

Molecular interaction between parkin and PINK1 in mammalian neuronal cells

Ji Won Um ^a, Christine Stichel-Gunkel ^c, Hermann Lübbert ^d, Gwang Lee ^b, Kwang Chul Chung ^{a,*}

^a Department of Biology, College of Life Science and Biotechnology, Yonsei University, 262 Seongsanno, Seodaemun-gu, Seoul 120-749, Republic of Korea

^b Brain Disease Research Center, Institute for Medical Science, Ajou University School of Medicine, Suwon 443-749, Republic of Korea

^c Biofrontera Bioscience GmbH, D-51377 Leverkusen, Germany

^d Department of Animal Physiology, Ruhr-University Bochum, D-44780 Bochum, Germany

ARTICLE INFO

Article history:

Received 24 October 2008

Revised 9 December 2008

Accepted 11 December 2008

Available online 8 January 2009

Keywords:

Parkin's disease

Parkin

PINK1

Lewy bodies

Proteasome

ABSTRACT

Parkin's disease (PD) is characterized by the deterioration of dopaminergic neurons in the pars compacta of substantia nigra and the formation of intraneuronal protein inclusions. The etiology of PD is not known, but the recent identification of several mutation genes in familial PD has provided a rich understanding of the molecular mechanisms of PD pathology. Mutations in *PTEN-induced putative kinase 1* (*PINK1*) and *parkin* are linked to early-onset autosomal recessive forms of familial PD. Here we show molecular and functional interactions between parkin and PINK1. Parkin selectively binds to PINK1 and upregulates PINK1 levels. In addition, PINK1 reduces the solubility of parkin, which induces the formation of microtubule-dependent cytoplasmic aggregates. Our findings reveal that parkin and PINK1 affect each other's stability, solubility and tendency to form aggregates, and have important implications regarding the formation of Lewy bodies.

© 2008 Elsevier Inc. All rights reserved.

Introduction

Parkin disease (PD) is a devastating neurodegenerative disease characterized by a distinct set of motor symptoms, including muscle rigidity, tremor, postural instability and bradykinesia (Olanow and Tatton, 1999). Pathological hallmarks of PD include the progressive degeneration and death of dopaminergic neurons in the substantia nigra pars compacta and the presence of cytoplasmic inclusions known as Lewy bodies (LBs) (Moore et al., 2005a,b). The etiology of PD remains poorly understood. The familial form of PD is influenced by genetic mutations. Recently, several genetic loci have been implicated in the pathogenesis of familial forms of PD. Mutations in at least six genes are individually linked to familial form of PD, including autosomal dominant mutations in *α-synuclein*, *ubiquitin C-terminal hydrolase L1* and *leucine-rich repeat kinase 2* (*LRRK2*) and autosomal recessive mutations in *parkin*, *PTEN-induced putative kinase 1* (*PINK1*) and *DJ-1* (Thomas and Beal, 2007). Although the loci of these genes are different, there are sufficient similarities in the phenotypes of their patients to strongly implicate the functional links of these diverse target proteins.

Parkin functions as an E3 ubiquitin ligase, which ubiquitinates several target proteins and enhances degrading them via ubiquitin–proteasome system (UPS) (Shimura et al., 2000). Several substrates of parkin have been reported to date. Dysfunction of parkin has been implicated to cause an accumulation of UPS-destined parkin substrates, which leads to cellular toxicity. The *PINK1* gene encodes a

serine/threonine kinase (Silvestri et al., 2005; Abou-Sleiman et al., 2006; Beilina et al., 2005). PINK1 phosphorylates TNF receptor-associated protein 1, which leads to cell protection against oxidative stress-induced apoptosis (Pridgeon et al., 2007). In cultured cells, the overexpression of PINK1 protects cells against apoptotic stimuli (Valente et al., 2004; Petit et al., 2005). In *Drosophila*, loss of PINK1 leads to mitochondrial defects and degeneration of muscle and dopaminergic neurons (Clark et al., 2006; Park et al., 2006).

Neurodegeneration in PD is accompanied by abnormal protein accumulation, aggregation and the sequestration of a wide range of proteins into LBs. LBs contain various proteins, including the most abundant *α-synuclein* (Spillantini et al., 1997), neurofilaments (Schmidt et al., 1991), ubiquitin and ubiquitinated proteins (McNaught et al., 2002; Lennox et al., 1989). LBs also contain the components of the UPS, including proteasomal subunits, ubiquitination/de-ubiquitination enzymes and proteasome activators (Li et al., 1997; Lowe et al., 1990). It has been found that some PD-associated gene products are functionally linked to one another. Such interactions can lead to the activation of common pathogenic pathways of neuronal degeneration and the emergence of PD. For example, parkin directly ubiquitinates or cleave *α-synuclein* (Shimura et al., 2001; Kim et al., 2003). Parkin also interacts with pathogenic DJ-1 mutant (Moore et al., 2005a,b) and LRRK2 (Smith et al., 2005). In addition, PINK1 physically interacts with DJ-1 and the resulting PINK1/DJ-1 complex protects cells against stress (Tang et al., 2006).

The intracellular levels of parkin and PINK1 are modulated via UPS (Tang et al., 2006; Choi et al., 2000; Muqit et al., 2006; Lin and Kang, 2008). In PD patients, proteasomal activity has been reported to be significantly reduced (McNaught et al., 2003), which results in the

* Corresponding author. Fax: +82 2 312 5657.

E-mail address: kchung@yonsei.ac.kr (K.C. Chung).

abnormal accumulation of parkin and PINK1 proteins. This hypothesis was further supported by the finding that PINK1 and parkin are localized in LBs (Gandhi et al., 2006; Schlossmacher et al., 2002). In addition, a number of recent studies by using *Drosophila melanogaster* and mammalian cells indicated that parkin and PINK1 share a common pathway to maintain mitochondrial function (Clark et al., 2006; Exner et al., 2007; Park et al., 2006; Yang et al., 2006) as well as to regulate its morphology towards mitochondrial fission (Deng et al., 2008; Poole et al., 2008). In the present study, we investigated the biological and functional relationship between PINK1 and parkin. We show that PINK1 physically interacts with parkin in cultured cells and brain tissues, which results in the alteration of parkin solubility and PINK1 levels.

Results

Parkin interacts with PINK1

To investigate the relationship between parkin and PINK1, we determined whether parkin binds to PINK1 in mammalian cells using a co-immunoprecipitation assay. After HEK293 cells were transfected with Flag-tagged PINK1 and Myc-tagged parkin, immunoprecipitation was performed with an anti-Myc antibody, followed by immunoblotting with an anti-Flag antibody. Consistent with previous reports (Silvestri et al., 2005; Abou-Sleiman et al., 2006; Beilina et al., 2005), PINK1 appeared in two bands, as shown by Western blot analysis (Fig. 1A). The upper band represents full-length PINK1, whereas the lower band represents the processed form

of PINK1 from which the N-terminal mitochondrial-targeting motif has been removed.

In addition, we found that parkin binds to PINK1 (Fig. 1A). Based on a previous report showing that PINK1 is degraded by the ubiquitin–proteasome pathway (Tang et al., 2006; Muqit et al., 2006; Lin and Kang, 2008), cells were treated with proteasomal inhibitor, MG132, and co-immunoprecipitation was performed. As expected, the levels of PINK1 protein were significantly accumulated; enhanced binding of parkin to PINK1 was observed (Fig. 1B). Moreover, PINK1 was precisely colocalized with parkin, primarily in the cytoplasm (Fig. 1C).

To determine whether endogenous parkin binds to endogenous PINK1 in mammalian cells, a rabbit polyclonal PINK1 antibody against 157–170th residues was prepared and its specificity was checked. As shown in Supplementary material, Fig. S1, the antibody recognized endogenous PINK1 band with the expected size in human dopaminergic neuroblastoma SH-SY5Y cells. The band mobility was similar to that observed with transfected Myc-tagged wild-type (WT) PINK1 (Supplementary material, Fig. S1A). In order to show the antibody specificity, when cell lysates were immunoblotted with preimmune serum, there was no band with the similar size (Supplementary material, Fig. S1B). In addition, when cells were transfected with either PINK1-siRNA or control-siRNA, the level of endogenous PINK1 was remarkably reduced by PINK1-siRNA, but not by nonspecific control-siRNAs (Supplementary material, Fig. S1C). These data confirmed that antiserum raised by PINK1 antigen can specifically detect the PINK1 protein. By using this anti-PINK1 antibody co-immunoprecipitation assay of SH-SY5Y cell lysates was performed, followed by the immunoblotting with anti-parkin antibody. As shown

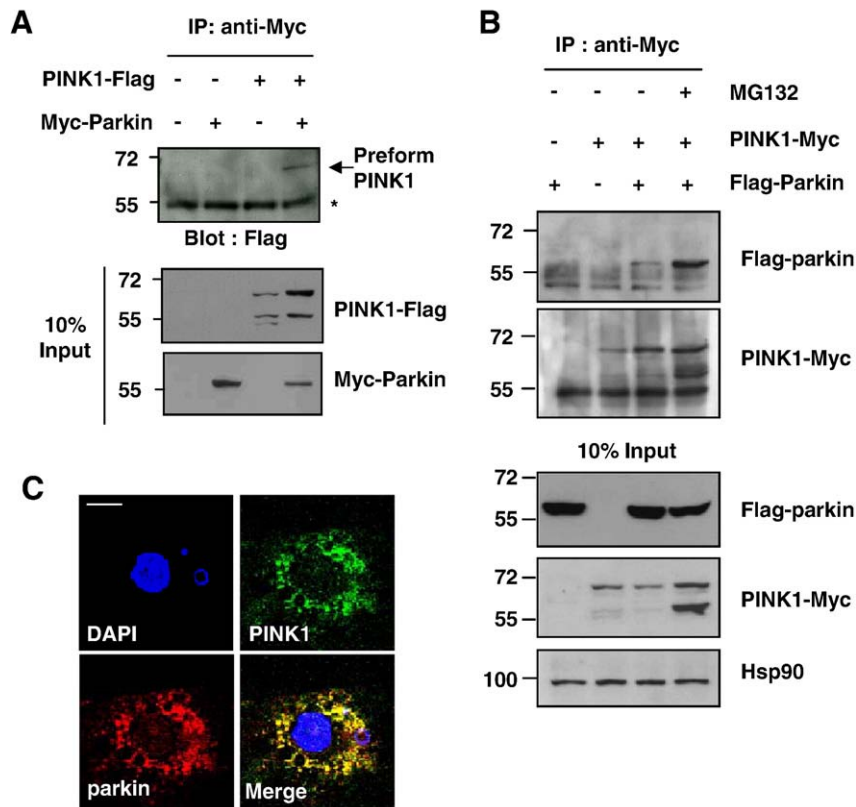


Fig. 1. Parkin interacts with PINK1. (A) Where indicated, HEK293 cells were transfected with plasmids encoding Myc-tagged parkin and/or Flag-tagged PINK1. Immunoprecipitation (IP) was performed with an anti-Myc antibody, and the immunocomplexes were analyzed by Western blotting with an anti-Flag antibody, as indicated. The proper expression of transiently transfected proteins in cell lysates was identified with anti-Flag or anti-Myc antibodies. An asterisk indicates IgG heavy chains. (B) Where indicated, Myc-tagged PINK1 and/or Flag-parkin were transfected into HEK293 cells for 24 h. Cells were left untreated or treated with MG132 (10 μ M) for 6 h. IP was performed using an anti-Myc antibody; the immunocomplexes were analyzed by Western blotting with anti-Flag or anti-Myc antibodies. Equal sample loading was examined by immunoblotting with an anti-Hsp90 antibody. (C) COS7 cells were transfected with plasmids for GFP-tagged PINK1 and Myc-tagged parkin. After 24 h, cells were fixed, permeabilized and labeled with an anti-parkin antibody. Cells were then stained with TRITC-conjugated secondary antibodies and DAPI. Immunostained preparations were examined using confocal microscopy. Scale bar = 10 μ m.

