

Original Article

Parkin represses 6-hydroxydopamine-induced apoptosis via stabilizing scaffold protein p62 in PC12 cells

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Aim: Parkin has been shown to exert protective effects against 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in different models of Parkinson disease. In the present study we investigated the molecular mechanisms underlying the neuroprotective action of parkin *in vitro*.

Methods: HEK293, HeLa and PC12 cells were transfected with parkin, parkin mutants, p62 or si-p62. Protein expression and ubiquitination were assessed using immunoblot analysis. Immunoprecipitation assay was performed to identify the interaction between parkin and scaffold protein p62. PC12 and SH-SY5Y cells were treated with 6-OHDA (200 μ mol/L), and cell apoptosis was detected using PI and Hoechst staining.

Results: In HEK293 cells co-transfected with parkin and p62, parkin was co-immunoprecipitated with p62, and parkin overexpression increased p62 protein levels. In parkin-deficient HeLa cells, transfection with wild-type parkin, but not with ligase activity-deficient parkin mutants, significantly increased p62 levels, suggesting that parkin stabilized p62 through its E3 ligase activity. Transfection with parkin or p62 significantly repressed ERK1/2 phosphorylation in HeLa cells, but transfection with parkin did not repress ERK1/2 phosphorylation in p62-knockdown HeLa cells, suggesting that p62 was involved in parkin-induced inhibition on ERK1/2 phosphorylation. Overexpression of parkin or p62 significantly repressed 6-OHDA-induced ERK1/2 phosphorylation in PC12 cells, and parkin overexpression inhibited 6-OHDA-induced apoptosis in PC12 and SH-SY5Y cells.

Conclusion: Parkin protects PC12 cells against 6-OHDA-induced apoptosis via ubiquitinating and stabilizing scaffold protein p62, and repressing ERK1/2 activation.

Keywords: Parkinson disease; parkin; p62; ERK1/2; 6-OHDA; PC12 cells

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Introduction

Parkinson disease (PD) is the second most common age-associated neurodegenerative disease, affecting approximately 1% of 65-year-old people^[1]. The pathological features of PD include the selective loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the presence of Lewy bodies (LBs)^[2]. Both environmental and genetic factors are associated with PD. Exposure to pesticides and neurotoxins has been implicated in the etiology of PD^[3]. In recent years,

the role of genetic mutations in PD-related genes has provided crucial insight into the pathogenesis of PD. Several genes with loss-of-function mutations have been identified in familial PD^[4]. Among these genes, *PARK2*, which encodes parkin, is most commonly associated with the recessive forms of PD^[5]. Parkin functions as an E3 ubiquitin ligase, targeting ubiquitin to specific substrates. *PARK2* mutations lead to the loss of its function and therefore to an accumulation of its substrates in the brain; such accumulation is related to the occurrence of PD^[6]. It has been reported that parkin, but not its pathogenic mutants, can protect DA neurons against neurotoxicity^[7].

As an E3 ligase, parkin participates in mitophagy upon being transferred from the cytosol to the mitochondria to ubiquitinate mitochondrial substrates after carbonyl cyanide

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m-chlorophenylhydrazine (CCCP) treatment^[8]. The ubiquitinated mitochondria are then recognized by p62, which links ubiquitinated substrates/mitochondria to phagophores and leads to the engulfment of the ubiquitinated substrates/mitochondria by phagophores to form autophagosomes^[9, 10]. As a signaling adaptor protein, p62 has multiple domains^[11] and is associated with many diseases. Mutations in the gene encoding p62 lead to Paget's disease of bone (PDB)^[12]. Furthermore, the accumulation of p62 promotes tumorigenesis^[13].

