase activity and its mitochondrial localization sequence are prerequisites to induce translocation of the E3 ligase parkin to depolarized mitochondria and subsequent parkin-dependent autophagic degradation of damaged mitochondria [43]. PARK6 (G309D), (E417G) and (CΔ145) mutations investigated in this study are likely to impair PINK1/parkin-mediated mitophagy and cause an accumulation of damaged mitochondria, which may contribute to PD pathogenesis. PINK1-deficient SN dopaminergic cells prepared by us should be a valuable tool to investigate physiological and pathological importance of PINK1/parkin-mediated mitophagy in SN dopaminergic neurons.

Our results demonstrated that both basal level of mitochondrial superoxide and oxidative stressor H₂O₂-induced ROS generation were greatly increased in PINK1-null SN

dopaminergic neurons. PINK1 deficiency-induced ROS formation and oxidative stress is expected to cause depolarization of mitochondrial membrane potential and mitochondrial fragmentation [18,20,38]. Overexpression of wild-type PINK1 inhibited mitochondrial superoxide generation and oxidative stressor H₂O₂-induced ROS production in PINK1-deficient SN dopaminergic cells. In contrast, PARK6 mutant (G309D), (E417G) or (CΔ145) PINK1 was defective in decreasing mitochondrial level of superoxide and H₂O₂-induced ROS generation in PINK1^{-/-} dopaminergic neurons. These findings suggest that PINK1 exerts a neuroprotective effect on SN dopaminergic cells by attenuating ROS formation and oxidative stress and that PARK6 mutations-induced loss of PINK1 function could augment the vulnerability of dopaminergic neurons to oxidative stress-induced neurotoxicity. In accordance with our findings, fibroblasts and immortalized lymphoblast prepared from PD patients with (G309D) and (W437X) PINK1 mutations displayed an increased oxidative damage when compared with cells from control individuals [44,45].

A previous study by Shen and co-workers investigated the morphology and function aspects of mitochondria in the striatum of adult PINK1^{-/-} mice [46]. In accordance with our findings, mitochondrial dysfunction and enhanced oxidative stress were observed in adult PINK1-deficient mice [46]. Our study shows that mitochondria fragmentation is greatly increased in cultured PINK1-null dopaminergic neurons. However, electron microscopic studies did not find gross change in the ultrastructure or the total number of mitochondria in the striatum of adult PINK1-deficient mice [46]. This discrepancy is likely to result from PINK1 deficiency-induced region specific effect (substantia nigra versus striatum) or unknown compensatory mechanism(s) in adult PINK1^{-/-} mice.

The complex I of mitochondrial electron transport chain is thought to be the main source of ROS formation, and an impaired activity of mitochondrial complex I increases ROS generation and causes resulting oxidative stress [17,34]. A deficiency in mitochondrial complex I activity was found in the SN of PD patients [18-20]. Interestingly, reduced

activity of mitochondrial complex I was also observed in cultured fibroblasts prepared from PARK6 patients [47]. The pesticide rotenone is an inhibitor of mitochondrial complex I and induces cell death of SN dopaminergic neurons by causing oxidative stress. PINK1 deficiency-induced depolarization of mitochondrial membrane potential and elevated ROS formation is likely to enhance the susceptibility of SN dopaminergic neurons to mitochondrial toxin rotenone. Consistent with this hypothesis, rotenone-induced cell death of dopaminergic neurons was significantly enhanced in PINK1-deficient SN neuronal culture.

Our study suggests that PINK1 exerts neuroprotective effect on cultured SN dopaminergic cells. The absence of functional PINK1 could lead to neurotoxicity of SN dopaminergic cells and resulting parkinsonism. A recent post-mortem neuropathological examination of an early-onset PARK6 patient, a carrier of two heterozygous compound PINK1 mutations, reported neuronal loss in the substantia nigra pars compacta [48]. The present study shows that PINK1 deficiency enhances the vulnerability of SN dopaminergic cells to mitochondrial toxin rotenone. However, the number of TH-positive dopaminergic neurons in SN culture of PINK1^{-/-} mouse was similar to that in SN neuronal culture of wild-type mouse. Neuronal loss of SN dopaminergic cells and PARK6 motor dysfunction was not observed in 6 to 9-month-old PINK1-deficient mice prepared by us (data not shown). Similar to our results, previous TH-staining study using adult PINK1^{-/-} mice, generated by targeting deletion of exons 4-7 or exons 4-5 of PINK1 gene, showed no abnormality in morphology and number of SN dopaminergic neurons [35,36]. The absence of dopaminergic neuronal loss in the SN and PARK6 neurological phenotype in adult PINK1-null mice could result from several possible mechanisms, including the redundancy of mitochondrial Ser/Thr protein kinases, the lack of appropriate environmental triggers, functional compensation or species-dependent PINK1 function.