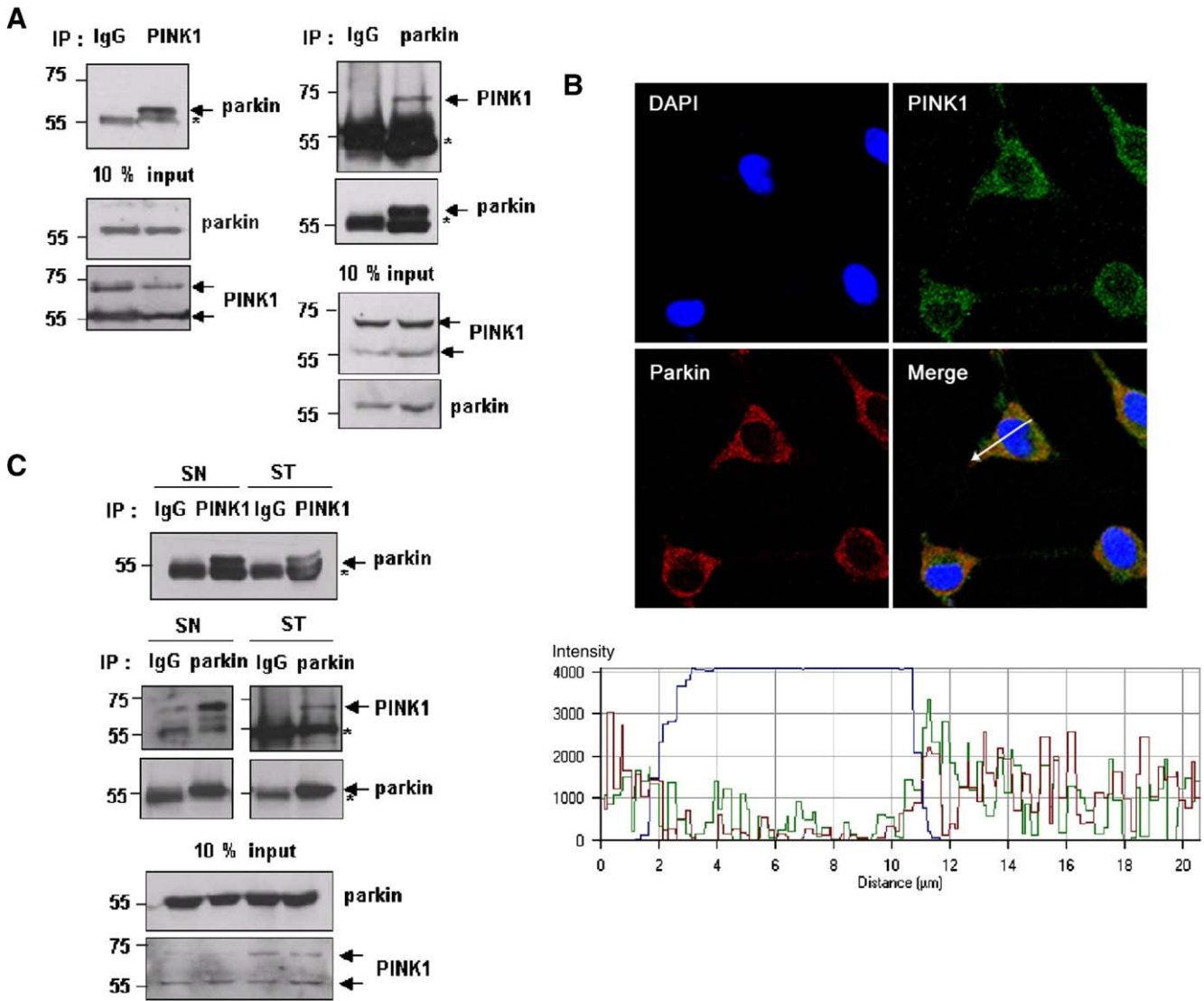


Fig. 2. Endogenous parkin binds to endogenous PINK1. (A) Where indicated, the immunoprecipitation of SH-SY5Y cell lysates was performed with anti-PINK1 or anti-parkin antibodies, followed by immunoblot analysis with anti-Parkin (Cell signaling) or anti-PINK1 antibody. As a control, cell lysates were immunoprecipitated with preimmune IgG (IgG), as indicated. The expression of parkin and PINK1 in cell extracts was determined by immunoblotting with anti-parkin (Cell signaling) or anti-PINK1 antibodies. An asterisk indicates IgG heavy chains. (B) SH-SY5Y cells were fixed, permeabilized and labeled with anti-parkin (Cell signaling) and anti-PINK1 antibodies. Cells were then stained with TRITC-conjugated and FITC-conjugated secondary antibodies and DAPI. Immunostained preparations were examined with a confocal microscope (LSM 510 META by Carl Zeiss). The white arrow indicates the regions in which intensity profiles (bottom) along a line were determined using confocal microscopic analysis software. (C) Immunoprecipitation of rat brain lysates from substantia nigra (SN) or striatal regions (ST) was performed with anti-PINK1 or anti-parkin antibodies, followed by immunoblot analysis with anti-Parkin (Cell signaling) or anti-PINK1 antibodies. As a control, cell lysates were immunoprecipitated with preimmune IgG (IgG), as indicated. The expression of parkin and PINK1 in cell lysates was determined by immunoblotting with anti-parkin (Cell signaling) or anti-PINK1 antibodies. An asterisk indicates IgG heavy chains.

in Fig. 2A, endogenous parkin interacts with endogenous PINK1 (Fig. 2A). The specific interaction between PINK1 and parkin was also observed in PC12 cells (Supplementary material, Fig. S2A). Confocal microscopic analysis revealed that parkin was colocalized with PINK1 in SH-SY5Y cells (Fig. 2B). Furthermore, the parkin/PINK1 complex was predominantly localized in the cytoplasm, while only very small amounts were found in the nucleus (Fig. 2B).

We then examined whether the association between parkin and PINK1 also occurs in the mammalian CNS. As shown in Fig. 2C, parkin selectively binds to PINK1 in the substantia nigra and striatal regions of rat brain, whereas no obvious interaction band was found for immunocomplex samples prepared with preimmune IgG as a control. The binding of parkin to PINK1 was also observed in rat CNS cortical tissues (Supplementary material, Fig. S2B). Taken together, these results suggest that the physical association of PINK1 with parkin is not merely an artifact observed in cell culture and occurs in the mammalian CNS.

To clarify whether PINK1 binds to parkin directly or indirectly, we conducted an *in vitro* GST pull down assay with recombinant GST fused PINK1 and *in vitro* translated parkin. We found that parkin can interact with GST-PINK1 directly, but not with GST as a negative control (Supplementary material, Fig. S3).

We then investigated whether the interaction between PINK1 and parkin depends on PINK1 kinase activity. Towards this end, we used a triple kinase-dead (KD) mutant (K219A/D362A/D384A) of PINK1 that has been already confirmed to have no kinase activity (Beilina et al., 2005). In contrast to wild type PINK1, this KD kinase-deficient PINK1 mutant fails to bind to parkin (Fig. 3A), indicating that PINK1 kinase activity is required to the association with parkin. To identify the PINK1-binding domain of parkin, several deletion mutants of parkin were cotransfected with GFP-tagged PINK1 into HEK293 cells. As shown in Fig. 3C, PINK1 interacts with deletion parkin mutants spanning 1–415 or 77–465 amino acids. However, 217–465 and 295–465 mutants of parkin containing RING domains or its 1–100 mutant

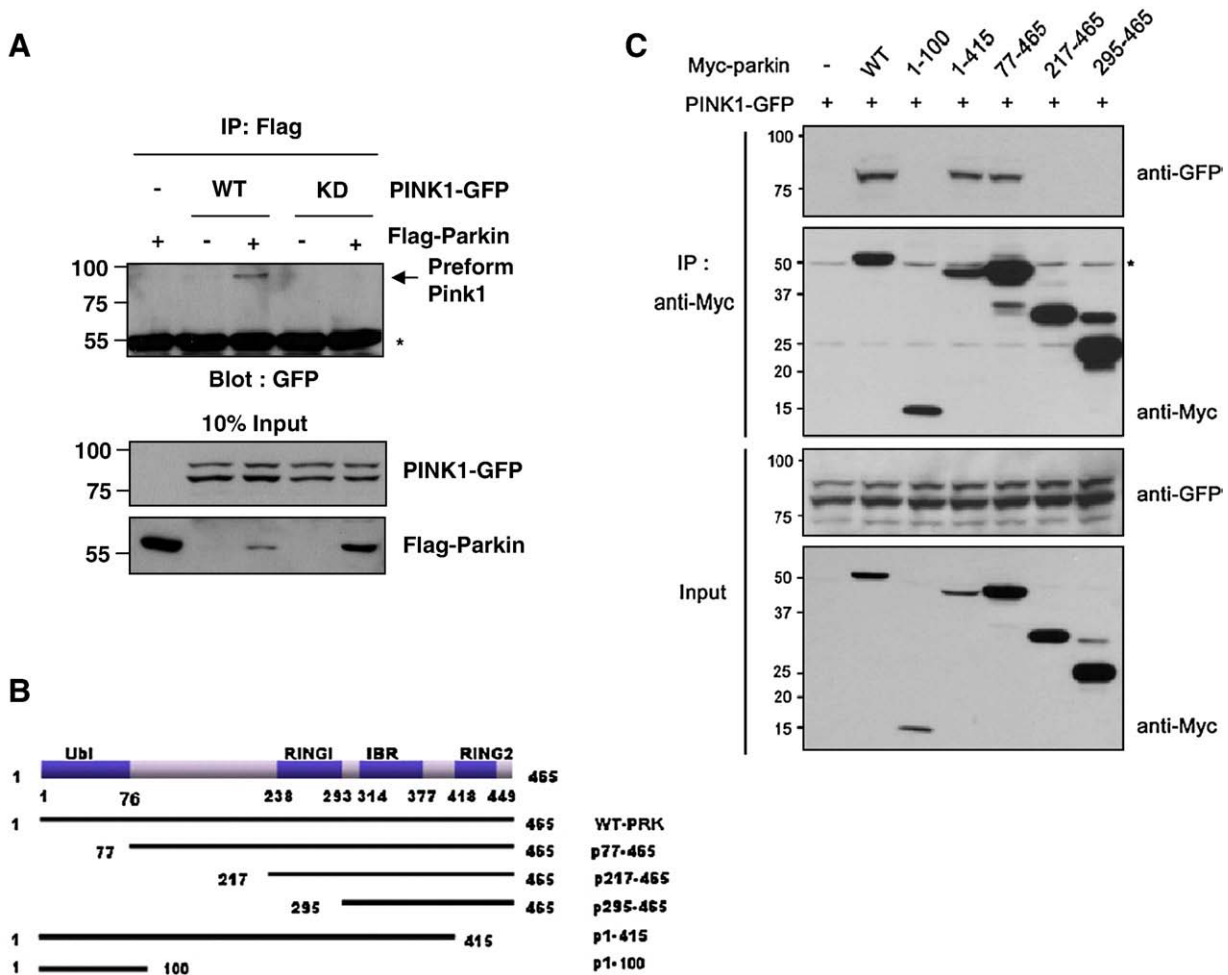


Fig. 3. Identification of PINK1-binding domain within parkin. (A) Where specified, HEK293 cells were transfected with Flag-tagged wild type parkin or GFP-tagged wild type (WT) or kinase dead mutant PINK1 (KD). Cell lysates were immunoprecipitated with an anti-Flag antibody, followed by immunoblot analysis with anti-GFP IgG. An asterisk indicates IgG heavy chains. (B) Diagram depicting wild type and deletion parkin mutants. Functional domains are denoted, including Ubiquitin-like (UBL), RING1, In-Between Ring1 (IBR) and RING2 domain. (C) After HEK293 cells were transfected with plasmid encoding GFP-PINK1 alone or together with Myc-tagged wild type parkin or several deletion mutants (77–465; 1–100; 1–415; 217–465; 295–465), immunoprecipitation (IP) was performed with anti-Myc antibody, and the immunocomplexes were analyzed with anti-GFP or anti-Myc antibody. The proper expression of transiently transfected protein in cell lysates was identified with anti-GFP or anti-Myc antibodies. An asterisk indicates IgG heavy chains.

