Programmed cell death-2 isoform1 is ubiquitinated by parkin and increased in the substantia nigra of patients with autosomal recessive Parkinson's disease

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ABSTRACT

Mutations in *parkin* gene are responsible for autosomal recessive Parkinson's disease (ARPD) and its loss-of-function is assumed to affect parkin ubiquitin ligase activity. Accumulation of its substrate may induce dopaminergic neurodegeneration in the substantia nigra (SN) of ARPD. Here, we show that parkin interacts with programmed cell death-2 isoform 1 (PDCD2-1) and promotes its ubiquitination. Furthermore, accumulation of PDCD2-1 was found in the SN of ARPD as well as in sporadic PD, suggesting that common failure of the ubiquitin–proteasome system is associated with neuronal death in both ARPD and sporadic PD.

Structured summary:
MINT-6805975, MINT-6806032, MINT-6806051, MINT-6806070: *PDCD2* (uniprotkb:Q16342) *physically interacts* (MI:0218) with *Parkin* (uniprotkb:O60260) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-6805947: *Parkin* (uniprotkb:O60260) *physically interacts* (MI:0218) with *PDCD2* (uniprotkb:Q16342) by *two hybrid* (MI:0018)
MINT-6806000: *PDCD2* (uniprotkb:Q16342) *physically interacts* (MI:0218) with *ubiquitin* (uniprotkb:P62988) by *anti tag coimmunoprecipitation* (MI:0007)

1. Introduction

Mutations in the *parkin* gene are linked to autosomal recessive Parkinson's disease (ARPD) [1]. Parkin functions as an E3 ubiquitin (Ub) ligase in the ubiquitin–proteasome system (UPS) [2], which maintains intracellular protein quality. The E3 Ub ligase attaches Ub to specific substrate(s), as well as activates E1 and conjugating E2 enzymes. Polyubiquitinated proteins are degraded by the 26S proteasome. Homozygous mutations of the *parkin* gene result in loss-of-function and result in failure of the UPS. In this context, degeneration of dopaminergic neurons in the substantia nigra (SN) of ARPD is presumed to be induced by accumulation of protein substrate(s). Thus, identification of the substrate(s) for parkin is important for understanding the mechanism of neurodegeneration of ARPD.

Programmed cell death-2 (PDCD2) protein was isolated from a human fetal lung cDNA library and characterized as highly homologous to *Rp-8*, a protein associated with apoptosis [3–5]. PDCD2 exists in two isoforms [6]. The PDCD2 isoform 1 (PDCD2-1) comprises 344 amino acids residues containing the MYND zinc-finger domain [4,6]. In present study, we found that parkin interacts with PDCD2-1 and controls its degradation. We also demonstrated high levels of PDCD2-1 in the SN of patients with ARPD and those with sporadic Parkinson's disease (sPD).

2. Materials and methods

2.1. Yeast two-hybrid screening

The parkin ubl-linker (1-238) cDNA was linked to the cDNA for the GAL4-DNA binding domain in pGBD-ubl-linker, and yeast PJ69-2A cells were transformed with pGBD-ubl-linker, and subsequently with the library of SH-SY5Y cDNAs fused to the cDNA for the GAL4 activation domain (GAL4AD). The transformed yeast were selected and confirmed as described by the manufacturer (Clontech, Shiga, Japan).

2.2. Plasmids and antibodies

The full-length and mutant parkins were cloned into 3XMyc-pcDNA3.1(+) vector at the *Bam*HI site. Mutagenesis to introduce

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point mutations was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The pCMV/3XFLAG-PDCD2-1 was prepared by polymerase chain reaction (PCR) using appropriately designed primers with restriction site (*Bam*HI). The PCR product was inserted into the p3XFLAG-CMV™ vector (Sigma–Aldrich, St. Louis, MO). HA-Ub was a kind gift from Prof. Keiji Tanaka (Tokyo Metropolitan Institute of Medical Science, Tokyo). Rabbit polyclonal PDCD2 antibody was kindly provided by Prof. Philip A. Sharp (Massachusetts Institute of Technology, Cambridge, MA) [7]. Rabbit polyclonal anti-parkin and mouse monoclonal GAPDH antibodies were obtained from Cell Signaling Technology (Beverly, MA) and Chemicon (Temecula, CA), respectively. Monoclonal (9E10), horseradish peroxidase (HRP)-conjugated monoclonal (9E10), and polyclonal (A-14) anti-Myc, HRP-conjugated anti-FLAG (M2), biotin-conjugated anti-HA (3F10) antibodies were purchased from Santa Cruz biotechnology (Santa Cruz, CA), Sigma–Aldrich, and Roche (Mannheim, Germany), respectively.