In summary, our results indicate that wild-type PINK1 is required for maintaining hyperpolarized mitochondrial membrane potential and normal mitochondrial morphology and inhibits ROS formation of cultured SN dopaminergic neurons. The present study further provides the first evidence that PARK6 mutant (G309D), (E417G) or (CΔ145) PINK1 is defective in attenuating ROS production and maintaining mitochondrial membrane potential and morphology of SN dopaminergic neurons. Wild-type PINK1 is believed to function as a mitochondrial Ser/Thr protein kinase, suggesting that PINK1 maintains mitochondrial membrane potential, regulates mitochondria fission/fusion and inhibits ROS generation by phosphorylating mitochondrial proteins of SN dopaminergic neurons. PARK6 mutant (G309D), (E417G) and (CΔ145) PINK1 studied in the present study are found in the Ser/Thr kinase domain of PINK1 and believed to cause an impairment of Ser/Thr kinase activity [11,12,49]. Therefore, it is very likely that an impaired Ser/Thr kinase activity caused by PARK6 (G309D), (E417G) and (CΔ145) mutations results in the loss of PINK1-mediated mitochondrial functions. A previous study reported that PINK1 inhibits oxidative stress-induced apoptotic cell death by phosphorylating mitochondrial chaperone TRAP1 [15]. Further quantitative phosphoproteomic analysis is required to identify other putative mitochondrial substrates of PINK1 and investigate the molecular mechanisms of PINK1 phosphorylation-mediated neuroprotective effects in SN dopaminergic cells.

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Figures and Figure Legends

Figure 1. Generation of PINK1-deficient mice. (A) Knockout vector targeting the exon 1 of PINK gene is composed of the short 5' homologous sequence, PGK-Neo selection cassette and the long 3' homologous sequence. Following the homologous recombination, the exon 1 is replaced by PGK-Neo selection cassette. (B) For the Southern blot analysis, tail genomic DNA prepared from wild-type (+/+), heterozygous PINK1^{+/-} or homozygous PINK1^{-/-} mice was digested with XbaI and hybridized with a 3' DNA probe. The expected sizes of DNA fragment in wild-type allele and targeted allele are 16 kb and 9 kb, respectively. (C) Northern blot analysis using brain total RNA of wild-type mouse detected an expected 2.3 kb PINK1 mRNA, which was greatly decreased in PINK1^{+/-} mouse and absent in PINK1^{-/-} mouse.