containing ubiquitin-like domain did not bind to PINK1 (Fig. 3C). These data implied that the 101–216th region of parkin appears to be crucial for the interaction with PINK1, despite the multiple domains of parkin could be required for PINK1-association.

Parkin and PINK1 levels are mutually modulated

Interestingly, it was consistently found that cotransfection of parkin plus PINK1 causes a concomitant reduction of parkin with a significant increase of PINK1. To further examine this phenomenon, HEK293 cells were transfected with Myc-parkin, GFP-tagged wild type PINK1, or its KD mutant. Subsequently, 1% NP-40-soluble (S) and insoluble fractions (P) were prepared from cell lysates. Overexpression of parkin plus PINK1 resulted in marked increase of 1% NP-40-insoluble parkin level with a concomitant decrease of its soluble level to a similar extent, compared to cells overexpressing parkin or PINK1 alone (Fig. 4A). Interestingly, parkin solubility was reduced by wild type PINK1, but not by KD mutant (Fig. 4A). Moreover, PD-linked PINK1 mutant (G309D) had no effect on parkin solubility (Fig. 4B). Furthermore, parkin upregulated the levels of wild type PINK1 in soluble and particulate fraction, but not with KD- or PD-linked mutants (Figs. 4A and B). After HEK293 cells were cotransfected with GFP, PINK1, and parkin, Western blotting with anti-GFP antibody showed that GFP expression was not affected by parkin and PINK1

(Supplementary material, Fig. S4). In order to determine whether this finding may be an artifact caused by protein overexpression, the change of endogenous PINK1 levels was analyzed in mouse neuroblastoma N2A cells to overexpress parkin. This cell line was simply chosen and used to elevate the transfection efficiency of parkin. As shown in Fig. 4C, the level of premature form of endogenous PINK1 was higher in both soluble and particulate fractions by the ectopic overexpression of parkin. In addition, when PINK1 was overexpressed, endogenous parkin level in soluble fraction was reduced, whereas its insoluble level increased (Fig. 4D). Taken together, these data indicate that PINK1 reduces parkin solubility, which depends on the kinase activity, whereas parkin is capable of upregulating the PINK1 levels.

In addition to protein solubility, we further explored whether the subcellular localizations of two proteins are altered by co-expression. After cell lysates were fractionated into the cytosolic, mitochondrial, and nuclear fractions, a small amount of PINK1 within the cytosol appeared to be relocated into mitochondrial by parkin, but not any remarkable change of their localization (Supplementary material, Fig. S5). These results indicate that the solubility of parkin and PINK1, but not their subcellular locations, are mutually affected by ectopic coexpression of these two proteins.

Based on the previous reports that oxidative stresses and S-nitrosylation induce the reduction of parkin solubility (LaVoie et al., 2007; Yao et al., 2004), we examined whether PINK1-induced

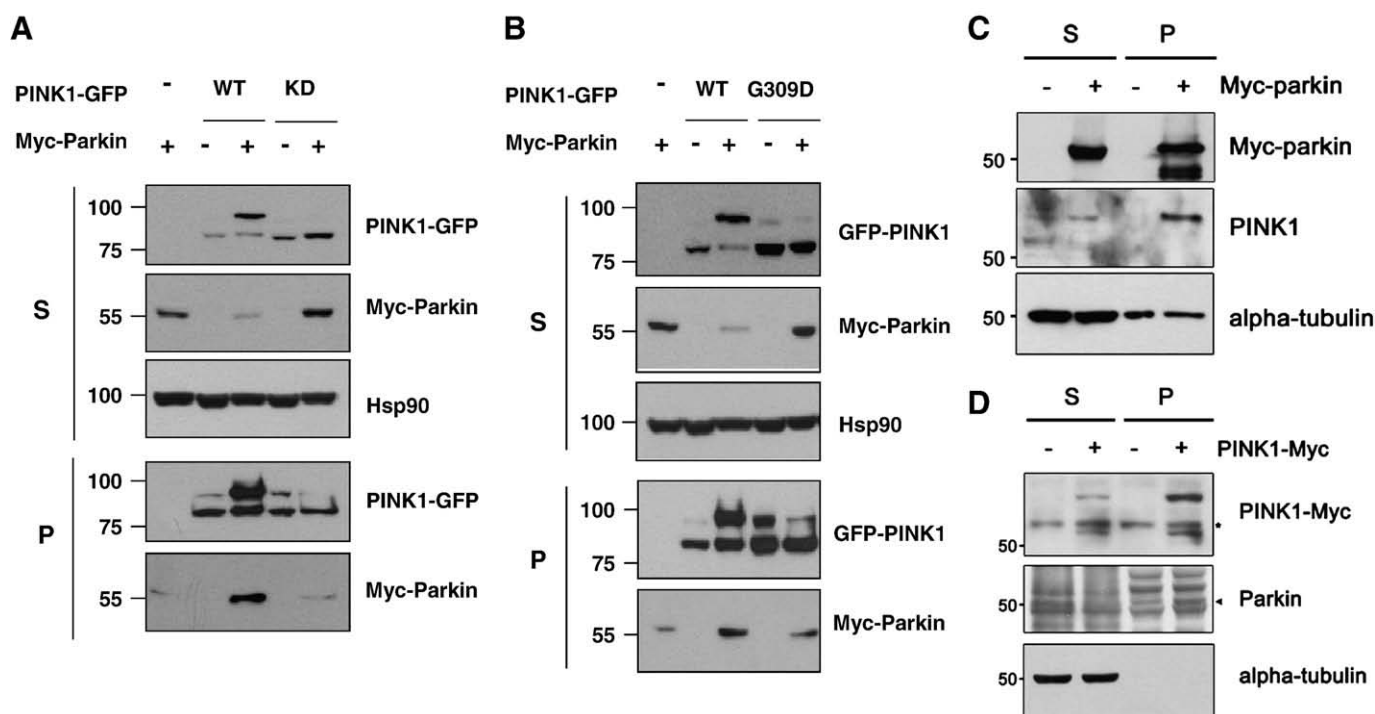


Fig. 4. Parkin solubility is altered by PINK1; PINK1 levels are upregulated by parkin. (A, B) After HEK293 cells were transfected with plasmids encoding Myc-tagged parkin, GFP-tagged wild type PINK1 (WT) or its mutants (A; KD, B; G309D), cell extracts were lysed with NP-40 (S) and SDS (P)-containing buffer. Each fraction was analyzed by immunoblotting with anti-GFP or anti-Myc antibodies. To demonstrate equal loading, cell lysates were analyzed with anti-Hsp90 antibody, as indicated. (C, D) After N2A cells were transfected with Myc-tagged parkin (C) or Myc-tagged PINK1 (D), the cells were lysed with 1% NP-40 (S) and 2% SDS (P)-containing buffer. Each fraction was analyzed by immunoblot with anti-Myc, anti-PINK1 or anti-parkin (Chemicon) antibodies. As a control for equal protein loading, cell lysates were immunoblotted with anti- α -tubulin antibody.

reduction of parkin solubility might be caused by such stresses. As shown in [Supplementary material, Fig. S6](#), the PINK1-overexpression still reduced the parkin-solubility in the presence of antioxidants, such as NAC or PTIO, suggesting that PINK1-induced reduction of parkin level is not mediated via the oxidative protein modification.

PINK1-induced attenuation of parkin solubility promotes parkin aggregation

Next we examined whether the alteration of parkin solubility is associated with the propensity of PINK1 to form intracellular aggregates. The localization of parkin was determined using fluorescent microscopy. When PINK1 was coexpressed with GFP as a control, GFP proteins were expressed evenly throughout the cytoplasm and nucleus ([Fig. 5A](#)). When PINK1-Myc was coexpressed with GFP-tagged wild type parkin, parkin aggregates formed ([Fig. 5B](#)). Additional experiments revealed that the formation of parkin aggregates was caused by wild type PINK1, but not by KD- or two PD-linked (i.e. G309D, L347P) mutants of PINK1 ([Fig. 5C](#)). This is consistent with previous results ([Figs. 4A and B](#)). When the number of cells expressing parkin diffusely, in a punctated manner, or localized to the nucleus were counted, wild type PINK1 significantly increased the number of cells showing punctated localization of parkin aggregates ([Fig. 5D](#)). This result indicates that PINK1 regulates parkin solubility and consequently induces parkin-containing aggregates.

The morphology of PINK1-induced parkin aggregates appeared similar to that of aggresomes ([Kopito, 2000](#)). It has been reported that microtubule-dependent retrograde transport plays a role in aggresome formation. To test whether the parkin aggregates were indeed aggresomes, we examined the effect of microtubule-depolymerizing drug, nocodazole, on the formation of PINK1-induced parkin aggregates. As shown in [Fig. 6A](#), when parkin and PINK1 were coexpressed, there were no parkin aggregates in nocodazole-treated cells. In addition, PINK1-induced reduction in parkin solubility was

not observed in the cells treated with nocodazole ([Fig. 6B](#)). This finding suggests that PINK1-induced formation of parkin aggregates is dependent on the intact microtubule structure. Furthermore, when cells were immunostained with anti-parkin or anti-ubiquitin antibodies as an aggresome marker, parkin was colocalized with ubiquitin in the form of aggresome in the presence of PINK1, whereas ubiquitin was evenly localized in the cytoplasm and nucleus in control cells transfected with GFP-parkin alone ([Fig. 6C](#)). These data strongly indicated that parkin aggregates are indeed aggresomes.