Previous studies have suggested that the PB1 domain of p62 interacts with extracellular signal-regulated kinase (ERK) and maintains low ERK activity under normal conditions^[11, 14, 15]. Consistent with these findings, ERK1/2 activity is enhanced in p62-deficient mice^[15]. ERK1/2 is a member of the family of mitogen-activated protein kinases (MAPKs) and mediates different cellular events through signaling pathways^[16, 17]. Usually, the activation of ERK1/2 has a positive effect on neuronal cell survival^[18, 19]. However, persistent ERK1/2 activation is toxic to DA neurons^[20]. ERK1/2 activation is observed in both cellular and animal models after the administration of neurotoxins such as 6-hydroxydopamine (6-OHDA) and levodopa, which elicit oxidative injury^[21-23]. The inhibition of ERK1/2 activity ameliorates DA neuron loss and PD-associated phenotypes^[24]. These studies suggest the involvement of ERK1/2 activation in PD pathogenesis. Interestingly, parkin protects DA neurons against rotenone-induced cell death by repressing ERK activation^[25]. However, the mechanism mediating the effects of parkin on ERK1/2 is completely unknown.

Here, we demonstrate that parkin ubiquitinates and stabilizes p62, leading to the inhibition of ERK1/2 activity, thereby protecting DA neurons against 6-OHDA-induced cell death.

Materials and methods

Plasmids

The EGFP, EGFP-parkin, EGFP-parkin K161N, EGFP-parkin T240R, Flag, Flag-parkin, and EGFP-p62 constructs have been previously described^[26, 27].

Cell culture, transfection and treatment

HeLa, HEK293 or PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Los Angeles, CA, USA) supplemented with 10% fetal bovine serum (Gibco), 100 µg/mL penicillin and 100 µg/mL streptomycin. SH-SY5Y cells were grown in DMEM/F12 (Gibco). All of these cells were maintained at 37 °C with 5% CO₂. To achieve transient overexpression, the cultured cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were harvested for immunoblot analyses or immunoprecipitation assays. PC12 or SH-SY5Y cells transfected with p62 or parkin were treated with 6-OHDA (Sigma, St Louis, MO, USA) at 200 µmol/L. Twenty-four hours later, the treated cells were collected for immunoblot analyses. For propidium iodide (PI; Sigma) staining, cells transfected with EGFP or EGFP-parkin were treated with 6-OHDA for 12 h. The cells were then stained with PI and observed using an

inverted fluorescent microscope (IX71, Olympus, Tokyo, Japan).

Immunoblot analysis and antibodies

The cells were lysed in cell lysis buffer containing 50 mmol/L Tris-HCl (pH7.5), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, and a complete protein inhibitor cocktail (Roche, Mannheim, Germany). The samples were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The following primary antibodies were used: monoclonal anti- α -tubulin antibody, anti-GAPDH antibody (Millipore), monoclonal anti-Flag antibody (Sigma), monoclonal anti-p-ERK1/2 antibody, anti-GFP antibody, anti-ubiquitin (Ub) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-ERK1/2 antibody, and anti-p62 antibody (Santa Cruz Biotechnology). Anti-mouse or anti-rabbit IgG-HRP was used as the secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Immunoreactive bands were detected using an ECL detection kit (Thermo, Rockford, IL, USA).

Immunoprecipitation

HEK293 cells were co-transfected with EGFP or EGFP-p62 and Flag or Flag-parkin. Twenty-four hours after transfection, the cells were collected and sonicated in cell lysis buffer. Then, the cellular debris was removed by centrifugation at 12000×g for 15 min at 4 °C. After discarding the precipitate, the supernatants were incubated with monoclonal anti-GFP (Roche) and Protein-G agarose overnight at 4 °C. After incubation, the immunoprecipitates were washed five times with cell lysis buffer. Then, the proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE.

RNA interference

Double-stranded oligonucleotides were designed against the 5'-CATGTCCTACGTGAAGGATGATT-3' region of p62. Non-specific oligonucleotides served as a negative control. The oligonucleotides were transfected with RNAiMAX (Invitrogen) into confluent cells. Briefly, a mixture of Opti-MEM, RNAiMAX and siRNA was incubated for 20 min at room temperature before transfection. Twelve hours after transfection, the medium was replaced with fresh complete medium. The cells were collected 72 h after transfection for further analysis.