Figure 1

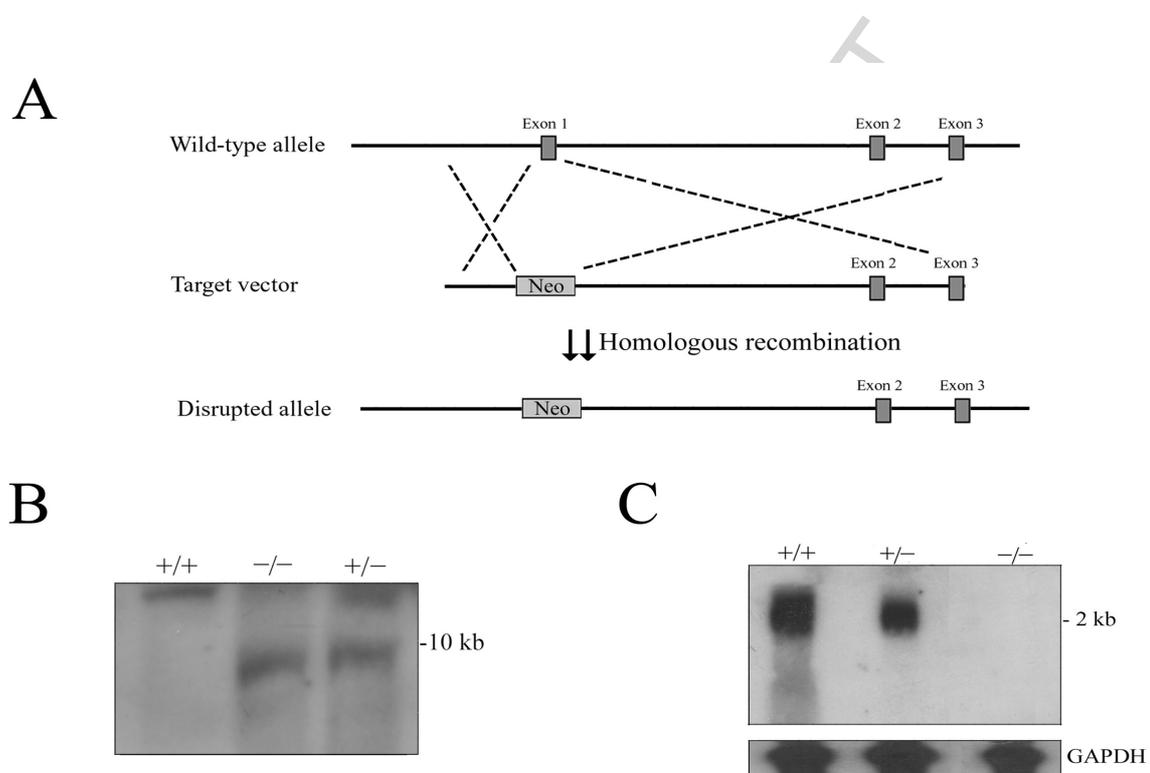


Figure 2. Cultured substantia nigra dopaminergic neurons of wild-type or PINK1^{-/-} mouse. (A) RT-PCR analysis demonstrated that in contrast to neuronal culture of substantia nigra (SN) or neocortex (CTX) prepared from wild-type mouse, PINK1 mRNA was absent in cultured substantia nigra or neocortical neurons of PINK1^{-/-} mouse. (B) Multipolar or oval tyrosine hydroxylase-positive dopaminergic neurons were found in primary SN neuronal culture prepared from wild-type or PINK1^{-/-} mouse. Scale bar is 25 μ m.

Figure 2

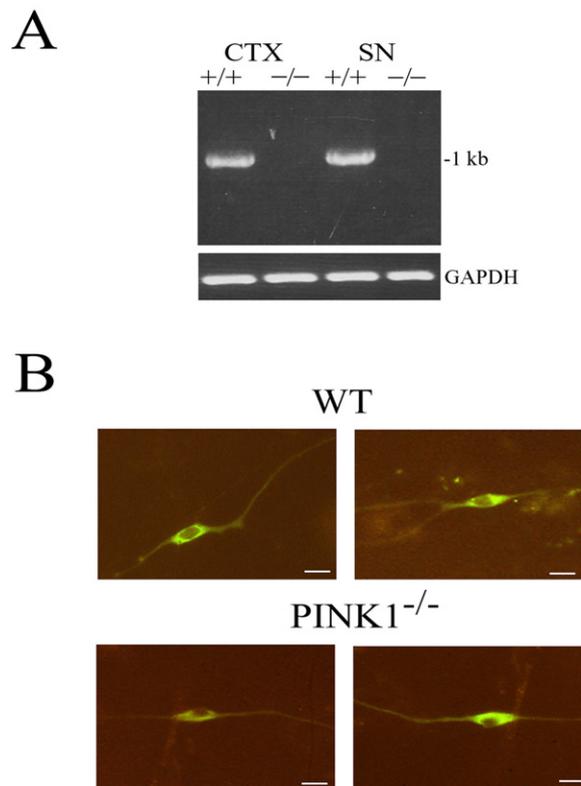


Figure 3. PINK1 deficiency causes a reduction in mitochondrial membrane potential of SN dopaminergic neurons. (A) Confocal TMRM staining was performed to visualize hyperpolarized mitochondrial membrane potential ($\Delta\Psi_m$) of a multipolar wild-type or PINK1^{-/-} SN dopaminergic neuron. Note that TMRM fluorescence intensity was significantly decreased in a PINK1-deficient SN dopaminergic cell. Scale bar is 10 μ m. (B) A reduction in TMRM fluorescence intensity and depolarization of $\Delta\Psi_m$ was observed from PINK1^{-/-} SN dopaminergic neurons. Each bar shows the mean \pm S. E. value of 45-50 neurons. * P < 0.01.