2.3. Cell culture and transfection

COS1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml) at 37 °C under 5% CO_2. COS1 cells were transiently transfected with expression vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the protocol supplied by the manufacturer.

2.4. Immunoprecipitation (IP) and western blotting

Twenty-four hours after transfection, the cells were washed with phosphate-buffered saline (PBS) and then lysed on ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, and 10% glycerol) containing protease inhibitor (Complete Mini EDTA-free, Roche Diagnostics, Penzberg, Germany) for 20 min. Lysates were centrifuged at 4 °C at 15 000 rpm. The protein concentration in the supernatant was determined using the DC protein assay kit (Bio-Rad, Hercules, CA). The supernatant fractions were incubated with the primary antibodies and then treated with protein G sepharose 4 fast flow (Amersham, Sweden). Immunoprecipitates were washed three times with lysis buffer then boiled in 3X SDS loading buffer (30 μl) for 5 min. Each sample (10 μl) was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The separated proteins were transferred onto a polyvinylidene difluoride microporous membrane (Bio-Rad, Hercules, CA) using transfer buffer (40 mM CAPS, 30 mM Tris, and 15% MeOH). The transferred membrane was blocked with 5% skim milk and incubated overnight with the primary antibody at 4 °C. After incubation with HRP-conjugated secondary antibodies, the reaction was visualized using a chemiluminescence reagent (ECL-plus, Amersham). The intensity of the signal was analyzed by LAS-1000plus (Fuji film, Tokyo).

Table 1
Clinical summary of the subjects included in the study.

Case	Diagnosis	Age	Sex	Duration	Mutation
1	Control	45	Female	–	–
2	Control	57	Male	–	–
3	Control	70	Female	–	–
4	sPD	66	Male	10	–
5	sPD	81	Female	13	–
6	sPD	62	Male	12	–
7	ARPD	63	Male	30	Exon 3 deletion
8	ARPD	68	Male	35	Exon 3 deletion
9	ARPD	62	Male	38	Exon 4 deletion

2.5. Ubiquitination assay

COS1 cells were transfected with 3XMyc-parkin, 3XFLAG-PDCD2-1, and HA-Ub in various combinations. After 24 h, the cells were incubated in a medium with 50 μM MG132 (Peptide Institute Inc., Osaka, Japan) for additional 30 min. The cell lysate was immunoprecipitated with anti-FLAG M2 affinity gel (Sigma) and products were analyzed with biotin-conjugated anti-HA, HRP-conjugated anti-FLAG, and HRP-conjugated anti-Myc antibodies.

2.6. Brain samples

Fresh frozen brains were obtained from the Department of Neurology, Juntendo University School of Medicine. The subjects were three patients with ARPD, three with sPD, and three control subjects. Table 1 provides a summary of the clinical profile of the subjects. The diagnosis was confirmed by neuropathological examination of the brain tissues as sPD, and excluding neurodegenerative disorders in control subjects. Gene analysis of *parkin* was preformed as reported previously [1]. The study protocol was approved by the Human Ethics Review Committee of Juntendo University School of Medicine. Approximately, 0.5 g of frozen midbrain were placed into 3.5 ml ice-cold homogenisation buffer [DTT, 50 mM Tris–HCl (pH 7.4), 0.1% SDS, 1% Triton X 100, and 1% sodium deoxycholate] and homogenized in the presence of a mixture of protease inhibitors (Complete Mini EDTA-free). The protein concentration in each sample was determined using the DC protein assay kit (Bio-Rad).