Statistical analysis

Densitometric analyses of immunoblots from three independent experiments were performed using Photoshop 7.0 (Adobe, San Jose, CA, USA). The data were analyzed using Origin 6.0 (Originlab, Northampton, MA, USA). The quantitative data are presented as the mean±SEM. Statistical significance was assessed via one-way ANOVA and significance was set at $P < 0.05$.

Results

Parkin interacts with and ubiquitinates p62

Parkin is an E3 ubiquitin ligase that ubiquitinates its target proteins^[28]. It has been reported that both parkin and p62

function in mitophagy^[29]. p62 recognizes the substrates that are ubiquitinated by parkin; then, with the help of LC3, p62 delivers these substrates to the autophagosomes for degradation. As parkin is an E3 ligase that binds to its substrates, we hypothesized that parkin and p62 directly interact with each other. Thus, we performed immunoprecipitation assays. In cells that were co-transfected with EGFP-p62 and Flag-parkin, Flag-parkin was co-immunoprecipitated when EGFP-p62 was precipitated with anti-EGFP antibody (Figure 1A). However, Flag-parkin was not co-immunoprecipitated in cells that were

co-transfected with EGFP (Figure 1A). In addition, the ubiquitination level of p62 was significantly increased in cells transfected with EGFP-parkin (Figure 1B).

Parkin stabilizes p62 through its E3 ubiquitin ligase activity

Ubiquitination usually acts as a degradation signal for some proteins. We hypothesized that the increased ubiquitination of p62 by parkin promotes its degradation. We overexpressed EGFP or EGFP-parkin in parkin-deficient HeLa cells, which harbor parkin exon deletions^[30], to identify the effects of parkin on p62 degradation. Interestingly, endogenous p62 was not decreased but was significantly increased following the overexpression of EGFP-parkin (Figure 2A). We next investigated whether the increase in p62 is associated with the E3 ligase activity of parkin. We transfected cells with wild-type EGFP-parkin or two ligase activity-deficient mutants (EGFP-parkin K161N and EGFP-parkin T240R). Our results showed that EGFP-parkin, but not EGFP-parkin K161N or EGFP-