Figure 3

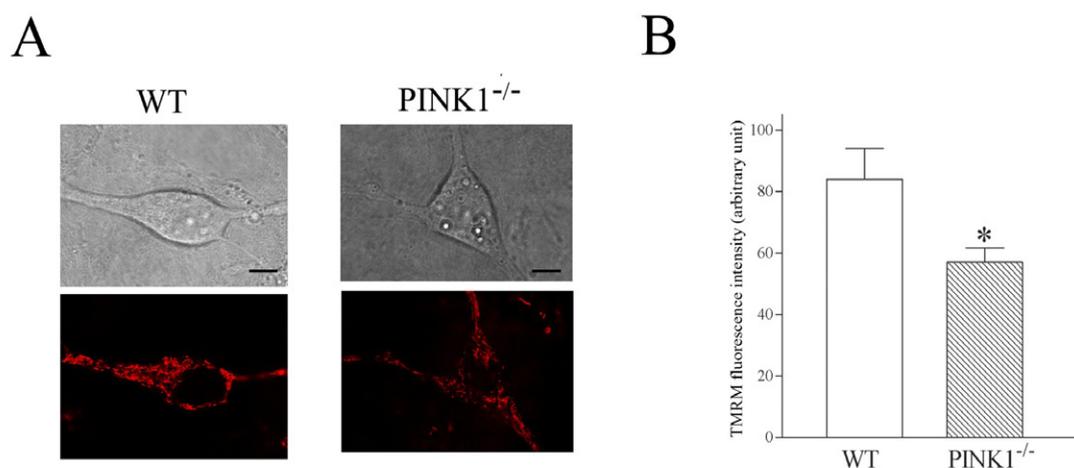


Figure 4. Expression of wild-type and PARK6 mutant PINK1 in the mitochondria of cultured PINK1-deficient SN neurons. Three days after the infecting cultured PINK1-deficient SN dopaminergic neurons with recombinant adenoviruses containing cDNA of HA-tagged wild-type or PARK6 mutant PINK1, subcellular fractionation and subsequent immunoblotting analysis using anti-HA antibody indicated that wild-type, (G309D), (E417G) or (C Δ 145) PINK1 was highly expressed in the mitochondrial extract of PINK1-null SN neurons. Cytochrome-c oxidase subunit IV (COX-IV) was used as an internal control for mitochondrial fraction.

Figure 4

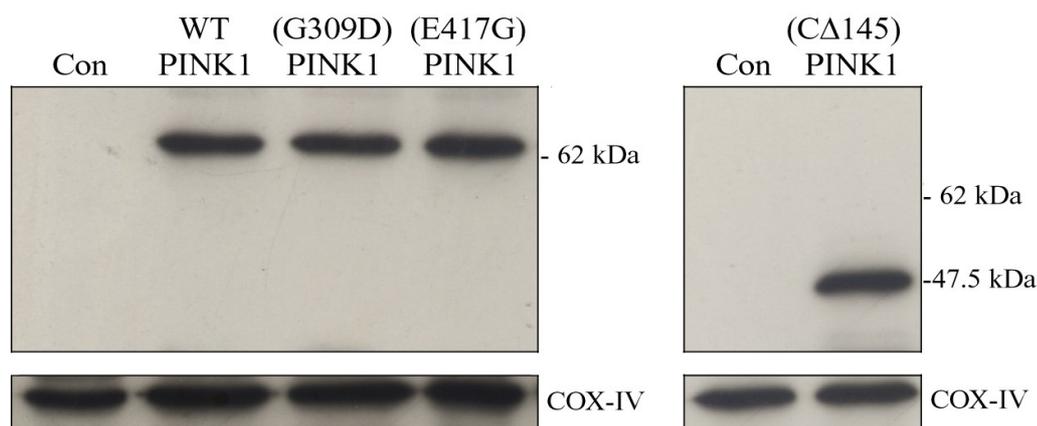


Figure 5. PARK6 mutant PINK1 fails to restore the mitochondrial membrane

potential in PINK1-deficient SN dopaminergic neurons. (A) Compared to reduced TMRM fluorescence intensity and depolarized $\Delta\Psi_m$ observed in an oval-shaped PINK1^{-/-} dopaminergic neuron, overexpression of wild-type PINK1 (+PINK1) increased TMRM fluorescence intensity and restored hyperpolarized $\Delta\Psi_m$ in a multipolar PINK1-null dopaminergic cell. In contrast, PARK6 mutant (G309D), (E417G) or (C Δ 145) PINK1 was defective in enhancing TMRM fluorescence signal and reversing depolarized $\Delta\Psi_m$ of PINK1^{-/-} SN dopaminergic neurons. Scale bar is 7 μ m. (B) In contrast to wild-type PINK1, mutant (G309D), (E417G) and (C Δ 145) PINK1 did not significantly increase TMRM fluorescence intensity in PINK1-deficient SN dopaminergic cells. Each bar represents the mean \pm S. E. value of 45-50 neurons. * P < 0.01 compared to PINK1^{-/-} dopaminergic neurons.

