PINK1 positively regulates HDAC3 to suppress dopaminergic neuronal cell death

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Received September 16, 2014; Revised September 16, 2014; Accepted October 8, 2014

Deciphering the molecular basis of neuronal cell death is a central issue in the etiology of neurodegenerative diseases, such as Parkinson’s and Alzheimer’s. Dysregulation of p53 levels has been implicated in neuronal apoptosis. The role of histone deacetylase 3 (HDAC3) in suppressing p53-dependent apoptosis has been recently emphasized; however, the molecular basis of modulation of p53 function by HDAC3 remains unclear. Here we show that PTEN-induced putative kinase 1 (PINK1), which is linked to autosomal recessive early-onset familial Parkinson’s disease, phosphorylates HDAC3 at Ser-424 to enhance its HDAC activity in a neural cell-specific manner. PINK1 prevents H₂O₂-induced C-terminal cleavage of HDAC3 via phosphorylation of HDAC3 at Ser-424, which is reversed by protein phosphatase 4c. PINK1-mediated phosphorylation of HDAC3 enhances its direct association with p53 and causes subsequent hypoacetylation of p53. Genetic deletion of PINK1 partly impaired the suppressive role of HDAC3 in regulating p53 acetylation and transcriptional activity. However, depletion of HDAC3 fully abolished the PINK1-mediated p53 inhibitory loop. Finally, ectopic expression of phosphomimetic-HDAC3S424E substantially overcomes the defective action of PINK1 against oxidative stress in dopaminergic neuronal cells. Together, our results uncovered a mechanism by which PINK1–HDAC3 network mediates p53 inhibitory loop in response to oxidative stress-induced damage.

INTRODUCTION

HDAC3 belongs to a multimolecular complex that contains the NCoR and SMRT protein subunits, which are required for the physiological action of many nuclear hormone receptors (1,2). In addition, HDAC3 also functions as a corepressor for many sequence-specific transcription factors, including NF-κB, SMAD7 and c-Jun (3–5). Through a physical interaction with these transcription factors, HDAC3 is recruited to specific promoters, where this enzyme induces transcriptional repression through histone deacetylation. On the other hand, HDAC3 also deacetylates non-histone substrates, such as GATA-2, NF-κB, SRY and YY1 (6). For instance, HDAC3 regulates the duration of NF-κB action through deacetylation of RelA and promotion of its interaction with inhibitory-κBα (IκBα), thus leading to nuclear export of NF-κB and termination of NF-κB signaling.
as well as replenishment of the cytoplasmic pool of RelA-IκBα (7). Intriguingly, HDAC3 interacts indirectly with p53 via formation of a complex with the MAGE-A tumor antigen, and this interaction confers resistance to chemotherapeutic agents (8). Moreover, a recent study demonstrated that suppression of HDAC3 expression leads to an increase in p53 acetylation (9). Thus, these findings raised the possibility that HDAC3 directly deacetylates the lysine residues of p53; however, whether HDAC3 regulates p53 function via non-epigenetic regulation has not yet been examined.

Evidence supports a relationship between HDAC3 and apoptosis. Apoptotic stimuli, such as osmotic stress and FAS ligand, trigger the caspase 7-dependent C-terminal cleavage of HDAC3 in mammalian cells and eventually lead to apoptosis induction by inhibition or enhancement of HDAC3 activity (10,11). Although these studies demonstrated the potential engagement of HDAC3 in caspase 7-dependent apoptosis, the opposing effects of caspase 7-mediated cleavage of HDAC3 on its deacetylase activity are somewhat controversial. Furthermore, either pharmacological inhibition or depletion of HDAC3 significantly enhanced growth arrest, p53 levels and apoptosis in human cancer cells (9,12,13). Consistent with this notion, it has been shown that a meta-analysis of a variety human solid tumors showed that HDAC3 may be one of the most frequently upregulated genes in cancer cells (14), suggesting that cancer cells may resist apoptotic cell death, at least in part, through HDAC3-mediated mechanisms. Thus, diverse stimuli may alter the function of HDAC3 via post-translational modifications, which presumably lead to suppression of cellular apoptosis via interference with pro-apoptotic molecules, such as p53. The precise mechanism by which molecules or signaling cascades modulate the anti-apoptotic functions of HDAC3 has not yet been determined.