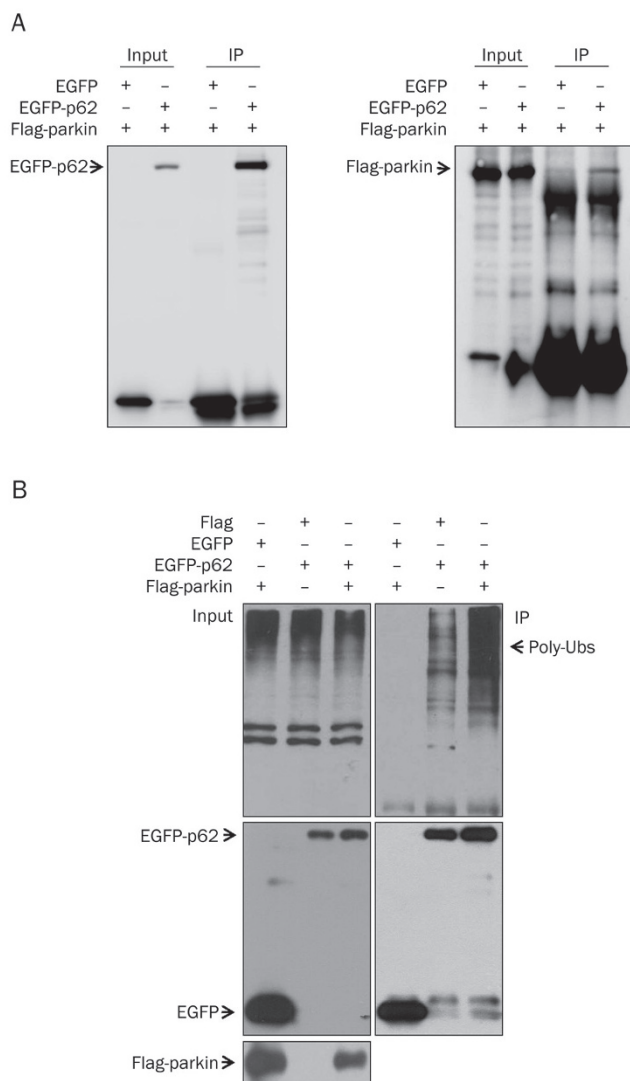


Figure 1. Parkin interacts with and ubiquitinates p62. (A) HEK293 cells were co-transfected with Flag-parkin and EGFP or EGFP-p62, respectively. The cells were collected 24 h after transfection and immunoprecipitated with an anti-GFP antibody. The inputs and immunoprecipitates were subjected to immunoblot analysis using an anti-Flag or anti-GFP antibody. (B) HEK293 cells were co-transfected with EGFP or EGFP-p62 and Flag or Flag-parkin. Twenty-four hours after transfection, the cells were collected for immunoprecipitation analysis using an anti-GFP antibody. The inputs and immunoprecipitates were subjected to immunoblot analysis using anti-Flag, anti-EGFP and anti-ubiquitin (Ub) antibodies.

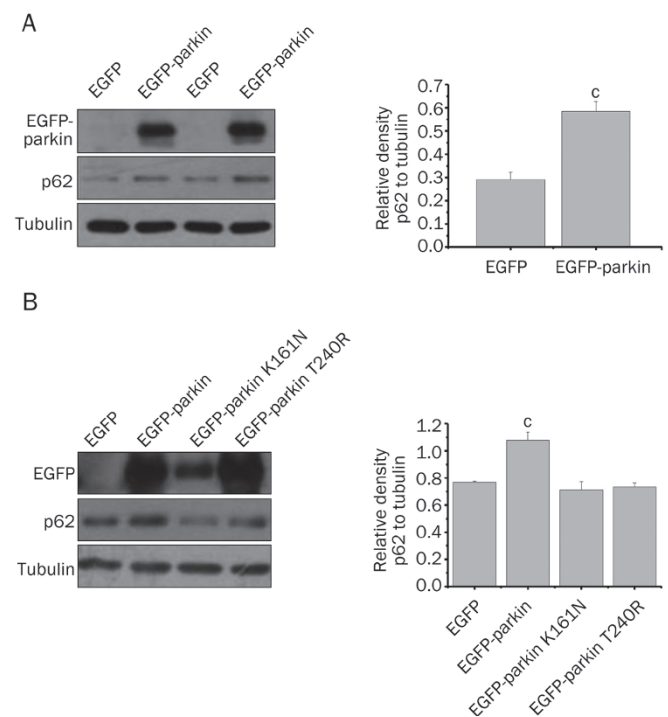


Figure 2. Parkin stabilizes p62 through its E3 ubiquitin ligase activity. (A) HeLa cells were transfected with EGFP or EGFP-parkin for 24 h. Cell lysates were subjected to immunoblot analysis with anti-GFP, anti-p62 and anti-tubulin antibodies. The quantitative analysis of the relative density of p62 compared with the loading control (tubulin) is shown in the right panel. The data are presented as the mean±SEM from three independent experiments. [°]*P*<0.01 vs EGFP group, one-way ANOVA. (B) HeLa cells expressing EGFP, EGFP-parkin, EGFP-parkin K161N or EGFP-parkin T240R. Twenty-four hours after transfection, the cells were subjected to immunoblot analysis with the indicated antibodies. The band density of p62 relative to tubulin is shown in the right panel. The data are presented as the mean±SEM from three independent experiments. [°]*P*<0.01 vs EGFP group, one-way ANOVA.

parkin T240R, increased p62 levels (Figure 2B). These results suggest that parkin stabilizes p62 through its E3 ligase activity.