Figure 5

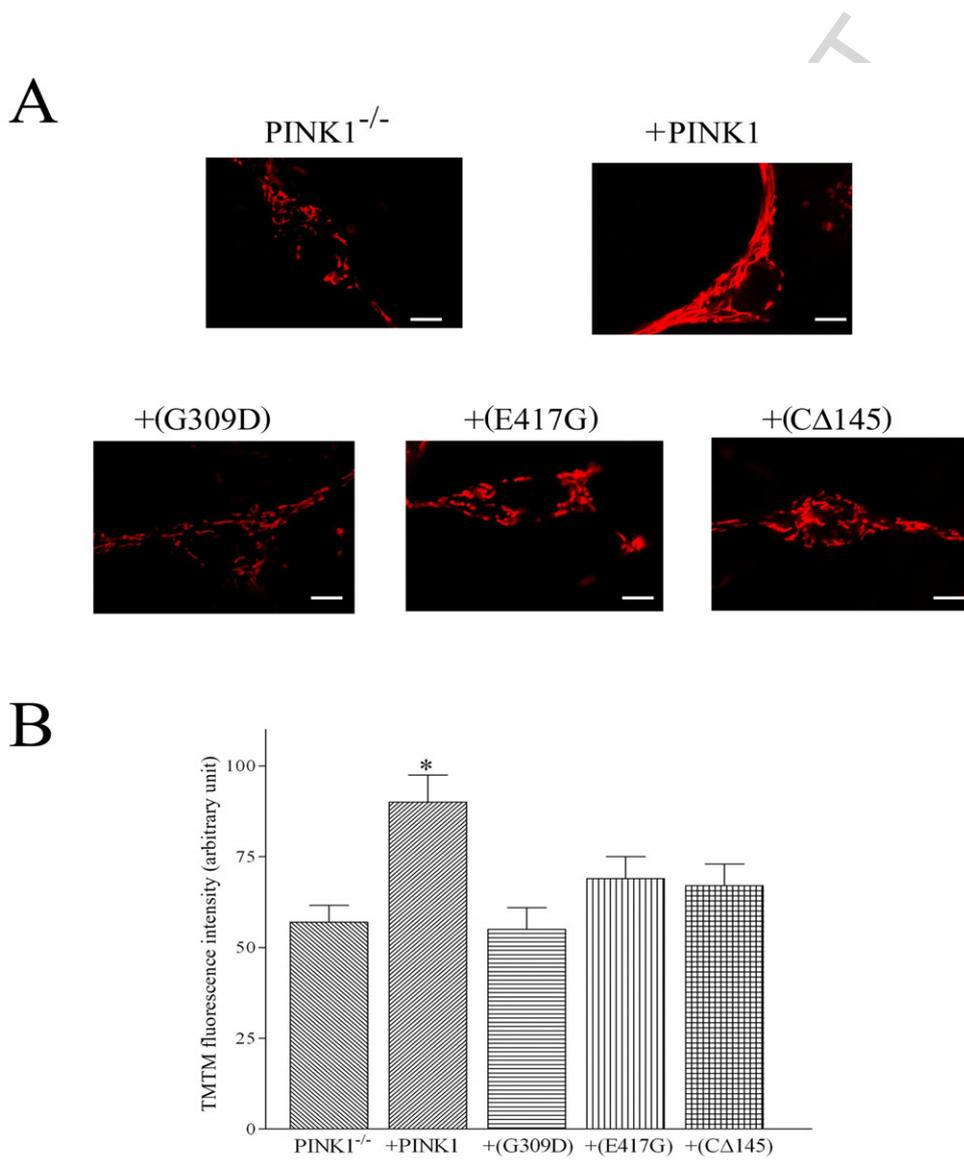


Figure 6. PINK1 deficiency promotes mitochondrial fragmentation in SN dopaminergic cells, which is rescued by wild-type PINK1 but not by PARK6 mutant PINK1. (A) Confocal MitoTracker Green staining was performed to visualize mitochondrial morphology. In contrast to filamentous and long thread-like mitochondria of a representative wild-type SN dopaminergic neuron, fragmented and shortened (<1.5 μm) mitochondria were observed from a PINK1^{-/-} dopaminergic cell. Overexpression of wild-type PINK1 restored long thread-like morphology of mitochondria in a PINK1-deficient dopaminergic neuron. Fragmented mitochondria were still observed in PINK1-null SN dopaminergic cells overexpressing PARK6 mutant (G309D), (E417G) or (C Δ 145) PINK1. Scale bar is 4 μm . (B) Percentage of dopaminergic neurons with fragmented mitochondrial was greatly increased in PINK1^{-/-} SN neuronal culture. In contrast to wild-type PINK1, mutant (G309D), (E417G) and (C Δ 145) PINK1 failed to rescue mitochondrial fragmentation in PINK1-deficient dopaminergic neurons. In each experiment, mitochondrial morphology of 20 wild-type or PINK1-null dopaminergic neurons was analyzed using the LAS AF software. For each dopaminergic cell, lengths of 30 well separated mitochondria were measured. Dopaminergic neuron was defined as the cell with mitochondrial fragmentation when $\geq 10\%$ of mitochondria measured were truncated and shortened (<1.5 μm). Each bar represents the mean \pm S. E. value of 5 experiments. * P < 0.01 compared to wild-type dopaminergic neurons. # P < 0.01 compared to PINK1^{-/-} dopaminergic neurons.