PTEN-induced putative kinase 1 (PINK1) is a Parkinson’s disease (PD)-associated protein kinase encoded by Park6. Loss-of-function mutations in Park6 are associated with autosomal-recessive and some sporadic forms of familial PD (15). PINK1 contains a unique mitochondrial-targeting motif and plays a role in mitochondrial function, including oxidative stress-induced cell death, mitochondrial calcium signaling and mitophagy (16). Thus, mammalian PINK1 is important for maintenance of mitochondrial function, and this protein protects neuronal cells against both intrinsic and environmental stress (17,18). In addition, PINK1 localizes to the cytoplasm as well as to the mitochondrial fraction (19). Intriguingly, a PINK1 mutant that contains a deletion of the putative mitochondrial-targeting motif was protective against MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced toxicity, suggesting the involvement of an alternative pathway mediated by cytoplasmic PINK1 in protection from neuronal cell death (20). Moreover, PINK1 was shown to suppress neuronal cell death via inhibition of the caspase 3/7-dependent apoptotic pathway (21). Despite the established roles of mitochondrial PINK1, the molecular mechanism by which cytoplasmic PINK1 influences and regulates neuronal cell apoptosis has not been fully elucidated.

In this study, we demonstrate that cytoplasmic PINK1 directly binds to and phosphorylates HDAC3 to enhance its HDAC activity. We also show that PINK1 prevents H2O2-induced HDAC3 cleavage via phosphorylation of HDAC3 at Ser-424. Furthermore, we show that PINK1-mediated phosphorylation of HDAC3 at Ser-424 enhances its association with p53. Finally, we demonstrated that HDAC3 is critical for PINK1-mediated effects on the p53 inhibitory loop. Our data reveal that HDAC3 is required for the antagonistic action of PINK1 against oxidative stress-induced DNA damage.

RESULTS

PINK1 phosphorylates Ser-424 of HDAC3 in the cytoplasm

To gain insight into the functional role of HDAC3 in the regulation of p53-mediated apoptosis, a yeast two-hybrid assay was performed to identify HDAC3-interacting proteins (Supplementary Material, Table S1). Strains encoding PINK1, but not strains harboring the empty vector pGBK7T, displayed a strong interaction with HDAC3 (Supplementary Material, Fig. S1A). Endogenous co-immunoprecipitation (IP) analysis showed that of class I HDACs, only HDAC3 selectively interacts with PINK1 but not the PINK1-interacting proteins, Parkin and DJ-1 (Supplementary Material, Fig. S1B). Importantly, HDAC3 specifically interacted with an intact form of PINK1 (~63 kDa) in the cytosolic fraction but not in the mitochondrial fraction. As expected, HDAC3 was also associated with NCoR in the nuclear fraction (Fig. 1A). GST pull-down analyses demonstrated that the C3 region of PINK1 (402–510 amino acids) robustly interacted with N-terminal of HDAC3 (HDAC3-N2) (106–211 amino acids) (Supplementary Material, Fig. S1C and D). Consistent with recent observations that PINK1 has non-mitochondrial-associated functions (20,22–24), these results strongly suggest a possible role of the PINK1–HDAC3 network in the cytoplasm.

PINK1 is a serine/threonine kinase that phosphorylates several target proteins, including mitofusin and Parkin (25,26). Thus, we next examined whether PINK1 directly phosphorylates HDAC3 protein. An in vitro kinase assay demonstrated that wild-type PINK1 (PINK1WT), but not the inactive and pathogenic PINK1G309D mutant, phosphorylated the C-terminal domain of HDAC3 (Supplementary Material, Fig. S2A). To determine the amino acid residues essential for PINK1-mediated phosphorylation of HDAC3, we used the NetPhosK 1.0 program for sequence analyses to predict seven putative phosphorylation sites in the HDAC3 C-terminal domain. In vitro kinase assays following site-directed mutagenesis showed that mutation of S424A in HDAC3 resulted in the complete loss of phosphorylation, providing evidence that Ser-424 of HDAC3 is critical for PINK1-dependent phosphorylation (Supplementary Material, Fig. S2B).