Parkin represses ERK1/2 activity by regulating p62

Previous studies have reported that p62 influences ERK1/2 activation^[14] and that parkin represses ERK activation^[25]. We

hypothesized that the effects of parkin on ERK1/2 are mediated by its regulation of p62. First, we examined the effects of p62 on ERK1/2 activation. In HeLa cells transfected with EGFP or EGFP-p62, pan-ERK1/2 levels were not changed, but ERK1/2 phosphorylation was decreased in cells transfected with EGFP-p62 (Figure 3A). By contrast, in p62-knockdown

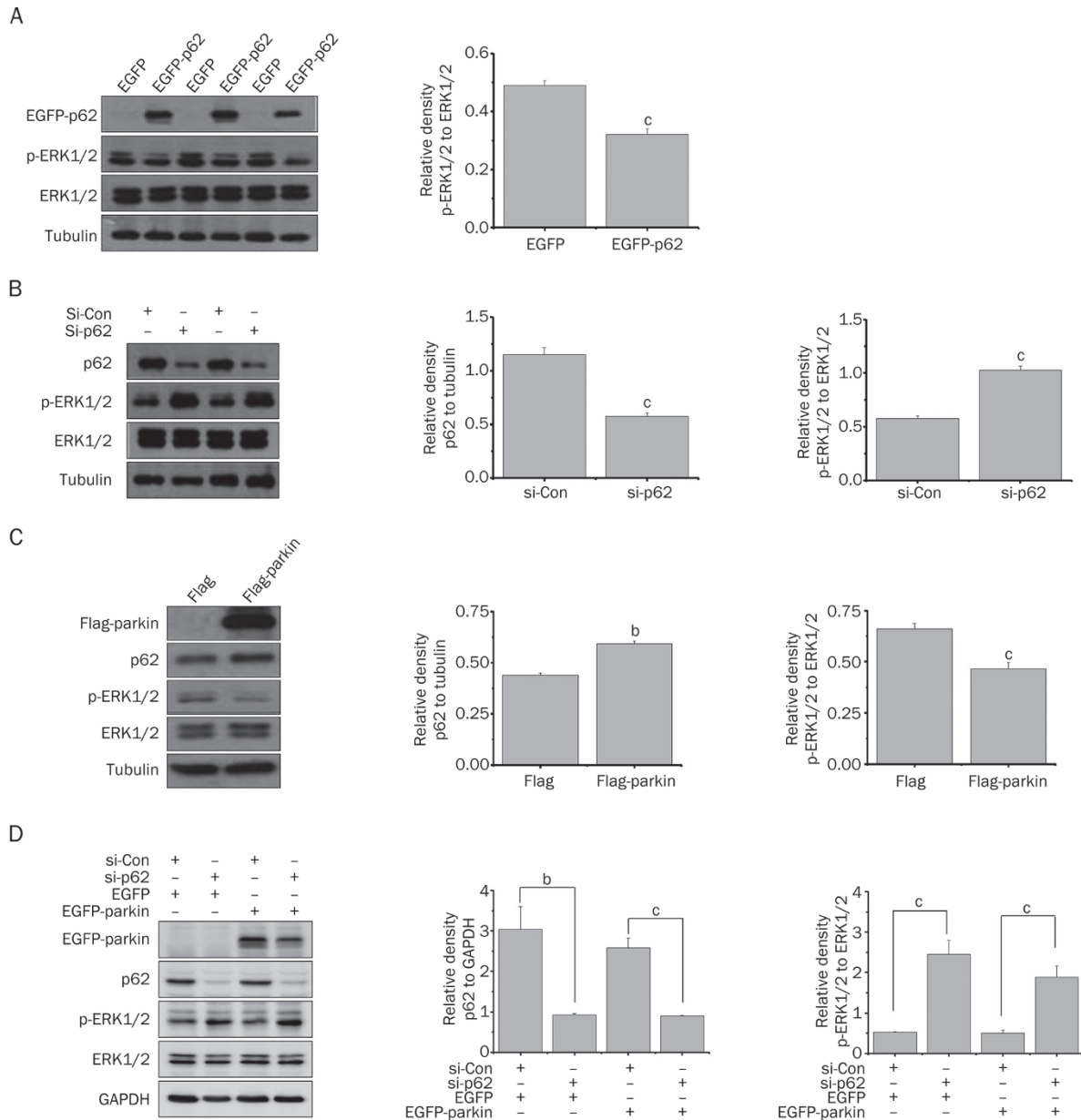


Figure 3. Parkin represses ERK1/2 activity by regulating p62. (A) HeLa cells were transfected with EGFP or EGFP-p62. Cell lysates were subjected to immunoblot analysis with anti-GFP, anti-pERK1/2, anti-ERK1/2 and anti-tubulin antibodies. The band density of p-ERK1/2 relative to pan-ERK1/2 is shown in the right panel. Mean±SEM. ^cP<0.01 vs EGFP group. (B) HeLa cells were transfected with si-con or si-p62 for 48 h followed by immunoblot analysis with the indicated antibodies. The band densities of p-ERK1/2 relative to pan-ERK1/2 and p62 relative to tubulin are shown in the middle and right panels. Mean±SEM. ^cP<0.01 vs si-con group. (C) HeLa cells were transfected with Flag or Flag-parkin for 24 h followed by immunoblot analysis. The band densities of p-ERK1/2 relative to pan-ERK1/2 and p62 relative to tubulin are shown. Mean±SEM. ^bP<0.05, ^cP<0.01 vs Flag group. (D) HeLa cells were first transfected with si-con or si-p62 for 24 h and then with EGFP or EGFP-parkin for another 24 h followed by immunoblot analysis. The band densities of p-ERK1/2 relative to pan-ERK1/2 and p62 relative to GAPDH are shown in the middle and right panels. Mean±SEM. ^bP<0.05, ^cP<0.01.

cells, ERK1/2 phosphorylation was increased (Figure 3B), suggesting that p62 inhibits ERK1/2 activation. Next, we examined the relationship between parkin, p62 and ERK1/2. In parkin-overexpressing cells, p62 was increased, whereas p-ERK1/2 was decreased (Figure 3C). Furthermore, in p62-knockdown cells, parkin failed to repress ERK1/2 activation (Figure 3D), suggesting that p62 mediates the parkin-dependent effects on ERK1/2 activation.