Figure 6

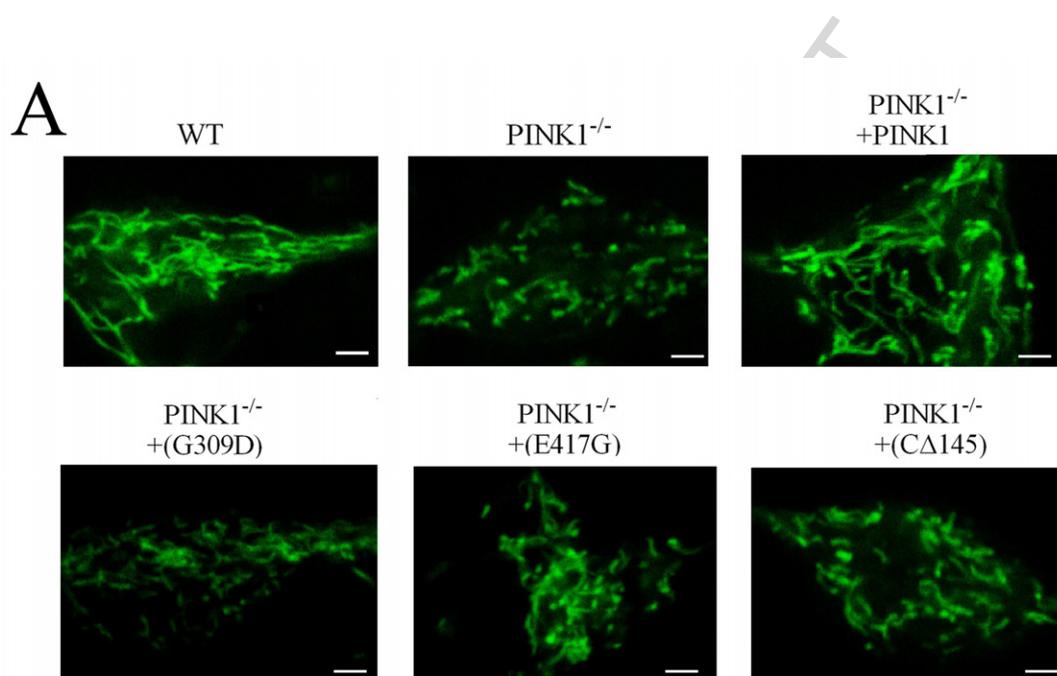
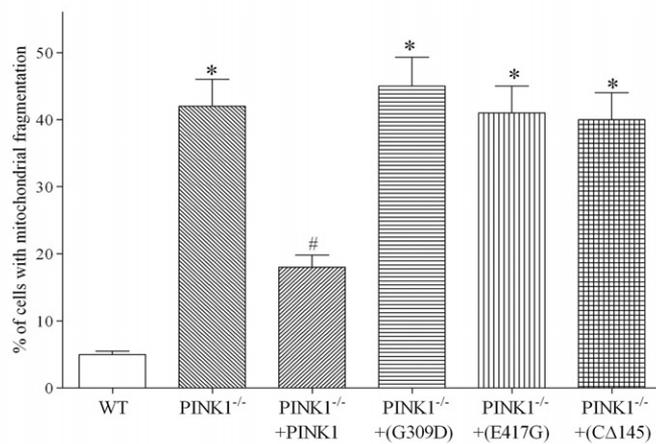
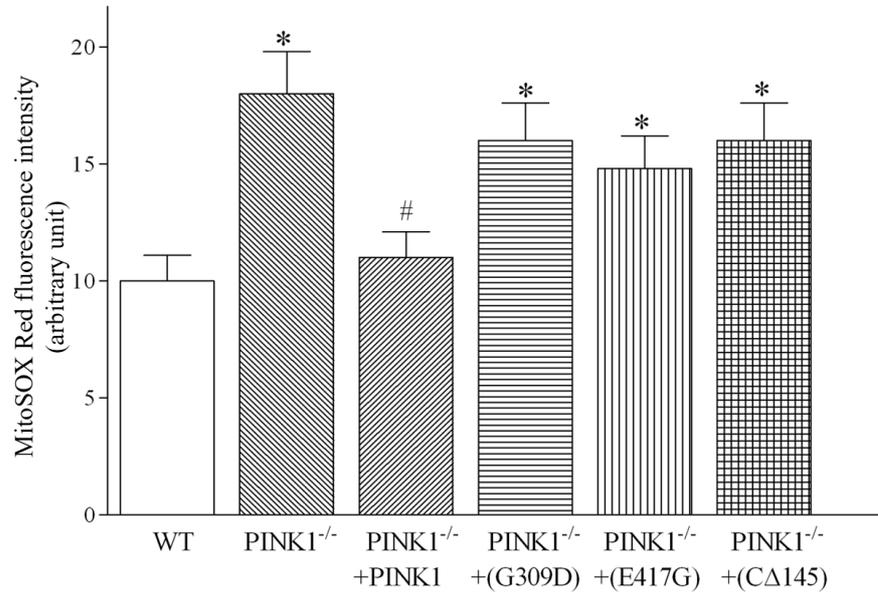
**B**