PINK1-mediated phosphorylation at Ser-424 of HDAC3 was further confirmed by detection of HDAC3 phosphorylation with the synthetic phospho-HDAC3S424 antibody. Phosphorylation of Flag-HDAC3WT, but not mutant Flag-HDAC3S424A, was detected after co-transfection with PINK1WT (Fig. 1B). We verified this finding in PINK1-deficient (PINK1−/−) mouse embryonic fibroblasts (MEFs). Importantly, the HDAC3 levels were similar in both PINK1−/− and PINK1+/+ MEFs; however, HDAC3 phosphorylation levels in PINK1−/− MEFs were greatly reduced compared with PINK1+/+ MEFs (Fig. 1C and Supplementary Material, Fig. S2C). Collectively, these data confirm that HDAC3 Ser-424 is phosphorylated in vivo by PINK1.
Figure 1. PINK1 phosphorylates HDAC3 to enhance its histone deacetylase activity. (A) Cytosolic interaction of PINK1 with HDAC3 was confirmed by co-immunoprecipitation analysis. *, Non-specific band. (B) Validation of PINK1-mediated HDAC3 phosphorylation at Ser-424 in SH-SY5 cells. SH-SY5 cells were transfected with the indicated plasmids. Whole-cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. (C) Genetic deletion of PINK1 significantly diminished the phosphorylation of HDAC3 at Ser-424. Indicated MEFs were fixed and then immunostained with the indicated antibodies. (D) PINK1 enhances the HDAC3 activity via phosphorylation of Ser-424. SH-SY5Y cells were transfected with the indicated plasmids. Whole-cell lysates were immunoprecipitated with anti-Flag antibody, and HDAC3 activity was measured. Error bars, SD (n = 3). (E) Overexpression of wild-type PINK1, but not of pathogenic PINK1 mutants, increased the enzyme activity of HDAC3. Whole-cell lysates were immunoprecipitated with anti-Flag antibody, and then HDAC3 activity was measured. Error bars, SD (n = 3). (F–G) Genetic ablation or knocking-down of PINK1 resulted in a decrease of endogenous HDAC3 activity. The enzyme activity of endogenous HDAC3 proteins in the indicated MEFs and SH-SY5Y cells were analyzed. The level of HDAC3 phosphorylation was analyzed by western blotting. Error bars, SD (n = 3).
PINK1 phosphorylates HDAC3 to enhance its HDAC activity in a neural cell-specific manner

Next, we investigated the effect of PINK1 on HDAC3 function. Intriguingly, we also found that PINK1WT overexpression enhances deacetylase activity of wild-type HDAC3 but not mutant HDAC3S424A. However, PINK1G309D had no effect on wild-type HDAC3 or mutant HDAC3S424A deacetylase activity (Fig. 1D and Supplementary Material, Fig. S3A). PD-related and kinase-dead PINK1 mutants, including K219M, E240K, L347P and L489P, failed to enhance HDAC3 activity and phosphorylation (Fig. 1E and Supplementary Material, Fig. S3B). Furthermore, genetic ablation and knocking-down of PINK1 resulted in decreased enzymatic activity and HDAC3 phosphorylation levels in both MEFs and SH-SY5Y cells, indicating the importance of PINK1-mediated phosphorylation in the enhancement of HDAC3 activity (Fig. 1F and G). In addition, overexpression of PINK1WT selectively increased HDAC3 activity but not the activities of other class I HDACs (Supplementary Material, Fig. S3C). These results collectively suggest that PINK1-mediated phosphorylation is required to enhance HDAC3 HDAC activity.