Accumulating evidences indicated that aggresomes are formed to avoid the accumulation of potentially toxic proteins in the cytoplasm and consequently allow cells to minimize misfolded protein-induced cytotoxicity ([Olanow et al., 2004; Kopito, 2000](#)). Based on those reports, we examined whether the formation of PINK1-induced parkin-aggresomes produces a cytoprotective effect. To this end, we measured the effect of parkin and/or PINK1 on the occurrence of proteasomal inhibitor-induced cell death. While the addition of MG132 caused cell death, reducing the cell viability by approximately 30%, parkin or PINK1 alone showed a relative increase of cell viability by ~10% ([Supplementary material, Fig. S7](#)). These results were consistent with the previous reports that parkin and PINK1 act as cellular protectors against proteasome inhibition-induced cell toxicity ([Yang et al., 2007; Valente et al., 2004](#)). Moreover, when parkin and PINK1 were expressed together, the cell viability additively increased by ~10% more ([Supplementary material, Fig. S7](#)). These data indicates that parkin and PINK1 synergistically protects cells against MG132-induced cell death, in which the formation of PINK1-induced parkin-positive aggresomes may play a role.

PINK1 is upregulated by overexpression of parkin

Next we determined which domain(s) of parkin are critical for the upregulation of PINK1. As shown in [Supplementary material, Fig.](#)

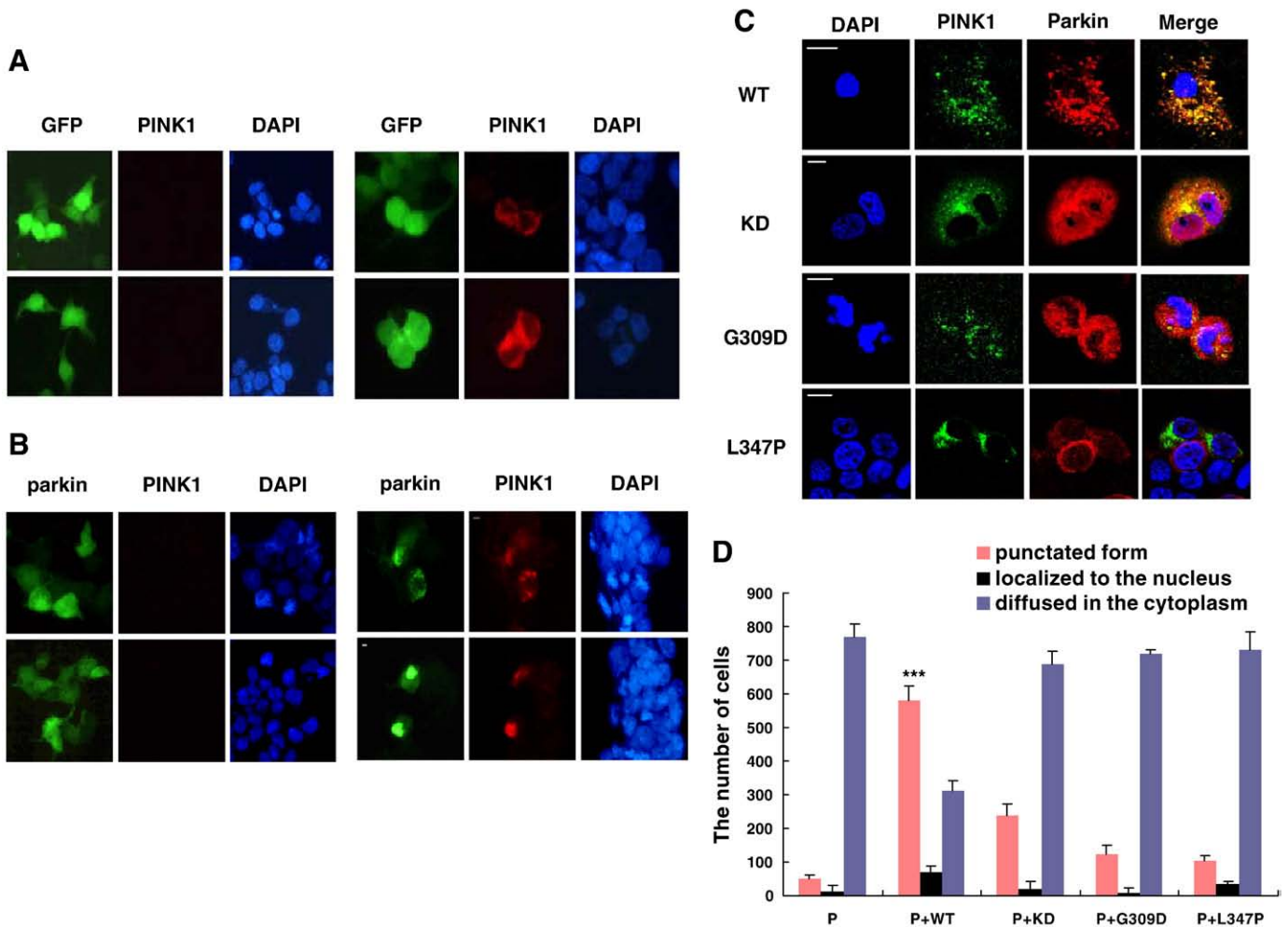


Fig. 5. PINK1-induced alteration in parkin solubility promotes the formation of intracellular parkin-positive inclusions. (A, B) COS7 cells were transfected with plasmids encoding Myc-tagged PINK1 and GFP-parkin (B) or GFP vector as a negative control (A). Twenty four hours later, cells were fixed, permeabilized and labeled with an anti-PINK1 antibody. Cells were then treated with TRITC-conjugated goat anti-rabbit IgGs and stained with DAPI. Immunostained preparations were visualized by fluorescent microscopy. (C) COS7 cells were transfected with plasmids encoding Myc-tagged parkin and PINK1-GFP (WT) or its mutants (KD, G309D, and L347P). Twenty four hours later, cells were fixed, permeabilized and labeled with an anti-parkin (Cell signaling) antibody. Cells were then treated with TRITC-conjugated goat anti-mouse IgGs and stained with DAPI. Immunostained preparations were visualized using confocal microscopy. Scale bars = 10 μ m. (D) The number of cells in which parkin proteins is observed in punctated form, diffused in the cytosol or localized to the nucleus was counted. The numbers of cells transfected with parkin alone or together with wild type PINK1, KD, G309D, L347P were 915, 955, 915, 899 and 918, respectively. Results are expressed as the mean and range of data from three independent experiments (***) $p < 0.0001$, compared with the cells transfected with parkin alone.

S8A, wild type parkin and its 77–465 or R42P mutants markedly increased PINK1 levels. However, other parkin deletion mutants (i.e. 1–415, 217–465 or 295–465) showed no significant effect on PINK1 level (Supplementary material, Fig. S8A). These results suggest that RING2 domain of parkin is critical for the upregulation of PINK1 as well as PINK1 binding. The RING2 domain, including a RING-IBR-RING structure, was reported to be necessary for the intrinsic ubiquitin E3 ligase activity of parkin (Capili et al., 2004), implicating that the enzymatic activity of parkin is crucial to the upregulation of PINK1.

Based on the previous finding that the levels of PINK1 protein, especially its cleaved form, are much higher in the presence of MG132, the current finding was further examined after the pretreatment of cells with MG132, followed by co-transfection with Myc-parkin and PINK1-Flag. As shown in Supplementary material, Fig. S8A, the increase of cleaved PINK1 levels was greatly enhanced by MG132, suggesting that parkin may regulate PINK1 levels by acting on the premature form of PINK1. We then tested whether the upregulation of PINK1 is specific to parkin, or could similarly be mediated by other ubiquitin E3 ligases. Interestingly, PINK1 levels were not significantly changed in response to NEDD4-1 or NEDD4-2,

which have HECT domains and act as ubiquitin E3 ligase, irrespective of the presence of MG132 (Supplementary material, Fig. S8B). Moreover, cIAP1, which acts as RING domain-dependent ubiquitin E3 ligase, had no effect on PINK1, (Supplementary material, Fig. S8C). These results demonstrate that the upregulation of PINK1 is a parkin-specific event.

Parkin decreases PINK1 ubiquitination

To investigate the mechanism by which parkin upregulates PINK1, *in vivo* ubiquitination assay of parkin and PINK1 was conducted in mammalian cells. First, to clarify the dominant protease system that regulates the stability of PINK1, the pattern of PINK1 proteolysis was compared in the absence or presence of several protease inhibitors, such as inhibitor(s) of proteasome, calpain, or lysosomal proteases. Lactacystin and epoxomicin are highly specific proteasome inhibitors; MG132 acts as a potent but less specific proteasome inhibitor, calpeptin is a calpain inhibitor and E-64 is used as a lysosomal protease inhibitor. Consistent with previous reports (Tang et al., 2006; Muqit et al., 2006; Lin and Kang, 2008), only proteasome inhibitors substantially restored the