Figure 7. PINK1 deficiency in SN dopaminergic neurons enhances mitochondrial superoxide formation, which is inhibited by wild-type PINK1 but not by PARK6 mutant PINK1. Confocal MitoSOX Red staining was performed to visualize the basal level of mitochondrial superoxide. MitoSOX Red fluorescence intensity and the formation of mitochondrial superoxide was significantly augmented in PINK1^{-/-} SN dopaminergic neurons. Infecting PINK1-null dopaminergic cells with recombinant adenoviruses containing the cDNA of wild-type PINK1 greatly inhibited MitoSOX Red signal and mitochondrial superoxide level. Overexpression of PARK6 mutant (G309D), (E417G) or (CΔ145) PINK1 was defective in inhibiting MitoSOX Red fluorescence intensity in PINK1-deficient SN dopaminergic neurons. Each bar represents the mean \pm S. E. value of 25-30 neurons. * P < 0.01 compared to wild-type dopaminergic neurons. # P < 0.01 compared to PINK1^{-/-} dopaminergic neurons.

Figure 7



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Figure 8. PINK1 deficiency augments H₂O₂-induced ROS formation in SN dopaminergic neurons, which is prevented by wild-type PINK1 but not by PARK6 mutant PINK1. (A) Confocal CM-H₂DCFDA imaging indicated that pretreating an oval-shaped wild-type dopaminergic neuron with oxidative stressor H₂O₂ (1 mM) for 30 min induced intracellular ROS formation and DCF fluorescence. (B) H₂O₂-induced increase in ROS production and DCF fluorescence was significantly augmented in a PINK1^{-/-} SN dopaminergic cell. Overexpression of wild-type PINK1 greatly reduced H₂O₂-evoked ROS generation and DCF fluorescence signal in a PINK1-deficient dopaminergic neuron. In contrast, infecting PINK1^{-/-} SN dopaminergic cells with adenoviruses containing the cDNA of PARK6 mutant (G309D), (E417G) or (CΔ145) PINK1 was defective in reducing H₂O₂-induced DCF fluorescence. Scale bar is 10 μm. (C) Summary of H₂O₂ (1 mM)-induced DCF fluorescence in wild-type dopaminergic neurons, PINK1^{-/-} dopaminergic cells and PINK1-deficient dopaminergic neurons overexpressing wild-type or PARK6 mutant PINK1. Each bar represents the mean ± S. E. value of 25 neurons. * P < 0.01 compared to wild-type dopaminergic neurons. # P < 0.01 compared to PINK1^{-/-} dopaminergic neurons.

Figure 8

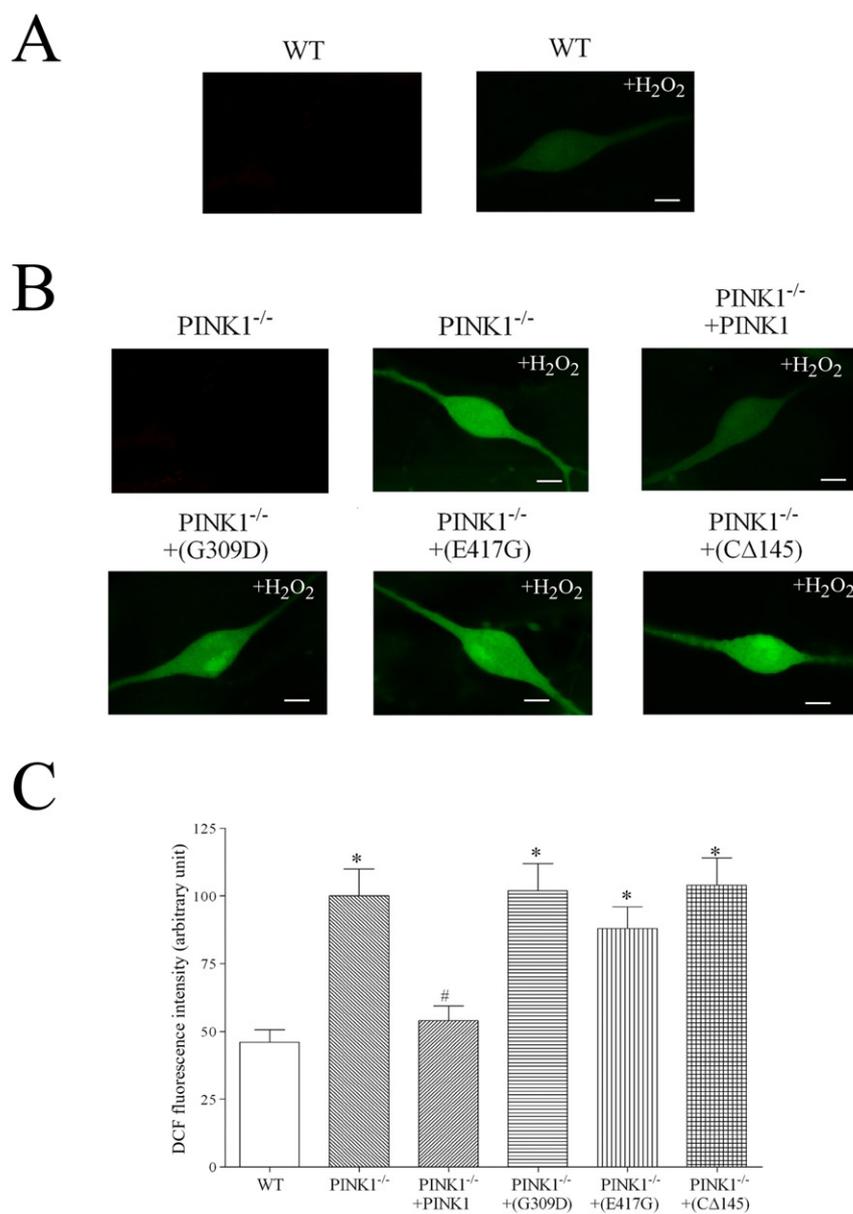
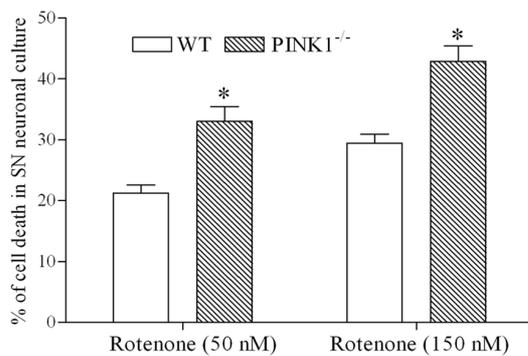