3. Results and discussion

3.1. Identification of PDCD2-1 as a parkin-binding protein

To identify parkin-binding protein, we screened the SHSY-5Y cDNA library using the ubl-linker parkin by the yeast two-hybrid method. PDCD2-1 was isolated as a potential clone that can interact with parkin (data not shown). Co-immunoprecipitation analysis was performed to confirm the interaction between parkin and PDCD2-1. The 3XFLAG-PDCD2-1 was co-transfected with either 3XMyc-parkin or 3XMyc control plasmid into COS1 cells. After lysis of the transfected cells, the supernatant (1 mg protein) was pulled-down with anti-Myc antibody (A-14). Immunoprecipitates were boiled in 3XSDS loading buffer (30 μl) and then each sample (10 μl) was subjected to SDS–PAGE, followed by western blotting with HRP-conjugated anti-FLAG antibody. The 3XFLAG-PDCD2-1 band was only detected in the sample with 3XMyc-parkin, but not in the 3XMyc plasmid (Fig. 1A). When co-immunoprecipitation was performed in reverse with anti-FLAG M2 affinity gel, only the 3XMyc-parkin band was noted in protein extracts with 3XFLAG-PDCD2-1 (Fig. 1B). These results suggest that wild parkin interacts with PDCD2-1.

3.2. PDCD2-1 binds to parkin at two sites

Parkin is a 465-amino acid protein, consisting of Ub-like domain (UBL) in the N-terminal and two RING finger motifs towards the C-terminal. Between the two RINGs, there is an in-between-RING (IBR) structure comprising a RING-box (Fig. 1C). To investigate the PDCD2-1 binding site(s) of parkin, we constructed various truncated and point mutants of parkin tagged with 3XMyc (Fig. 1C). Various mutants of 3XMyc-parkin and 3XFLAG-PDCD2-1 were co-transfected into COS1 cells. The supernatant (1 mg protein) was then immunoprecipitated with anti-Myc antibody (A-14), and each sample (10 μl) was subsequently subjected to SDS–PAGE, and western blotting with HRP-conjugated anti-FLAG
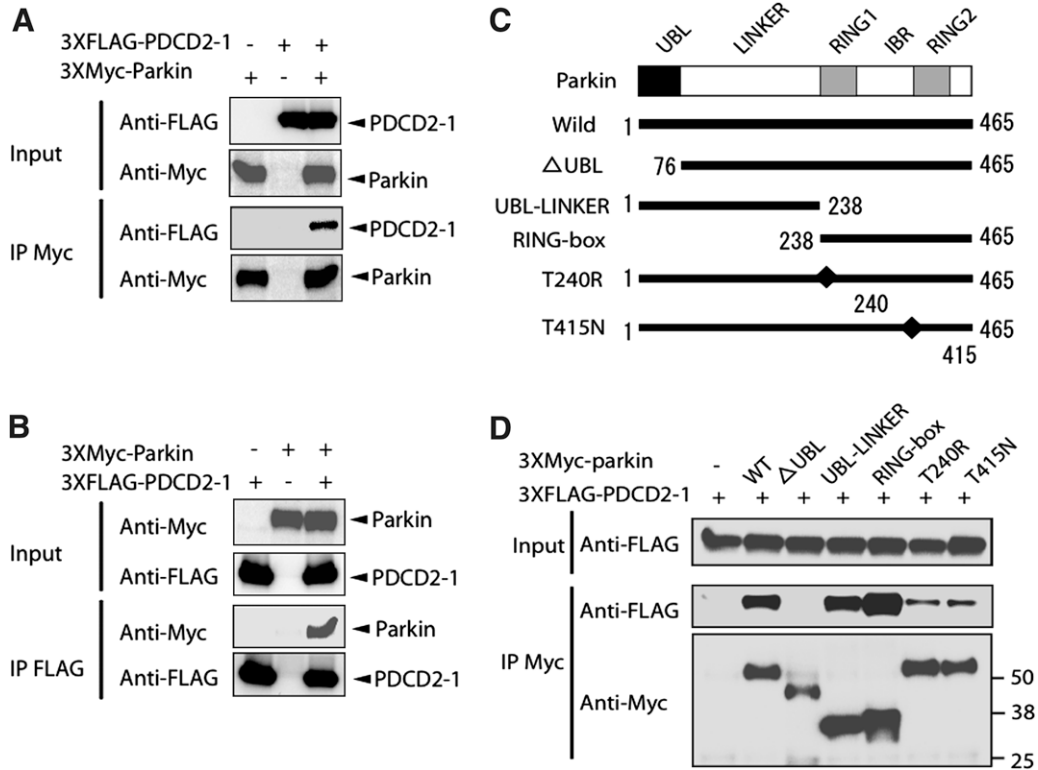
Fig. 1. Parkin interacts with PDCD2-1. (A) Lysates (1 mg protein) from COS1 cells co-transfected with 3XMyc-parkin and 3XFLAG-PDCD2-1 were subjected to immunoprecipitation (IP) with anti-Myc antibody followed by western blot analysis with HRP-conjugated anti-FLAG and anti-Myc antibodies. To confirm proper expression level of the transiently transfected protein, input lysates were also analyzed by western blotting with HRP-conjugated anti-FLAG and anti-Myc antibodies. (B) In reverse analysis, lysates (1 mg protein) from COS1 cells co-transfected with 3XMyc-parkin and 3XFLAG-PDCD2-1 were subjected to immunoprecipitation with anti-FLAG M2 affinity gel followed by Western blotting with HRP-conjugated anti-FLAG and anti-Myc antibodies. Input lysates were also analyzed by western blotting with HRP-conjugated anti-FLAG and anti-Myc antibodies. (C) The structure of parkin protein. Schematic representation of wild-type and various mutant parkins. UBL: Ub-like domain; IBR: inbetween RING structure. (D) Interaction between mutant parkin and PDCD2-1. Lysates (1 mg protein) from COS1 cells co-transfected with various 3XMyc tagged parkin domain constructs or control plasmid, together with 3XFLAG-PDCD2-1, were subjected to IP with anti-Myc antibody. IP products and input lysates were analyzed by western blotting with HRP-conjugated anti-FLAG and anti-Myc antibodies.