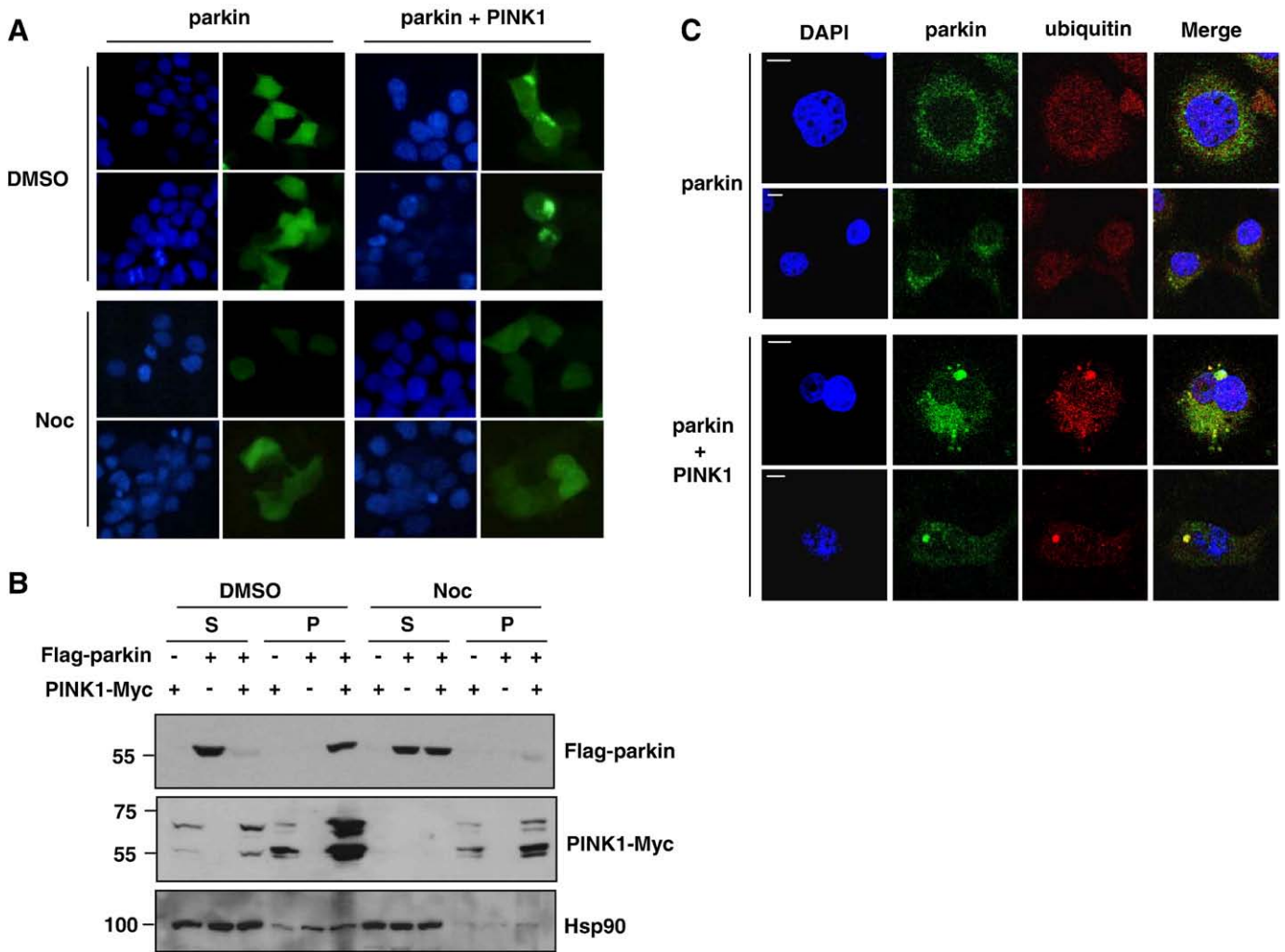


Fig. 6. Overexpression of PINK1 induces parkin to localize in aggregates. (A) HEK293 cells were transfected with Myc-PINK1 and GFP-parkin and treated with DMSO as a control or 1 μ g/ml nocodazole for 16 h. Then, cells were fixed and stained with DAPI. Immunostained preparations were visualized by fluorescent microscopy. (B) After HEK293 cells were transfected with Flag-parkin or PINK1-Myc alone, or together, cells were treated with DMSO as a control or 1 μ g/ml nocodazole (Noc) for 16 h. Cell lysates were fractionated into 1% NP-40-soluble and -insoluble fractions and analyzed by immunoblotting with anti-Flag or anti-Myc antibodies. To determine equal loading, cell lysates were analyzed by immunoblotting with anti-Hsp90 antibody. (C) COS7 cells were transfected with GFP-parkin alone or together with PINK1-Myc. Twenty four hours later, cells were fixed, permeabilized and labeled with an anti-ubiquitin antibody, followed by TRITC-conjugated goat anti-rabbit IgGs and stained with DAPI. Immunostained preparations were visualized by confocal microscopy. Scale bars = 10 μ m.

decrease in PINK1 levels in HEK293 cells, but neither calpain nor lysosomal protease inhibitors (Fig. 7A).

Interestingly, PINK1 ubiquitination was dramatically reduced by parkin overexpression, which led to the intracellular accumulation of PINK1 (Fig. 7B). To confirm that the reduction of PINK1 ubiquitination is specifically caused by parkin, we tested the effect of other E3 ubiquitin ligase containing RING domain, such as Siah-1. As shown in Fig. 7C, there was no significant change in ubiquitinated PINK1 levels in the absence or presence of Siah1, indicating that the reduction of PINK1-ubiquitination selectively depends on parkin. Furthermore, the self-ubiquitination of parkin was significantly reduced by PINK1, which was caused by the reduction in 1% NP-40 soluble parkin levels (Fig. 7B).

Increased steady state levels of PINK1 in cells stably overexpressing wild type parkin, but not its dominant-negative mutant

We next determined whether the overexpression of parkin affects the steady-state level of endogenous PINK1. Stable SH-SY5Y cell lines overexpressing either Myc-tagged wild type parkin (SH-SY5Y/WT-parkin) or deletion mutant (amino acids: 1–415) (SH-

SY5Y/1–415-parkin) was generated. Since the RING2 domain was previously reported to be necessary for the intrinsic E3 ligase activity (Capili et al., 2004), stable SH-SY5Y cell line overexpressing a RING2-deleted mutant was established as a ‘loss-of-parkin-function’ control. When immunocytochemistry was performed with anti-PINK1 and anti-parkin antibodies, PINK1 level increased only in SH-SY5Y/WT-parkin cells, but not in SH-SY5Y/1–415-parkin cells (Fig. 8A). In addition, the measurement of fluorescent intensity from each protein indicated that the steady-state PINK1 level is significantly increased in SH-SY5Y/WT-parkin cells, compared with parental control or SH-SY5Y/1–415-parkin cells (Fig. 8B).

Western blot analysis using anti-PINK1 or anti-parkin antibodies showed that both premature and cleaved form levels of PINK1 are accumulated in SH-SY5Y/WT-parkin cells, compared with parental control or SH-SY5Y/1–415-parkin cells (Fig. 8C). However, when real time-PCR was performed, there was no change of endogenous PINK1 mRNA level in SH-SY5Y/WT-parkin cells compared with parental control cells (Fig. 8D), indicating that parkin does not affect on the transcription of PINK1.

Next we checked whether and how the PINK1 level is being altered in parkin KO mice (PaKO). The PaKO was generated by the

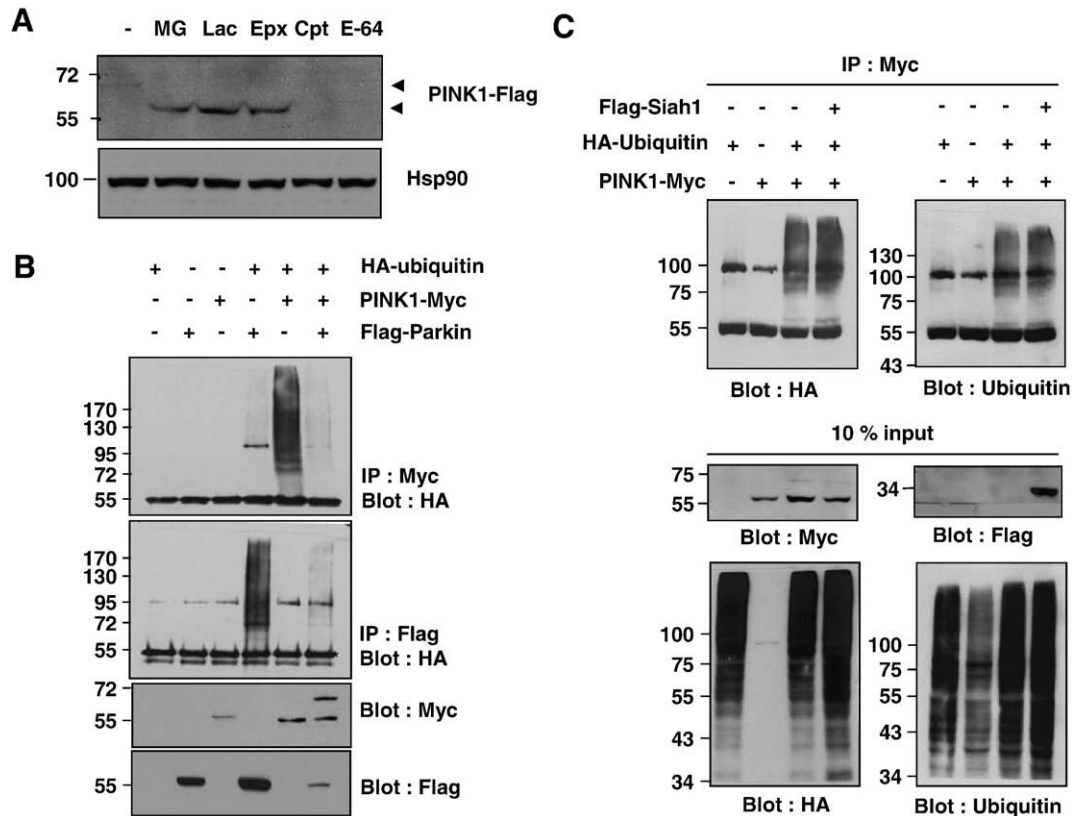


Fig. 7. Parkin decreases PINK1 ubiquitination and PINK1 reduces self-ubiquitination of parkin. (A) After HEK293 cells were transfected with plasmids encoding Flag-PINK1, cells were untreated or treated with either 10 μ M MG132 (MG), 10 μ M clasto-lactacystin β -lactone (Lac), 100 nM epoxomicin (Epx), 50 μ M calpeptin (Cpt) or 100 μ M E-64 for 6 h. Total cell lysates were resolved by SDS-PAGE and analyzed by Western blotting with anti-Flag antibodies. Equal loading of samples was examined by immunoblotting with anti-Hsp90 antibody. (B) After HEK293 cells were transfected with plasmids for either HA-ubiquitin or Myc-PINK1 alone, or together with Flag-Parkin, the cells were incubated in the presence of 10 μ M clasto-lactacystin β -lactone (Lac) for 6 h. Cell lysates were immunoprecipitated (IP) with anti-Myc or anti-Flag antibodies, followed by immunoblotting with anti-HA antibody. To determine the proper expression of Flag-parkin and PINK1-Myc, cell lysates were analyzed by immunoblotting with anti-HA, anti-Flag, or anti-Myc antibodies, as indicated. (C) After HEK293 cells were transfected with the plasmids for either HA-ubiquitin or Myc-PINK1 alone, or together with Flag-Siah1, cells were incubated in the presence of 10 μ M clasto-lactacystin β -lactone (Lac) for 6 h. The cell lysates were immunoprecipitated (IP) with anti-Myc antibody, followed by immunoblotting with anti-HA or anti-ubiquitin antibodies. To determine the proper expression of HA-ubiquitin, Flag-Siah1 and PINK1-Myc, cell lysates were analyzed by immunoblotting with anti-HA, anti-Flag or anti-Myc antibodies.

targeted deletion of exon 3, which results in a frameshift after 57th amino acid of parkin (Stichel et al., 2007). Western blotting of 1% NP-40-soluble fraction prepared from brain extracts showed that parkin level is completely abolished in three individuals of PaKO. In addition, parkin was not detected in NP-40-insoluble fraction. Furthermore, the levels of PINK1 in the NP-40-soluble and-insoluble fraction were down-regulated in the PaKO (Fig. 9A). When the intensity of PINK1 bands was quantified statistically, the soluble and insoluble PINK1 levels in the PaKO were reduced by 60% and 45%, respectively, compared with the control mice (Fig. 9B). Taken together, these results indicate that parkin overexpression causes the upregulation of endogenous PINK1 levels in dopaminergic neuroblastoma cells, and the deficiency of parkin induces the downregulation of PINK1 expression *in vivo*.

Discussion

There has been increasing evidences that mutation gene products in familial PD, such as PINK1, parkin and/or DJ-1, could interact and play an important role in mitochondrial function and resistance to oxidative stress in PD. For example, PINK1 physically interacts with DJ-1 and collaborates to protect cells against stress via complex formation (Tang et al., 2006). Co-expression of PINK1 and DJ-1 with PD-linked double mutation in cultured cells enhanced susceptibility to MPP⁺-induced cell death (Tang et al., 2006). In addition, parkin was reported to interact with a pathogenic DJ-1 mutant and to modulate the stability of the DJ-1 mutant (Moore et al., 2005a,b).

Moreover, recent studies from *Drosophila* demonstrated that PINK1 and parkin act in a linear genetic pathway required for the maintenance of mitochondrial function (Clark et al., 2006; Park et al., 2006), and regulates mitochondrial morphology by promoting mitochondrial fission (Deng et al., 2008; Poole et al., 2008). However, the possibility that PINK1 directly binds to parkin, and whether parkin could be a potential substrate for PINK1 kinase, or vice versa, has not been determined. The current study provides the first evidence of direct interaction between these two proteins. This study also confirms the previous finding that PINK1 and parkin are functionally linked and have a cytoprotective activity against proteasomal dysfunction-induced cell toxicity, possibly through a common mechanism.