Because CK2 also phosphorylates the Ser-424 residue of HDAC3 and increase the activity of HDAC3 (27), we next examined whether CK2 and PINK1 function cooperatively or independently to phosphorylate HDAC3 in a tissue-specific manner. To test this, brain lysates and SH-SY5Y cells were treated with either the CK2-specific inhibitor 4,5,6,7-tetramobenzotiazole (TBB) or siRNA against CK2, and enzymatic activity and HDAC3 phosphorylation levels were assessed. Intriguingly, TBB treatment had no effect on the level of HDAC3 phosphorylation and enzyme activity in brain regions from B6 mice. However, TBB treatment of heart, liver and lung lysates significantly reduced HDAC3 phosphorylation and enzyme activity (Fig. 2A and Supplementary Material, Fig. S4A). Strikingly, immunoblotting analysis demonstrated that PINK1 protein is highly expressed in neuronal cells when compare with non-neuronal cells (Fig. 2B). Furthermore, CK2 knockdown efficiently reduced HDAC3 phosphorylation in MCF7, A549 and U2OS cells, whereas the inhibition of CK2 had no effect on HDAC3 phosphorylation in SH-SY5Y and primary neuronal cells derived from mouse brain compared with shPINK1 treatment (Fig. 2C and D). In accordance with above results, we found that knocking-down of PINK1 specifically reduces the HDAC3 activity in SH-SY5Y cells but not A549 cells. As consistently, CK2 knockdown selectively reduced the HDAC3 activity in A549 cells (Fig. 2E and Supplementary Material, Fig. S4B). Collectively, these results suggest that PINK1 phosphorylates HDAC3 to enhance its HDAC activity in a neural cell-specific.

PINK1-mediated HDAC3 phosphorylation prevents caspase 7-dependent HDAC3 cleavage

Accumulating evidence suggests that upon exposure to apoptotic stimuli, C-terminal cleavage of HDAC3 at Glu-391 is crucial for efficient apoptosis induction (10,11). H2O2 treatment induced HDAC3 cleavage and reduced HDAC3 phosphorylation. Importantly, overexpression of PINK1WT, but not PINK1G309D, dramatically reversed the HDAC3 cleavage, activity and phosphorylation, whereas PINK1 knockdown exacerbated the negative effect of H2O2 on HDAC3 function (Fig. 3A). Additionally, overexpression of PINK1WT, but not mutant PINK1G309D, blocked H2O2-induced cleavage of Flag-HDAC3WT but not of the phosphor-mutant HDAC3S424A (Fig. 3B). As expected, the caspase 3/7 inhibitor Z-DEVD completely blocked H2O2-induced HDAC3 cleavage (Supplementary Material, Fig. S5A). It is also noteworthy that the level of an intact form of PINK1 (~63 kDa) is not affected by H2O2 treatment when compared with CCCP and MG132 treatment (Supplementary Material, Fig. S5B). Moreover, overexpression of HDAC3 had no effect on the CCCP-induced accumulation of PINK1 in the mitochondria, indicating that mitochondria are not the target of the PINK1–HDAC3 interaction (Supplementary Material, Fig. S5C). As expected, the endogenous interaction between HDAC3 and PINK1 was greatly diminished by H2O2 treatment (Supplementary Material, Fig. S5D). We further verified the protective role of PINK1 against HDAC3 cleavage and activity in PINK1+/+ and PINK1−/− MEFs (Fig. 3C and Supplementary Material, Fig. S5E). Collectively, these data suggest that PINK1 protects HDAC3 cleavage upon oxidative stress via phosphorylation of HDAC3 at Ser-424.

Protein phosphatase PP4c was previously shown to dephosphorylate HDAC3 at Ser-424 (27). We therefore examined whether PP4c is required for H2O2-induced dephosphorylation of HDAC3, and subsequently decreases HDAC3 activity. siRNA against PP4c significantly enhanced the levels of HDAC3 phosphorylation and activity in SH-SY5Y cells (Fig. 3D). Conversely, PP4c overexpression dosage-dependently reduced the phosphorylation and activity of HDAC3 (Supplementary Material, Fig. S6). Importantly, PP4c knockdown abrogated the H2O2-induced reduction of HDAC3 phosphorylation and enzyme activity as well as the induction of HDAC3 cleavage (Fig. 3E). Finally, PP4c overexpression reversed the positive effect of PINK1 on HDAC3 function, indicating that reversible phosphorylation of HDAC3 at Ser-424 is mediated by PINK1 and PP4c (Fig. 3F).