antibody. Truncated parkin lacking the UBL domain did not interact with PDCD2-1 (Fig. 1D, lane 3), suggesting that the UBL domain is necessary for such interaction. Furthermore, PDCD2-1 interacted with the RING-box domain (Fig. 1D, lane 5). However, in the presence of the LINKER domain, no interaction was noted between UBL-domain-negative parkin and RING-box domain as evident by the similarity of the amount of FLAG in lane 2 to that in lane 4 (Fig. 1D). Thus, the linker domain may structurally block the bind-
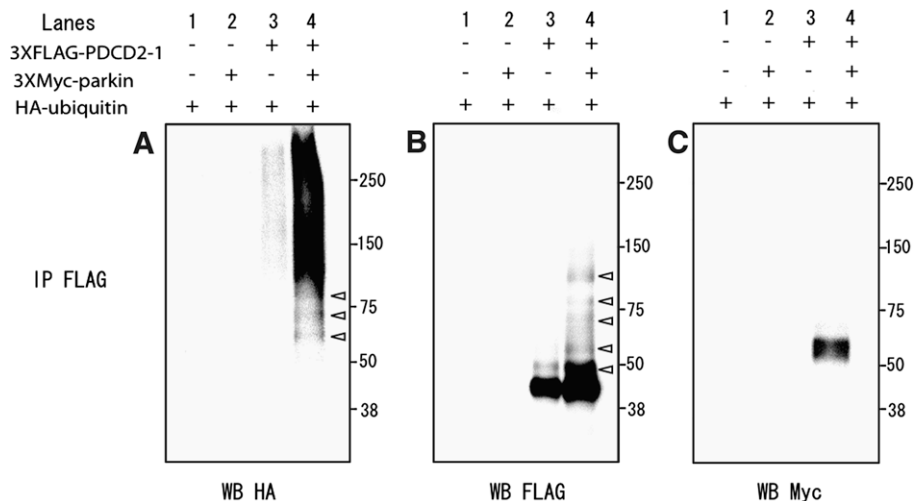

Fig. 2. Parkin ubiquitinates PDCD2-1. COS1 cells transfected with 3XFLAG-PDCD2-1, 3XMyc-parkin, and HA-Ub, were treated with MG132 for 30 min, and lysates (2 mg protein) were immunoprecipitated with anti-FLAG M2 affinity gel. The immunoprecipitated products of the ubiquitination assays were detected with biotin-conjugated anti-HA (A), HRP-conjugated anti-FLAG (B) and HRP-conjugated anti-Myc (C) antibodies. The ubiquitination of PDCD2-1 was promoted with transfection of parkin. White *arrowheads* indicate ubiquitination of PDCD2-1.

ing to RING-box. Interestingly, the ARPD causing missense mutation within the RING-box such as T240R and T415N, showed reduced binding to PDCD2-1 (Fig. 1D).