p62 represses 6-OHDA-induced ERK1/2 activation

6-OHDA is a neurotoxin that induces massive oxidative stress, leading to the specific degeneration of DA neurons^[31-33]. It has been reported that 6-OHDA-induced oxidative stress promotes sustained ERK1/2 activation^[34]. We determined whether p62 or parkin could inhibit ERK1/2 activation in 6-OHDA-treated cells. In cells that were transfected with EGFP-p62 (Figure 4A) or EGFP-parkin (Figure 4B), the overexpression of EGFP-parkin or EGFP-p62 decreased 6-OHDA-induced ERK1/2 activation, suggesting that parkin and p62 have similar effects on ERK1/2 activation. To further identify the protective effects of parkin on 6-OHDA-induced cell death, we transfected SH-SY5Y cells with EGFP or EGFP-parkin and treated the cells with or without 6-OHDA (Figure 4C). Parkin overexpression significantly decreased 6-OHDA-induced cell death, as shown by the decreased number of dead cells stained with PI.

Discussion

Genetic mutations and environmental toxins play important roles in DA neuronal degeneration. 6-OHDA is a neurotoxin that induces the specific degeneration of DA neurons and is usually used to prepare PD animal models^[35]. It has been reported that 6-OHDA produces reactive oxygen species (ROS) and impairs mitochondrial function^[36]. Although ERK activation usually promotes cell survival, oxidative stress induced by 6-OHDA activates MAPK family members such as ERK1/2 and promotes DA neuronal death^[34, 37, 38]. A previous study showed that persistent glutamate-induced ERK1/2 activation promotes cell death in a hippocampal cell line and primary neurons^[39]. Abnormal activation of ERK1/2, along with interactions with abnormal mitochondria, is present in the substantia nigra in the brains of PD patients^[40]. Moreover, the inhibition of ERK1/2 activation could reduce 6-OHDA-induced cell death^[37]. Thus, abnormal ERK1/2 activation plays a critical role in PD pathogenesis.