PINK1 suppresses H2O2-induced p53 acetylation via enhancement of the interaction between phosphorylated HDAC3 and p53

HDAC3 was found to interact with p53 through MAGE-A (8); therefore, we hypothesized that PINK1-mediated phosphorylation of HDAC3 would increase its association with p53, and subsequently decrease p53 acetylation levels. As expected, H2O2 treatment efficiently increased p53 acetylation and stability. However, overexpression of PINK1WT with HDAC3WT, but not with the inactive mutant HDAC3S424A, efficiently suppressed H2O2-enhanced p53 acetylation and stability (Fig. 4A). The interaction between Flag-HDAC3WT and p53 was robustly reduced by H2O2 treatment. Interestingly, PINK1WT overexpression efficiently enhanced the interaction between Flag-HDAC3WT and p53 but not between p53 and mutant HDAC3S424A, leading to p53 hypoacetylation and destabilization (Fig. 4B; lane 4 versus lane 10). We observed a direct interaction between HDAC3 and the DNA-binding domain of p53 by GST pull-down analysis, and HDAC3 directly deacetylated p53 in vitro (Supplementary Material, Fig. S7A and B). The association of wild-type HDAC3 with p53 was not affected by PINK1G309D (Fig. 4B; lane 4 versus lane 6). In addition, wild-type PINK1
substantially reduced the association of p53 with p300 enhanced by H2O2 treatment when compared with PINK1G309D (Fig. 4B). Taken together, these data demonstrate that PINK1-mediated phosphorylation of HDAC3 at Ser-424 enhances its association with p53.

Next, we examined the knocking-down effect of HDAC3 on PINK1-mediated hypoacetylation of p53. As expected, overexpression of PINK1WT efficiently suppressed H2O2-induced p53 acetylation when compared with PINK1G309D. However, depletion of HDAC3 mostly impaired the PINK1-mediated...
Figure 3. PINK1 prevents oxidative stress-induced HDAC3 cleavage via phosphorylation of HDAC3 at Ser-424. (A) PINK1 suppressed H_{2}O_{2}-induced HDAC3 cleavage and reduction of HDAC3 phosphorylation. SH-SY5Y cells were transfected with the indicated sets of plasmids or siRNA against PINK1 and were stimulated with H_{2}O_{2} (10 μM) for 12 h. Error bars, SD (n = 3). Arrow indicates cleaved-HDAC3. (B) PINK1-mediated phosphorylation was required for blockade of HDAC3 cleavage at Glu-391. SH-SY5Y cells were transfected with either wild-type or mutant HDAC3S424A and treated with H_{2}O_{2}. (C) Reconstitution of PINK1 prevents HDAC3 cleavage after H_{2}O_{2} treatment. MEFs were electroporated with the indicated sets of plasmids. (D) PP4c knockdown increased HDAC3 activity and phosphorylation. SH-SY5Y cells were transfected with the indicated siRNA against PP4c. The cell lysates were prepared and subsequently analyzed by western blotting with the indicated antibodies. HDAC3 activity was then measured. Error bars, SD (n = 3). (E) PP4c is required for H_{2}O_{2}-induced dephosphorylation of HDAC3. Error bars, SD (n = 3). (F) PP4c antagonized the positive action of PINK1 in the regulation of HDAC3 activity and phosphorylation. The level of HDAC3 phosphorylation was analyzed by western blotting. Error bars, SD (n = 3).
inhibition of p53 acetylation (Fig. 4C). However, genetic depletion of PINK1 partially decreased the restoration effect of HDAC3 on the inhibition of p53 acetylation when compared with the presence of PINK1, demonstrating the essential role of HDAC3 in PINK1-mediated p53 hypoacetylation (Supplementary Material, Fig. S8A). Intriguingly, we also found that PINK1 suppressed the p53 acetylation and stabilization induced by other DNA damaging agents (Supplementary Material, Fig. S8B).

Next, we examined the role of the PINK1–HDAC3 network in inhibition of the function of p53 via deacetylation of its lysine residues. Overexpression of either PINK1WT or HDAC3WT failed to suppress the transcriptional activity of the acetylation-mimetic mutant p53K320/372/382Q compared with p53WT. As expected, the acetylation-defective mutant p53K320/372/382R had no activity in PUMA gene transcription. We conclude that HDAC3-mediated deacetylation has a critical role in suppressing p53 activity (Fig. 4D). It is also noteworthy that p53 is required for caspase 3/7-dependent cleavage of HDAC3 and the negative action of PINK1/HDAC3 in oxidative stress-induced apoptosis (Supplementary Material, Fig. S8C). Collectively, these data indicate that PINK1–HDAC3 network suppresses the p53 activity via inhibition of p53 acetylation.