3.3. Ubiquitination and degeneration of PDCD2-1 are promoted by parkin

Next, we used the ubiquitination assay to determine whether PDCD2-1 is a substrate of parkin. COS1 cells were co-transfected with various combinations of 3XFLAG-PDCD2-1, 3XMyc-parkin, and HA-Ub. After treatment of cells with MG132 for 30 min, they were lysed and each supernatant (2 mg protein) was immunoprecipitated with anti-FLAG M2 affinity gel. Immunoprecipitates were boiled in 3XSDS loading buffer (30 μl) and then each sample (10 μl) was subjected to SDS–PAGE, followed by western blotting with biotin-conjugated anti-HA, HRP-conjugated anti-FLAG, and HRP-conjugated anti-Myc antibodies. Ubiquitination of PDCD2-1 was detected by western blotting with biotin-conjugated anti-HA and HRP-conjugated FLAG antibodies. As shown in Fig. 2A–C, ubiquitination of PDCD2-1 was promoted by transfection of 3XMyc-parkin. Mild ubiquitination of PDCD2-1 was noted without transfection of 3XMyc-parkin (lane 3, Fig. 2A–C). These results may reflect the influence of endogenous parkin in COS1 cells. To examine whether parkin promotes PDCD2-1 degradation, COS1 cells were transfected with a fixed amount of 3XFLAG-PDCD2-1 and increasing amounts of 3XMyc-parkin. The total amounts of transfected plasmids were adjusted with 3XMyc plasmid to avoid the influence
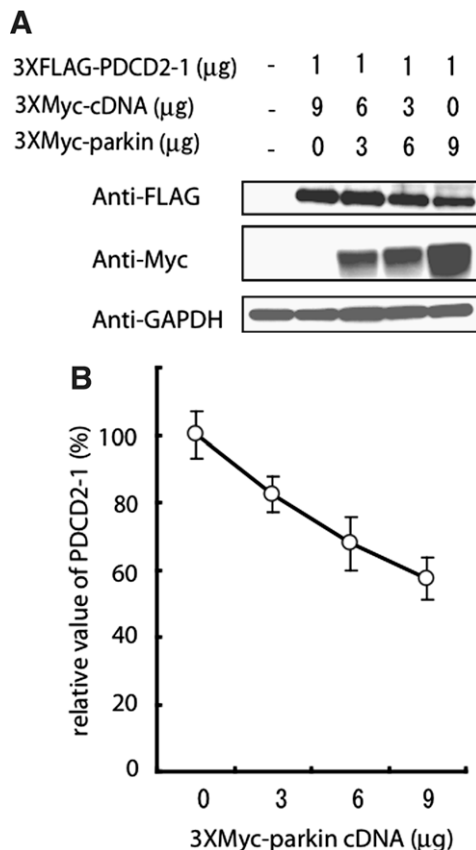
of transfection, and the experiment was performed three times. As is shown in Fig. 3A and B, the amount of co-transfected parkin modulated the level of PDCD2-1 in a dose-dependent manner; the relative value of PDCD2-1 decreased to approximately 60% at the highest dose of parkin.

3.4. Accumulation of PDCD2 in SN of ARPD and sPD

In the next step, we examined the level of PDCD2-1 in the SN of patients with ARPD and sPD and control subjects. The brain tissues were homogenized followed by western blotting with rabbit polyclonal anti-PDCD2 antibody. As shown Fig. 4A–C, the amount of PDCD2-1 was higher in all ARPD and sPD compared with the control subjects. Furthermore, PDCD2-1 level was lower in Cases 2 and 5 with high amount of parkin, compared with those with low amount of parkin Fig. 4A–C.