We showed that parkin is colocalized with PINK1 in the cytoplasm (Fig. 1C), and that a considerable amount of PINK1 is localized in cytosol as well as in mitochondria (Supplementary material, Fig. S5). These results are well supported by the previous reports that PINK1 localizes either in both cytosol and mitochondria (Silvestri et al., 2005; Beilina et al., 2005; Pridgeon et al., 2007) or only in cytosol (Takatori et al., 2008; Weihofen et al., 2008). We also demonstrated that parkin causes an increase of PINK1 level in NP-40-soluble and -insoluble fraction (Fig. 4). This could occur because the ubiquitinated PINK1 level becomes dramatically reduced by parkin (Fig. 7B). Based on the previous report that PINK1 is degraded via a proteasomal system (Tang et al., 2006; Muqit et al., 2006; Lin and Kang, 2008), PINK1 may be presumed to be ubiquitinated by an unknown endogenous E3 ubiquitin ligase,

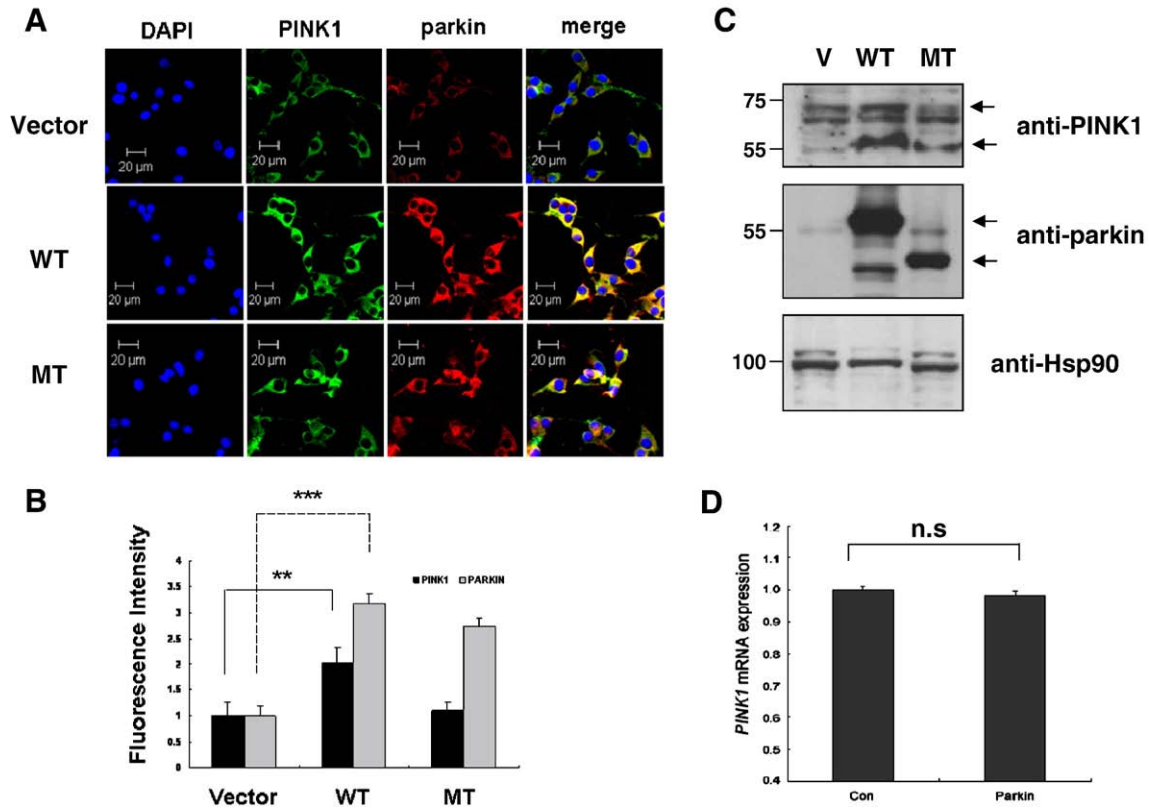


Fig. 8. PINK1 is upregulated in SH-SY5Y cells stably expressing wild type parkin. (A) SH-SY5Y cells stably expressing pcDNA3.1 vector as a control (Vector), Myc-tagged wild type parkin (WT) or its deleted mutant (amino acids: 1–415, MT) were fixed, permeabilized and labeled with anti-parkin (Cell signaling) or anti-PINK1 antibodies, followed by staining with FITC-conjugated goat anti-rabbit IgGs or TRITC-conjugated goat anti-mouse IgGs. Immunostained preparations were visualized by confocal microscopy. (B) The graph indicates the relative level of each protein, calculated by dividing the fluorescent intensity of each protein by the intensity of DAPI in randomly chosen fixed areas ($n = 10$) of three independent experiments ($***p < 0.001$, $**p < 0.01$). The fluorescent intensity was measured with a confocal microscopic analysis program. (C) Cell lysates were analyzed by immunoblotting with anti-PINK1 or anti-parkin (Cell signaling) antibodies. To determine equal loading, cell lysates were analyzed by immunoblotting with anti-Hsp90 antibody. (D) Total mRNAs were prepared from SH-SY5Y cells stably expressing pcDNA3.1 vector as a control (Con) or Myc-tagged wild type parkin (Parkin). The levels of PINK1 mRNA in each sample were measured by real time PCR. Values are normalized to β -actin level in each sample, and experiments were performed in triplicates \pm S.D. "n.s" means statistically not significant.

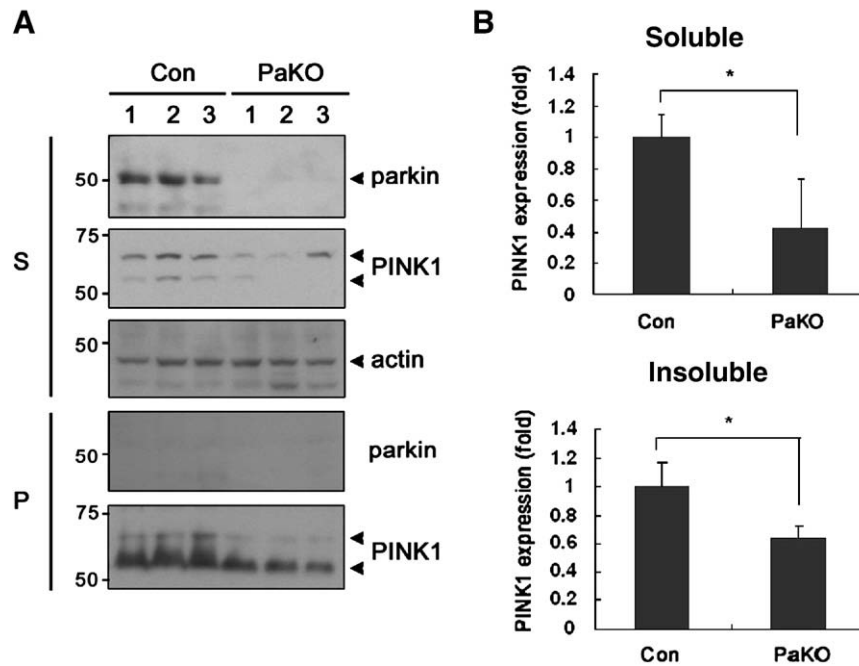


Fig. 9. Down-regulation of PINK1 in parkin KO mice. (A) Detergent-soluble and -insoluble fraction of brain lysates from parkin KO mice (PaKO; $n = 3$) or age-matched control mice (Con; $n = 3$) were resolved by SDS-PAGE, followed by immunoblotting with anti-PINK1 or anti-parkin (Cell signaling) antibodies. As a control for equal sample loading, cell lysates were immunoanalyzed with anti- α -tubulin antibody. (B) The PINK1 level in the soluble and insoluble fraction was quantified using ImageJ program (NIH), and normalized by α -tubulin content. The graph represents the mean of three independent experiments ($*p < 0.05$).

which could be negatively modulated by parkin in a direct or indirect way. This speculation was further supported by the current finding that 1–415 mutant of parkin interacts with PINK1 but fails to upregulate the level of PINK1 (Fig. 3C and S8), indicating that RING2 domain of parkin is critical for the up-regulation of PINK1 as well as PINK1-binding. As the RING2 domain, including a RING-IBR-RING structure, was reported to be necessary for the intrinsic ubiquitin E3 ligase activity of parkin (Capili et al., 2004), these results strongly suggest that the enzymatic activity of parkin is crucial to the up-regulation of PINK1.

Moreover, PINK1 induced the formation of microtubule-dependent parkin-positive aggresomes in cytoplasm (Fig. 6). It has been reported that the solubility of parkin is reduced by various stresses such as proteasomal dysfunction, PD-linked drugs or oxidative stress (Wang et al., 2005; LaVoie et al., 2005; Wong et al., 2007; LaVoie et al., 2007). However, the reduction of parkin solubility by PINK1 still occurred under treatment of antioxidants (Fig. S6), which indicated that the molecular mechanism for PINK1-induced reduction of parkin solubility is not mediated by oxidative stress or S-nitrosylation.

Although the molecular mechanism underlying the regulation of parkin solubility by PINK1 remains to be elucidated, wild type PINK1 directly binds to and possibly phosphorylates parkin, which then induces a conformational change of parkin and lead to the formation of parkin aggregates. This speculation was supported by the finding that the KD mutant of PINK1 could not bind to parkin (Fig. 3A) nor reduces parkin solubility (Fig. 4A). Furthermore, Kim et al. recently reported that PINK1 directly phosphorylates parkin at T175 residue of Linker domain (Kim et al., 2008).

Understanding the role of protein inclusions in the pathogenesis of neurodegenerative diseases remains one of the key questions of neurodegeneration research (McNaught and Olanow, 2006). There have been reports that both PINK1 and parkin are localized to aggresomes under the conditions of proteasomal inhibition (Muqit et al., 2006; Junn et al., 2002). PINK1 is detected within a subset (5–10%) of LBs (Gandhi et al., 2006). In addition, parkin is co-localized with α -synuclein in LBs (Schlossmacher et al., 2002). There is increasing evidence to support the hypothesis that LBs may be formed by an aggresomal process *in vivo* (Olanow et al., 2004). Although the formation of protein inclusions are cytotoxic or cytoprotective depending on the cellular condition, several reports indicated that aggresomes are cytoprotective proteinaceous inclusions formed at the centrosome that segregate and facilitate the degradation of excess amounts of unwanted and possibly cytotoxic proteins (Olanow et al., 2004). Sequestration of misfolded proteins into aggresomes can allow cells to minimize misfolded protein-induced cytotoxicity (Kopito, 2000). In this context, we propose that parkin could be recruited into aggresomes and then ubiquitinate misfolded proteins in these inclusions. Concerning the neuroprotective roles of parkin and PINK1, the upregulation of PINK1 levels and the subsequent formation of parkin-mediated aggresomes could also be a cellular counteraction for survival against toxic stress. This speculation was further confirmed by the finding that parkin and PINK1 together exhibit an additive cytoprotection against MG132-induced cell death (Supplementary material, Fig. S7). Moreover, the current finding that parkin solubility is not changed by PD-linked PINK1 mutants (G309D, L347P) (Figs. 4B and 5C) suggests that cytoprotective parkin-aggresomes may be not formed when PD-linked PINK1 mutant proteins are present. Further characterization of the molecular mechanisms leading to mutual regulation of parkin and PINK1 levels will help to clarify the biological functions of PINK1.