HDAC3 is required for maintenance of the PINK1-mediated p53 inhibitory loop

To solidify above findings, we assessed the change of apoptotic events with either a depletion or overexpression of PINK1 and/or HDAC3. Consistently, HDAC3 knockdown completely abolished the inhibitory effect of PINK1 on the transcription of pro-apoptotic genes, Bax and Puma (Fig. 5A). Overexpression

Figure 4. PINK1 suppresses p53 acetylation via enhancement of the association of HDAC3 with p53. (A) PINK1 suppressed p53 acetylation in cooperation with wild-type HDAC3 but not inactive HDAC3. Whole-cell lysates were immunoblotted with the indicated antibodies. Arrow indicates cleaved-HDAC3. (B) PINK1 phosphorylated HDAC3 to enhance the association between HDAC3 and p53. Whole-cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. (C) Knocking-down of HDAC3 abrogated the PINK1-mediated hypoacetylation of p53. Stable shHDAC3-expressing SY-SH5Y cells were transfected with the indicated sets of plasmids and then treated with H2O2. (D) PINK1–HDAC3 network suppressed the p53 activity via inhibition of p53 acetylation. HCT116/p53+/− cells were infected with the indicated sets of plasmids. After 2 days, the cells incubated for 12 h in the presence or absence of H2O2 (100 μM). The levels of indicated genes were analyzed by real-time PCR. Error bars indicate SD (n = 3).
of HDAC3WT partly suppressed the H2O2-induced p53 activation even without PINK1, again verifying that PINK1 is upstream of HDAC3-mediated inhibition of p53 activity (Fig. 5B). Importantly, phosphor-mutant HDAC3S424A failed to suppress the p53 transcriptional activity similar to inactive HDAC3Y298F, verifying the role of PINK1 in the anti-p53 function of HDAC3 (Fig. 5B). p53 mediates transcriptional activation upon binding to the promoter region of its target genes (28). We next explored the possibility that PINK1 suppresses the recruitment of p53 to the promoter region of its target gene in a HDAC3-dependent way. Overexpression of PINK1WT prevents H2O2-induced recruitment of the p53 and p300 to the promoter region of p53-target gene, Bax. However, the PINK1G309D mutant failed to inhibit the H2O2-enhanced recruitment of p53 to the promoter region of Bax (Fig. 5C). Notably, PINK1 failed to suppress the recruitment of p53–p300 complex to the promoter region of Bax, as shown in Fig. 5D. This indicates that PINK1 acts downstream of HDAC3 in the p53 inhibitory loop.

Figure 5. HDAC3 is essential for the PINK1-mediated p53 inhibitory loop. (A) PINK1 failed to overcome the knockdown effect of HDAC3 on p53 activity. Stable shHDAC3-expressing SY-SH5Y cells were transfected with the indicated sets of plasmids. The levels of each gene were analyzed using real-time PCR. Error bars, SD (n = 3). (B) Genetic deletion of PINK1 partially impaired the effects of HDAC3 on p53 activity. The levels of each gene were analyzed using real-time PCR. (C) Restoration of PINK1 reduced the recruitment of p53–p300 complex to the promoter region of the Bax gene. The results of ChIP assay are shown as the percentage of input. Error bars, SD (n = 3; *P < 0.01, **P < 0.001 versus Con + H2O2). (D) Depletion of HDAC3 abrogated the restoration effect of PINK1 on p53–p300 complex binding to the Bax promoter region. Error bars, SD (n = 3; *P < 0.01, **P < 0.001 versus Con + H2O2, #P < 0.01, ##P < 0.001 versus siCon + rPINK1).
Bax gene in the depletion of HDAC3 (Fig. 5D). Collectively, these results demonstrate that HDAC3 is required for PINK1-mediated suppression of p53 function.