Figure 9. PINK1 deficiency enhances rotenone-induced cell death of cultured SN

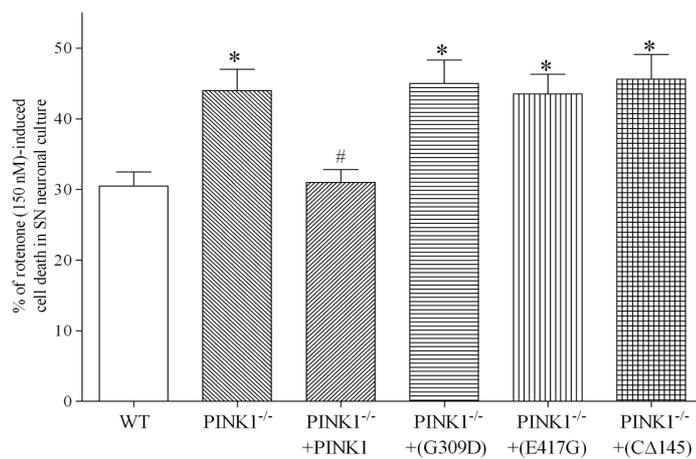
neurons. (A) Mitochondrial neurotoxin rotenone induced cell death of wild-type cultured SN neurons with a dose-dependent manner. Compared to wild-type SN neuronal culture, rotenone-induced cell death of dopaminergic neurons was significantly augmented in PINK1-deficient SN neuronal culture. (B) Overexpression of wild-type PINK1 significantly prevented the augmentation of rotenone (150 nM)-induced cell death in PINK1^{-/-} SN neuronal culture. Augmented rotenone-induced neuronal death was still observed in PINK1-null SN neurons overexpressing PARK6 mutant (G309D), (E417G) or (CΔ145) PINK1. Each bar shows the mean \pm S. E. value of 4-5 experiments. * P < 0.01 compared to wild-type cultured SN neurons. # P < 0.01 compared to cultured PINK1^{-/-} SN neurons.

Figure 9

A



B



Research highlights:

► Depolarization of mitochondrial membrane potential, mitochondrial fragmentation and augmented ROS formation were observed in cultured PINK1-deficient substantia nigra (SN) dopaminergic neurons. ► Mitochondrial toxin rotenone-induced cell death of dopaminergic neurons was augmented in PINK1-null SN neuronal culture.

► Overexpression of wild-type PINK1 restored hyperpolarized $\Delta\Psi_m$ and thread-like mitochondrial morphology and inhibited ROS formation in PINK1-null dopaminergic cells. ► PARK6 mutant (G309D), (E417G) or (C Δ 145) PINK1 failed to rescue mitochondrial dysfunction and inhibit oxidative stress in PINK1-deficient dopaminergic neurons.