The presented study demonstrates the functional relevance and cooperativity of PINK1 and parkin. The two proteins affect each other's stability, solubility and tendency to form cytoprotective aggresomes. Our report suggests that both of PINK1 and parkin

appear to play an important role to regulate the formation of LBs, and advances the current understanding of PD pathogenesis.

Experimental methods

Materials

Peroxidase-, FITC-, TRITC-conjugated anti-rabbit and anti-mouse antibodies were purchased from Zymed Laboratories Inc. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), horse serum (HS) and LipofectAMINE PLUS reagents were obtained from Invitrogen. Protein A-Sepharose and Glutathione-Sepharose 4B were purchased from Amersham Pharmacia Biotech, and enhanced chemiluminescence (ECL) reagents were obtained from Perkin Elmer Life Sciences. Protein marker was from Fermentas. Clasto-Lactacystin β -lactone and MG132 were purchased from A. G. Scientific, while calpeptin and nocodazole were purchased from Calbiochem. Anti-HA, anti-GFP, anti-GST, anti-Hsp90, anti-Sp1, anti- α -tubulin, anti-ubiquitin and anti-Myc antibodies were purchased from Santa Cruz Biotechnology. Anti-Flag antibodies, N-acetylcysteine (NAC), PTIO, and E-64 were purchased from Sigma-Aldrich. A monoclonal anti-parkin antibody was purchased from Cell Signaling; polyclonal anti-parkin antibody from Chemicon; and anti-CoxVI antibody was purchased from Molecular Probes, Inc.

Rabbit polyclonal PINK1 antibodies were raised by immunizing two rabbits with synthetic peptides corresponding to unique amino acid residues at positions 157–170, which are well conserved between species such as rat, monkey, and human. Anti-serum was affinity-purified on columns against the synthetic peptides (ATGen, Korea). Mammalian expression vectors encoding for Myc-tagged human parkin, pcDNA3.1-Myc-parkin, and its deleted mutants spanning amino acids 77–465, 1–415, 217–465, 295–465 were kindly provided by K. Tanaka (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). A mammalian expression vector encoding GFP-tagged wild type parkin was a gift from H. Rhim (The Catholic University, Seoul, Korea). Plasmids encoding for Myc-tagged PINK1, PINK1-GFP (wild type, kinase-inactive mutant, G309D, and L347P) and recombinant GST-PINK1, were gifts from M.R. Cookson (National Institute on Aging, MD, USA). Plasmids encoding PINK1-Flag and Flag-parkin were provided by M. Mouradian (University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, NJ, USA). Plasmid encoding Flag-Siah1 was kindly provided by W.S. Park (The Catholic University, Seoul, Korea). Plasmids encoding HA-NEDD4-1 and NEDD4-2 were provided by P.M. Snyder (University of Iowa College of Medicine, Iowa City, USA), and Flag-clAP1 by T.H. Lee (Yonsei University, Seoul, Korea).

Cell culture and preparation of cell lysates

Human embryonic kidney 293 (HEK293) cells, African green monkey kidney COS7 cells, murine neuroblastoma N2A cells and dopaminergic neuroblastoma SH-SY5Y cells were maintained in DMEM containing 10% FBS and 100 U/ml of penicillin–streptomycin. Pheochromocytoma 12 (PC12) cells were maintained in DMEM containing 5% FBS and 10% HS. To prepare cell lysates, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and solubilized in lysis buffer containing Tris, pH 7.4, 1.0% Nonidet P-40 (NP-40; USB Corporation), 150 mM NaCl, 10% glycerol, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ aprotinin, 10 mM NaF, and 0.2 mM phenylmethylsulfonyl fluoride. To further purify the cell lysates into 1% NP-40-soluble (S) and -insoluble (P) fraction, cells were lysed in lysis buffer and sedimented at 16,000 $\times g$ for 30 min at 4 °C. Supernatants from the initial fractionation were saved as S fraction. The pellets were washed once with PBS before re-extraction with SDS buffer containing 2% SDS. After sedimentation

at 16,000 ×g for 30 min at 4 °C, the supernatants were saved as P fraction.

Generation of parkin stable cell lines

SH-SY5Y cells were transfected with pcDNA3.1 plasmid vector expressing either Myc-tagged wild type parkin or its deleted mutant (amino acids: 1–415) using LipofectAMINE PLUS reagents (Invitrogen). Two days later, the cells stably transfected with parkin were selected in DMEM containing 2 mg/ml of G-418 (Sigma-Aldrich). Consequently, at least 30 clonal cells were picked; the amount of parkin expression for each cell line was measured by Western blot analysis with anti-Myc or anti-parkin antibodies. All positive cell lines used for the experiments described here were maintained in DMEM containing 500 µg/ml of G-418 to prevent extrusion of the integrated constructs.

Immunoprecipitation and Western blot analysis

One microgram of suitable antibodies was incubated with cell extracts (1 mg) prepared in lysis buffer overnight at 4 °C. A 1:1 suspension of protein A-Sepharose beads (30 µl) was added and incubated for 2 h at 4 °C with gentle rotation. The beads were pelleted and washed five times with cell lysis buffer. The immunocomplexes were dissociated by boiling in SDS-PAGE sample buffer. Whole protein samples were separated on an SDS-PAGE gel and transferred to a nitrocellulose membrane (Amersham Biosciences). The membrane was blocked in TBST buffer containing 20 mM Tris, pH 7.6, 137 mM NaCl, 0.05% Tween 20 and 5% nonfat dry milk for 1 h at the room temperature and incubated overnight at 4 °C in 3% nonfat dry milk containing the appropriate primary antibodies. The membrane was washed several times in TBST and incubated with a secondary IgG-coupled horseradish peroxidase antibody. After 60 min, the membrane was washed several times with TBST and visualized with ECL reagents.

Generation of parkin knockout mice

The knockout (KO) mouse with an exon 3 deletion in the parkin gene has been generated by the protocols, as described previously (42). For the experiments, F4 parkin homozygous KO (−/−) mice and their non-transgenic parkin (+/+) littermates were used. All KO animal experiments were conducted and approved using the German guidelines of the animal care and use committee of the state Nordrhein-Westfalen.

Immunocytochemistry

Cells were fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 30 min, and washed with PBS. The samples were then blocked with 1% BSA for 30 min. Brain sections from 6-OHDA, rotenone or MG132-injected rats were permeabilized with 0.2% Triton X-100 in PBS for 30 min and washed with PBS. Sections were then treated with 3% hydrogen peroxide solution for 5 min, to inhibit endogenous peroxidase activity, and blocked with 1% BSA for 30 min. Sections were incubated overnight at 4 °C with anti-PINK1 (1:1000 dilution), anti-parkin (1:200 dilution), or anti-ubiquitin (1:100 dilution) antibodies. Then, either FITC-conjugated or TRITC-conjugated secondary antibodies (1:200 dilution) were used to detect the primary antibodies. Samples were stained with DAPI, mounted and observed using a Carl Zeiss LSM-510 META confocal microscope. The data were processed with the Carl Zeiss LSM Image Browser.

Preparation of rat brain lysates

Detergent-soluble and -insoluble fractions rat brain tissue, including the substantia nigral region and striatum, were prepared by

homogenization in lysis buffer, which contained 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP-40, and 10 mM Na-β-glycerophosphate. After homogenization, samples were rotated at 4 °C for 30 min for complete lysis. The homogenate was then centrifuged at 16,000 ×g for 20 min at 4 °C; the resulting pellet and supernatant fractions were collected. The pellet fraction was washed once in lysis buffer containing detergent and solubilized in lysis buffer containing 2% SDS.

Quantitative real time RT-PCR analysis of PINK1 mRNA levels

After total cellular mRNAs were isolated from SH-SY5Y cells, first strand cDNA was synthesized using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR for cDNA quantification was performed with SYBR Green PCR master mix (Applied Biosystems) and ABI 7300 Real-Time PCR System, according to the manufacturer's instruction. The data were analyzed by 7300 System Software v 1.4.0. The primers used were as follows: human PINK1 forward primer, 5'-GAGTATCTGATAGGGCAGTCCATT-3'; human PINK1 reverse primer, 5'-CCTCTCTGGATTTCTGTAAGTGA-3'; β-actin forward primer, 5'-TGTTACCAACTGGACGACA-3'; β-actin reverse primer, 5'-TCTCAGCTGCTGGAAG-3'.

Statistical analysis

Statistical significance of cell counting data or fluorescent intensity measurements was determined by unpaired Student's *t*-test using Sigma Plot 9.0. Values were expressed as mean S.E.M.

Acknowledgments

We thank K. Tanaka, H. Rhim, M.R. Cookson, M. Mouradian, W.S. Park, P.M. Snyder, and T.H. Lee for providing plasmids. This study was supported by grants from the Brain Research Center of the 21st Century Frontier Research Program Technology (M103KV010011-06K2201-01110 to K.C.C.) and from the Korea Science and Engineering Foundation (KOSEF) through National Research Laboratory Program (R04-2007-000-20014-0 to K.C.C.) funded by Ministry of Science, Republic of Korea. This work was also partly supported by the KOSEF grant (R11-2007-040-01005-0 and R01-2007-000-20089-0 to K.C.C.), a grant from the Korea Health 21 R and D Project (A080551 to K.C.C.), Ministry of Health and Welfare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2008.12.010.

References

- Abou-Sleiman, P.M., Muqit, M.M., McDonald, N.Q., Yang, Y.X., Gandhi, S., Healy, D.G., Harvey, K., Harvey, R.J., Deas, E., Bhatia, K., Quinn, N., Lees, A., Latchman, D.S., Wood, N.W., 2006. A heterozygous effect for PINK1 mutations in Parkinson's disease? *Ann. Neurol.* 60, 414–419.
- Beilina, A., Van Der Brug, M., Ahmad, R., Kesavapany, S., Miller, D.W., Petsko, G.A., Cookson, M.R., 2005. Mutations in PTEN-induced putative kinase 1 associated with recessive Parkinsonism have differential effects on protein stability. *Proc. Natl. Acad. Sci. U. S. A.* 102, 5703–5708.
- Capili, A.D., Edghill, E.L., Wu, K., Borden, K.L., 2004. Structure of the C-terminal RING finger from a RING-IBR-RING/TRIAD motif reveals a novel zinc-binding domain distinct from a RING. *J. Mol. Biol.* 340, 1117–1129.
- Choi, P., Ostrerova-Golts, N., Sparkman, D., Cochran, E., Lee, J.M., Wolozin, B., 2000. Parkin is metabolized by the ubiquitin/proteasome system. *NeuroReport* 11, 2635–2638.
- Clark, I.E., Dodson, M.W., Jiang, C., Cao, J.H., Huh, J.R., Seol, J.H., Yoo, S.J., Hay, B.A., Guo, M., 2006. *Drosophila* pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature* 441, 1162–1166.
- Deng, H., Dodson, M.W., Huang, H., Guo, M., 2008. The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 105, 14503–14508.