To corroborate our findings, we examined the restoration effects of HDAC3 on the reinstatement of PINK1 function in the suppression of oxidative stress-induced DNA damage. Importantly, restoration of shHDAC3-resistant plasmid, shHDAC3 WT, but not both inactive shHDAC3 Y298F and phosphor-mutant shHDAC3 S424A, into HDAC3-deleted SH-SY5Y cells substantially recovered the inhibitory function of PINK1 on H2O2-induced DNA damage (Fig. 6A). Restoration of PINK1 WT, but not PINK1 G309D mutant, into PINK1 WT/− MEFs efficiently reinforced the function of HDAC3 in the inhibition of DNA damage response (Fig. 6B). Importantly, phosphomimetic HDAC3 S424E mutant further overcame H2O2-induced DNA damage compared with HDAC3 WT. Moreover, phosphomimetic mutation at Ser-424 substantially reduced the H2O2-induced HDAC3 cleavage and enhanced the HDAC3 activity when compared with HDAC3 WT, thus verifying our notion that HDAC3 phosphorylation at Ser-424 by PINK1 potentiates its anti-apoptotic function against oxidative stress-induced DNA damage (Fig. 6C and Supplementary Material, Fig. S9A).

Finally, we verified the anti-apoptotic action of HDAC3 against oxidative stress-induced DNA damage in midbrain dopamine (DA) neuronal cells differentiated from neural precursor cells (29,30). Ectopic expression of HDAC3 WT robustly reduced the H2O2-induced DA neuronal cell death when compared with both HDAC3 Y298F and HDAC3 S424A (Fig. 6D and Supplementary Material, Fig. S9B). Furthermore, overexpression of phosphomimetic HDAC3 S424E mutant more efficiently suppressed the H2O2-induced DA neuronal cell death than HDAC3 WT. Similar results were also obtained with neural precursor cells (Supplementary Material, Fig. S9C). These data collectively demonstrated that the HDAC3 is required for anti-apoptotic role of PINK1 against oxidative stress-induced DNA damage in neuronal cells.

**DISCUSSION**

Aberrant p53 function plays a pivotal role in the development of neurodegenerative diseases, as elevated levels of p53 and p53-mediated neuronal cell death are observed in both cellular and animal models of PD (31,32). Therefore, identification of the molecules and/or molecular pathways involved in p53-mediated apoptosis has been of great interest in the field of human neuropathology (33). Both Parkin and DJ-1 have been directly implicated in the suppression of p53 expression and transcriptional activity, respectively (34,35). Until now, most studies about PINK1 highlighted the control of mitochondrial quality and mitophagy (36,37), which is also triggered by overexpression of the p53-target genes, Puma and Bax (38). Therefore, the loss of PINK1 function is thought to result in the accumulation of damaged mitochondria, which propels the cells toward p53-mediated apoptosis by induction of Puma and Bax expression; however, the role of PINK1 in the regulation of p53 expression or transcriptional activity remains sparse.

Recent evidence suggested a unique role for cytoplasmic PINK1 in cell signaling (20,23,39). Moreover, PINK1 is required for optimal insulin-like growth factor-1 and insulin-dependent Akt signal transduction (40). Thus, impaired survival signaling due to a pathogenic mutation of PINK1 may render the dopaminergic neurons vulnerable to stress-induced cell death. In accordance with these findings, we here propose that cytosolic PINK1-mediated survival networking was involved in control of p53-mediated apoptosis. Indeed, we observed that PINK1 specifically interacts with HDAC3 in the cytoplasm but not in the mitochondria fraction. Therefore, we here focus on the cytosolic events mediated by PINK1—HDAC3 networking. The observation that the exclusive interaction between PINK1 and HDAC3 occurred in the cytosol leads to the question of how cytosolic interaction between PINK1 and HDAC3 could affect acetylation of p53. It was previously shown that mutation of Ser-424 residue had no effect on nuclear localization of HDAC3 (27). We concur that the phospho-form of HDAC3 is mostly detected in the nucleus (Fig. 1C, second panel). It may be that PINK1 phosphorylates HDAC3 in the cytoplasm and that phospho-HDAC3 is then further modified or else associated with nuclear import carriers and is translocated into the nucleus, where it interacts with p53. This mechanism is similar to that reported for SMAD2/3 and STAT1/2 (41,42). Further work may identify the additional modifications or the nuclear import carrier proteins.