We demonstrated in the present study that PDCD2-1 is a parkin substrate. PDCD2-1 is expressed ubiquitously in various human tissues including the brain [4]. Previous reports described the involvement of PDCD2-1 in various processes such as apoptosis, inflammation, and cell proliferation [6,8–10]. Although the role of PDCD2-1 in the central nervous system remains to be elucidated, the expression of Rp-8, which is homologous to PDCD2-1, increases during cell death in rodent cerebellum [5]. Furthermore, antisense knockdown of parkin causes apoptosis of SH-SY5Y cells [11] and overexpression of parkin protects SH-SY5Y cells against apoptosis
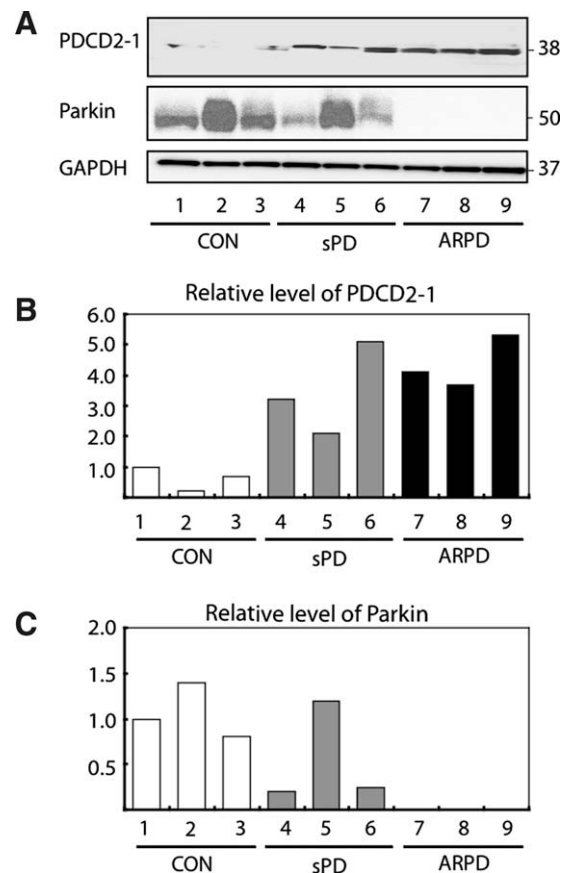

Fig. 3. Degradation of PDCD2 depends on parkin. (A) Lysates prepared from COS1 cells transfected with 3XFLAG-PDCD2-1 and with 3XMyc-parkin. The amount of transfected 3XFLAG-PDCD2-1 was fixed at 1 μg while the amount of transfected 3XMyc-parkin was increased to 0, 3, 6, and 9 μg. The total amounts of transfected plasmids were adjusted with 3XMyc plasmid to avoid the influence of transfection. (B) Data are expressed as mean ± S.E.M. Note the fall in the level of 3XFLAG-PDCD2-1 with increased expression of 3XMyc-parkin.

Fig. 4. (A) Quantitative analysis of PDCD2-1 and parkin levels normalized to GAPDH in control subjects, sPD, and ARPD. Homogenates of human midbrain from control subjects ($n = 3$), sPD ($n = 3$), and ARPD ($n = 3$) were immunoblotted with anti-PDCD2 (top panel) and anti-parkin (middle panel) antibodies. (B) PDCD2-1 levels were significantly higher in sPD and ARPD than control subjects. The levels of PDCD2-1 in the SN of sPD and ARPD were 2.2 to 5.1- and 3.8 to 5.7-fold higher than the control, respectively. (C) Expression of parkin was noted in control and sPD, but not in ARPD.

induced by dopamine or 6-hydroxydopamine [12]. These findings suggest that parkin is associated with apoptosis and its anti-apoptotic protective effect is dependent on its E3 activity.

Another important finding of this study was the increased levels of PDCD2-1 in the SN of ARPD and sPD. To date, several studies demonstrated that parkin is modified after translation [13,14]. Both in vivo and in vitro experiments have shown that parkin is s-nitrosylated and that such s-nitrosylation serves to inhibit the E3 ligase activity and protective function of parkin [13,14]. Furthermore, s-nitrosylated parkin is increased in sPD [13,14]. These results suggest the pathogenesis of sPD could also involve failure of parkin activity in the UPS. The UPS may be an important and common pathway of nigral neuronal death in both ARPD and sPD.

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