- Exner, N., Treske, B., Paquet, D., Holmström, K., Schiesling, C., Gispert, S., Carballo-Carbajal, I., Berg, D., Hoepken, H.H., Gasser, T., Krüger, R., Winklhofer, K.F., Vogel, F., Reichert, A.S., Auburger, G., Kahle, P.J., Schmid, B., Haass, C., 2007. Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by parkin. *J. Neurosci.* 27, 12413–12418.
- Gandhi, S., Muqit, M.M., Stanyer, L., Healy, D.G., Abou-Sleiman, P.M., Hargreaves, I., Heales, S., Ganguly, M., Parsons, L., Lees, A.J., Latchman, D.S., Holton, J.L., Wood, N.W., Revesz, T., 2006. PINK1 protein in normal human brain and Parkinson's disease. *Brain* 129, 1720–1731.
- Ii, K., Ito, H., Tanaka, K., Hirano, A., 1997. Immunocytochemical co-localization of the proteasome in ubiquitinated structures in neurodegenerative diseases and the elderly. *J. Neuropathol. Exp. Neurol.* 56, 125–131.
- Junn, E., Lee, S.S., Suhr, U.T., Mouradian, M.M., 2002. Parkin accumulation in aggresomes due to proteasome impairment. *J. Biol. Chem.* 277, 47870–47877.
- Kim, S.J., Sung, J.Y., Um, J.W., Hattori, N., Mizuno, Y., Tanaka, K., Paik, S.R., Kim, J., Chung, K.C., 2003. Parkin cleaves intracellular alpha-synuclein inclusions via the activation of calpain. *J. Biol. Chem.* 278, 41890–41899.
- Kim, Y., Park, J., Kim, S., Song, S., Kwon, S.K., Lee, S.H., Kitada, T., Kim, J.M., Chung, J., 2008. PINK1 controls mitochondrial localization of Parkin through direct phosphorylation. *Biochem. Biophys. Res. Commun.* 377, 975–980.
- Kopito, R.R., 2000. Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol.* 10, 524–530.
- LaVoie, M.J., Ostaszewski, B.L., Weihofen, A., Schlossmacher, M.G., Selkoe, D.J., 2005. Dopamine covalently modifies and functionally inactivates parkin. *Nat. Med.* 11, 1214–1221.
- LaVoie, M.J., Cortese, G.P., Ostaszewski, B.L., Schlossmacher, M.G., 2007. The effects of oxidative stress on parkin and other E3 ligases. *J. Neurochem.* 103, 2354–2368.
- Lennox, G., Lowe, J., Morrell, K., Landon, M., Mayer, R.J., 1989. Anti-ubiquitin immunocytochemistry is more sensitive than conventional techniques in the detection of diffuse Lewy body disease. *J. Neurol. Neurosurg. Psychiatry* 52, 67–71.
- Lin, W., Kang, U.J., 2008. Characterization of PINK1 processing, stability, and subcellular localization. *J. Neurochem.* 106, 464–474.
- Lowe, J., McDermott, H., Landon, M., Mayer, R.J., Wilkinson, K.D., 1990. Ubiquitin carboxyl-terminal hydrolase (PGP 9.5) is selectively present in ubiquitinated inclusion bodies characteristic of human neurodegenerative diseases. *J. Pathol.* 161, 153–160.
- McNaught, K.S., Olanow, C.W., 2006. Protein aggregation in the pathogenesis of familial and sporadic Parkinson's disease. *Neurobiol. Aging* 27, 530–545.
- McNaught, K.S., Shashidharan, P., Perl, D.P., Jenner, P., Olanow, C.W., 2002. Aggresome-related biogenesis of Lewy bodies. *Eur. J. Neurosci.* 16, 2136–2148.
- McNaught, K.S., Belzair, R., Isacson, O., Jenner, P., Olanow, C.W., 2003. Altered proteasomal function in sporadic Parkinson's disease. *Exp. Neurol.* 179, 38–46.
- Moore, D.J., West, A.B., Dawson, V.L., Dawson, T.M., 2005a. Molecular pathophysiology of Parkinson's disease. *Annu. Rev. Neurosci.* 28, 57–87.
- Moore, D.J., Zhang, L., Troncoso, J., Lee, M.K., Hattori, N., Mizuno, Y., Dawson, T.M., Dawson, V.L., 2005b. Association of DJ-1 and parkin mediated by pathogenic DJ-1 mutations and oxidative stress. *Hum. Mol. Genet.* 14, 71–84.
- Muqit, M.M., Abou-Sleiman, P.M., Saurin, A.T., Harvey, K., Gandhi, S., Deas, E., Eaton, S., Payne Smith, M.D., Venner, K., Matilla, A., Healy, D.G., Gilks, W.P., Lees, A.J., Holton, J., Revesz, T., Parker, P.J., Harvey, R.J., Wood, N.W., Latchman, D.S., 2006. Altered cleavage and localization of PINK1 to aggresomes in the presence of proteasomal stress. *J. Neurochem.* 98, 156–169.
- Olanow, C.W., Tatton, W.G., 1999. Etiology and pathogenesis of Parkinson's disease. *Annu. Rev. Neurosci.* 22, 123–144.
- Olanow, C.W., Perl, D.P., DeMartino, G.N., McNaught, K.S., 2004. Lewy-body formation is an aggresome-related process: a hypothesis. *Lancet Neurol.* 3, 496–503.
- Park, J., Lee, S.B., Lee, S., Kim, Y., Song, S., Kim, S., Bae, E., Kim, J., Shong, M., Kim, J.M., Chung, J., 2006. Mitochondrial dysfunction in *Drosophila* PINK1 mutants is complemented by parkin. *Nature* 441, 1157–1161.
- Petit, A., Kawarai, T., Paitel, E., Sanjo, N., Maj, M., Scheid, M., Chen, F., Gu, Y., Hasegawa, H., Salehi-Rad, S., Wang, L., Rogaeva, E., Fraser, P., Robinson, B., St George-Hyslop, P., Tandon, A., 2005. Wild-type PINK1 prevents basal and induced neuronal apoptosis, a protective effect abrogated by Parkinson disease-related mutations. *J. Biol. Chem.* 280, 34025–34032.
- Poole, A.C., Thomas, R.E., Andrews, L.A., McBride, H.M., Whitworth, A.J., Pallanck, L.J., 2008. The PINK1/Parkin pathway regulates mitochondrial morphology. *Proc. Natl. Acad. Sci. U. S. A.* 105, 1638–1643.
- Pridgeon, J.W., Olzmann, J.A., Chin, L.S., Li, L., 2007. PINK1 protects against oxidative stress by phosphorylating mitochondrial chaperone TRAP1. *PLoS Biol.* 5, e172.
- Schlossmacher, M.G., Frosch, M.P., Gai, W.P., Medina, M., Sharma, N., Forno, L., Ochiishi, T., Shimura, H., Sharon, R., Hattori, N., Langston, J.W., Mizuno, Y., Hyman, B.T., Selkoe, D.J., Kosik, K.S., 2002. Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies. *Am. J. Pathol.* 160, 1655–1667.
- Schmidt, M.L., Murray, J., Lee, V.M., Hill, W.D., Wertkin, A., Trojanowski, J.Q., 1991. Epitope map of neurofilament protein domains in cortical and peripheral nervous system Lewy bodies. *Am. J. Pathol.* 139, 53–65.
- Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minooshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., Suzuki, T., 2000. Familial Parkinson disease gene product, parkin, is an ubiquitin-protein ligase. *Nat. Genet.* 25, 302–305.
- Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S., Selkoe, D.J., 2001. Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. *Science* 293, 263–269.
- Silvestri, L., Caputo, V., Bellacchio, E., Atorino, L., Dallapiccola, B., Valente, E.M., Casari, G., 2005. Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive Parkinsonism. *Hum. Mol. Genet.* 14, 3477–3492.
- Smith, W.W., Pei, Z., Jiang, H., Moore, D.J., Liang, Y., West, A.B., Dawson, V.L., Dawson, T.M., Ross, C.A., 2005. Leucine-rich repeat kinase 2 (LRRK2) interacts with parkin, and mutant LRRK2 induces neuronal degeneration. *Proc. Natl. Acad. Sci. U. S. A.* 102, 18676–18681.
- Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R., Goedert, M., 1997. α -Synuclein in Lewy bodies. *Nature* 388, 839–840.
- Stichel, C.C., Zhu, X.R., Bader, V., Linnartz, B., Schmidt, S., Lübbert, H., 2007. Mono- and double-mutant mouse models of Parkinson's disease display severe mitochondrial damage. *Hum. Mol. Genet.* 16, 2377–2393.
- Takatori, S., Ito, G., Iwatsubo, T., 2008. Cytoplasmic localization and proteasomal degradation of N-terminally cleaved form of PINK1. *Neurosci. Lett.* 430, 13–17.
- Tang, B., Xiong, H., Sun, P., Zhang, Y., Wang, D., Hu, Z., Zhu, Z., Ma, H., Pan, Q., Xia, J.H., Xia, K., Zhang, Z., 2006. Association of PINK1 and DJ-1 confers digenic inheritance of early-onset Parkinson's disease. *Hum. Mol. Genet.* 15, 1816–1825.
- Thomas, B., Beal, M.F., 2007. Parkinson's disease. *Hum. Mol. Genet.* 16, 183–194.
- Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., Albanese, A., Nussbaum, R., González-Maldonado, R., Deller, T., Salvi, S., Cortelli, P., Gilks, W.P., Latchman, D.S., Harvey, R.J., Dallapiccola, B., Auburger, G., Wood, N.W., 2004. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* 304, 1158–1160.
- Wang, C., Ko, H.S., Thomas, B., Tsang, F., Chew, K.C., Tay, S.P., Ho, M.W., Lim, T.M., Soong, T.W., Pletnikova, O., Troncoso, J., Dawson, V.L., Dawson, T.M., Lim, K.L., 2005. Stress-induced alterations in parkin solubility promote parkin aggregation and compromise parkin's protective function. *Hum. Mol. Genet.* 14, 3885–3897.
- Weihofen, A., Ostaszewski, B., Minami, Y., Selkoe, D.J., 2008. Pink1 Parkinson mutations, the Cdc37/Hsp90 chaperones and Parkin all influence the maturation or subcellular distribution of Pink1. *Hum. Mol. Genet.* 17, 602–616.
- Wong, E.S., Tan, J.M., Wang, C., Zhang, Z., Tay, S.P., Zaiden, N., Ko, H.S., Dawson, V.L., Dawson, T.M., Lim, K.L., 2007. Relative sensitivity of parkin and other cysteine-containing enzymes to stress-induced solubility alterations. *J. Biol. Chem.* 282, 12310–12318.
- Yang, Y., Gehrke, S., Imai, Y., Huang, Z., Ouyang, Y., Wang, J.W., Yang, L., Beal, M.F., Vogel, H., Lu, B., 2006. Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of *Drosophila* Pink1 is rescued by Parkin. *Proc. Natl. Acad. Sci. U. S. A.* 103, 10793–10798.
- Yang, H., Zhou, H.Y., Li, B., Niu, G.Z., Chen, S.D., 2007. Downregulation of parkin damages antioxidant defenses and enhances proteasome inhibition-induced toxicity in PC12 cells. *J. Neuroimmune Pharmacol.* 2, 276–283.
- Yao, D., Gu, Z., Nakamura, T., Shi, Z.Q., Ma, Y., Gaston, B., Palmer, L.A., Rockenstein, E.M., Zhang, Z., Masliah, E., Uehara, T., Lipton, S.A., 2004. Nitrosative stress linked to sporadic Parkinson's disease: S-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. *Proc. Natl. Acad. Sci. U. S. A.* 101, 10810–10814.