Parkin is documented to mediate neuroprotection against 6-OHDA-induced neurotoxicity^[7, 21, 41, 42]. As parkin is an E3 ubiquitin ligase, its neuroprotective effect may be executed via its substrates. In the present study, we found that p62 is a substrate of parkin. Parkin directly interacts with p62 and ubiquitinates p62 via its E3 ligase activity. However, the ubiquitination of p62 by parkin does not promote p62 degradation. By contrast, the stability of p62 is increased, leading to upregulated p62 protein levels in cells. However, parkin mutants (K161N and T240R) that have lost E3 ligase activity fail to stabilize p62, suggesting that the ubiquitination of p62 by parkin does not provide a degradation signal. Polyubiquitination is

specifically linked to different lysine (K) sites in the substrate, primarily K48 or K63^[43]. A K48-linked polyubiquitin chain is considered to be a proteasome degradation signal. However, K63-linked polyubiquitin chains do not mediate proteasomal degradation, but instead drive other cellular processes, such as endocytic trafficking, translation and DNA repair^[44]. Recently, ESCRT0 (endosomal sorting complex required for transport) and other factors have been identified as molecules that selectively bind to K63-linked polyubiquitin chains and inhibit their binding to the proteasome^[45]. Thus, K63-linked polyubiquitin chains may block their substrates from being degraded by the proteasome. In our study, the p62 that is polyubiquitinated by parkin is not degraded, but rather is stabilized, suggesting that the polyubiquitin chains may be K63-linked.

p62 is an important scaffold protein that has multiple domains. Those domains enable p62 to regulate kinase-activated and ubiquitin-mediated signaling pathways^[46]. The PB1 domain of p62 interacts with some protein kinases, including ERK1/2, MEKK3 and MEK5^[11]. It has been reported that p62 is involved in regulating ERK1/2 activation. In p62-deficient mice, the basal level of ERK1/2 activity is enhanced^[14]. However, p62 overexpression dramatically inhibits ERK1/2 activation^[15]. We also observed that p62 overexpression represses and p62 knockdown activates the phosphorylation of ERK1/2. Furthermore, we found that parkin overexpression represses ERK1/2 activation, similar to p62 overexpression. Importantly, parkin overexpression fails to repress ERK1/2 activation in p62-knockdown cells, suggesting that p62 mediates the parkin-dependent effects on ERK1/2 activation. In addition, both parkin and p62 repress 6-OHDA-induced ERK1/2 activation in neuronal cells, further suggesting that parkin and p62 act on the same pathway. Thus, our study provides a mechanistic explanation of how parkin represses ERK1/2 activation.

In summary, this study provides evidence that p62 is a substrate of parkin. Parkin ubiquitinates and stabilizes p62 to repress ERK1/2 activation, thereby attenuating 6-OHDA-induced ERK1/2 activation.

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Author contribution

Feng GAO and Guang-hui WANG designed the experiments; Xiao-ou HOU and Jian-min SI performed the main experiments; Hai-gang REN, Dong CHEN and Hong-feng WANG prepared the plasmids; Zheng YING and Qing-song HU contributed analytic tools, Feng GAO and Guang-hui WANG analyzed the data; Xiao-ou HOU drafted the manuscript; and Feng GAO and Guang-hui WANG revised the manuscript.

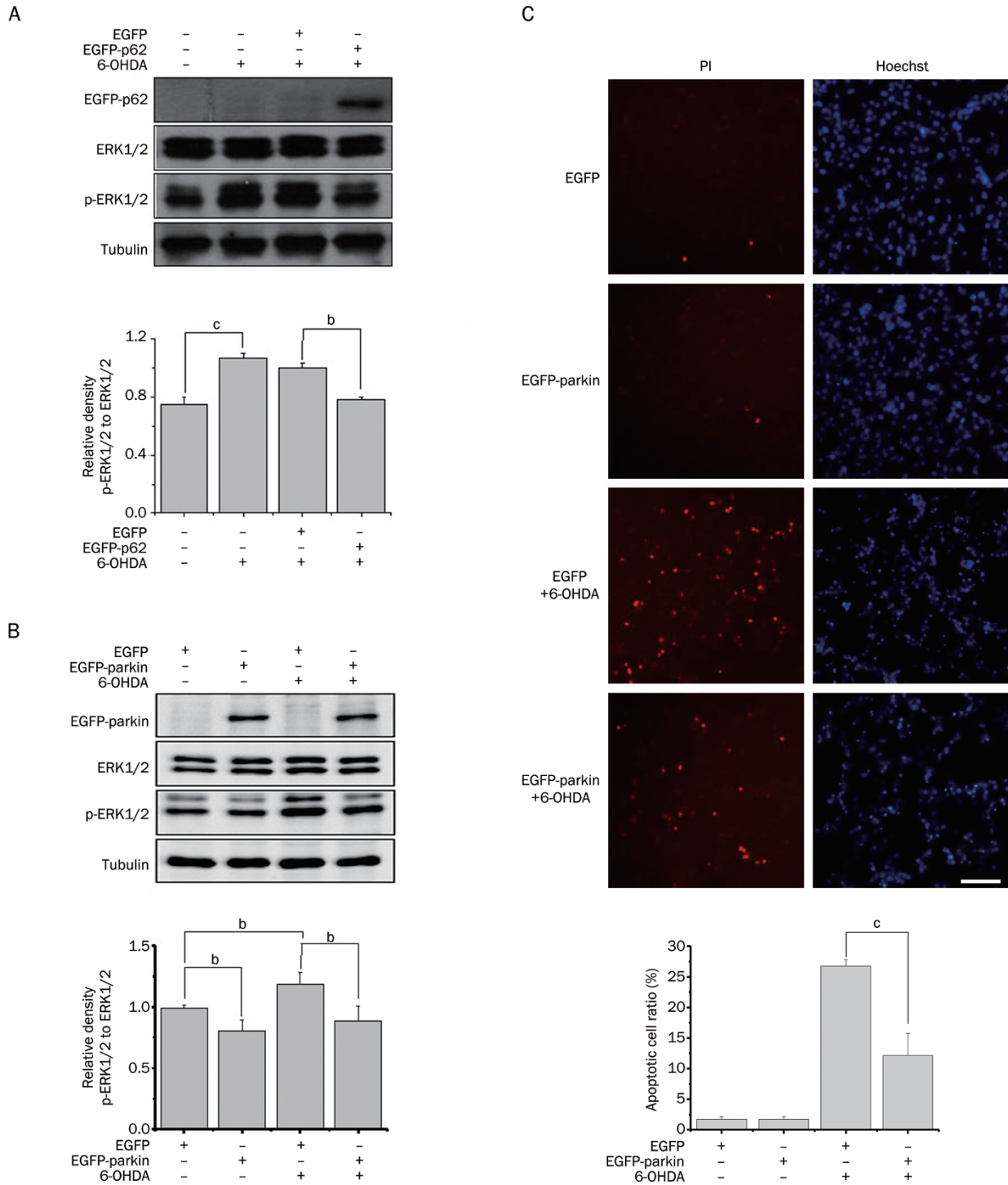


Figure 4. p62 and parkin inhibit 6-OHDA-induced ERK1/2 activation. (A) PC12 cells were transfected with EGFP or EGFP-p62 and then treated with 200 $\mu\text{mol/L}$ 6-OHDA for 24 h. The cell lysates were subjected to immunoblot analysis with the indicated antibodies. The band density of p-ERK1/2 relative to pan-ERK1/2 is shown in the lower panel. The values are the mean \pm SEM from three independent experiments. ^b $P < 0.05$, ^c $P < 0.01$, one-way ANOVA. (B) The cells were transfected with EGFP or EGFP-parkin for 24 h and treated with 6-OHDA for another 24 h. The total cell lysates were subjected to Western blotting analysis with the indicated antibodies. The band density of p-ERK1/2 relative to pan-ERK1/2 is shown in the lower panel. The values are the mean \pm SEM from three independent experiments. ^b $P < 0.05$, one-way ANOVA. (C) SH-SY5Y cells were transfected with EGFP or EGFP-parkin for 24 h and treated with 6-OHDA for another 12 h. The cells were stained with PI (red) and Hoechst (blue). The scale bar represents 20 μm . The percentage of apoptotic cells was calculated and plotted in the lower panel. ^c $P < 0.01$, one-way ANOVA.

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