Importantly, we provide evidence that PINK1-mediated phosphorylation directly affects HDAC3 function. First, PINK1 phosphorylates HDAC3 to enhance its HDAC activity. Both Src and casein kinase 2α (CK2α) are known to phosphorylate HDAC3 and consequently, to increase the deacetylase activity of this enzyme (27,43). In particular, CK2α phosphorylates HDAC3 at Ser-424, the same site as PINK1, and increases HDAC3 activity. We provide evidence that HDAC3 is phosphorylated by PINK1 in a neuronal cell-specific manner. Interestingly, phosphorylation of CK2α displayed no effect on either HDAC3 enzyme activity or its phosphorylation in brain lysates, neuronal cell lines or differentiated dopaminergic neuronal cell, whereas PINK1 knockdown dramatically impaired HDAC3 activity and phosphorylation in these cells. However, we cannot exclude the possibility that each kinase may phosphorylate HDAC3 to modulate the respective downstream events in a signaling-dependent manner because CK2 and PINK1 are involved in different signaling cascades (40,44). Second, caspase 7-dependent cleavage of HDAC3 was shown to be either active or inhibit the function of HDAC3; thus, the effect of C-terminal cleavage on HDAC3 function remains unclear. In this study, we clearly confirmed a negative effect of caspase 7-dependent cleavage on HDAC3 because we demonstrated an antagonistic role of PINK1 in HDAC3 cleavage via phosphorylation of its Ser-424. Third, PINK1-mediated phosphorylation of HDAC3 increased the binding of phosphorylated HDAC3 to p53. This association consequently resulted in the decrease of p53 acetylation and stability. Accumulated evidence indicates that inhibition of HDAC3 enhances p53 acetylation and stability in human cancer and normal cell lines (9,12,13). Consistent with these findings, we observed direct interaction between HDAC3 and p53 and direct deacetylation of p53 by HDAC3 in vitro. These data suggest that HDAC3 directly suppresses p53 activity via its deacetylation. Moreover, a ChIP assay demonstrated that HDAC3 was not recruited to the p53-binding responsive elements of Bax gene. Collectively, these results suggest that HDAC3 represses p53 activity by deacetylation of the p53 protein but not the epigenetic inhibition of p53-mediated transcription. However, since p53
Acetylation does not exclusively reflect p53 stability, further work is necessary to unravel the mechanism by which HDAC3 destabilizes p53 via deacetylation of specific lysine residues.

The most important finding of this study is that HDAC3 knockdown dramatically abolished the inhibitory action of PINK1 on p53-mediated apoptosis. Indeed, PINK1 overexpression failed to overcome the knockdown effect of HDAC3 on p53-mediated DNA damage in both SH-SY5Y. Of note, restoration of wild-type HDAC3 efficiently reinstated the inhibitory function of PINK1 in p53-mediated apoptosis. However, the phosphomutant HDAC3S424A failed to restore the action of PINK1 in the suppression of oxidative stress-induced DNA damage. These results clearly demonstrated that HDAC3 is essential for the PINK1-mediated p53 inhibitory loop via deacetylation of p53.

Taken together, our findings show that the PINK1–HDAC3 signaling network is important for the control of neuronal survival because it attenuates oxidative stress-induced damage. Thus, defects or mutations in PINK1 in PD patients may lead, in part, to an imbalanced decrease of HDAC3 activity and further vulnerability to p53-dependent neuronal apoptosis, which is thought to contribute to neurodegeneration. HDAC inhibitors were previously shown to suppress neurotoxicity (45,46). These results contradict our findings, although it must be taken into account that current inhibitors lack specificity. Although they function in vitro, they have a substantial potential for adverse side effects in vivo. In contrast, inhibitor of HDACs was recently shown to enhance neurotoxicity of DNA damage (47). Moreover, a very recent finding using neural-specific

Figure 6. Restoration of HDAC3 recovered the ineffectiveness of PINK1 in the suppression of p53-mediated DA neuronal cell apoptosis. (A) Restoration of HDAC3 recovered the inhibitory action of PINK1 on oxidative stress-induced DNA damage. DNA damage of cells was determined by Tunnel assay. Error bars, SD (n = 3). (B) Restoration of PINK1 into PINK1−/− MEFs efficiently potentiated the anti-apoptotic action of HDAC3. Both PINK1+/+ and PINK1−/− MEF cells were electroporated with indicated plasmids. DNA damage of MEFs was determined by Tunnel assay. Error bars, SD (n = 3). (C) Phosphomimetic mutation at Ser-424 potentiates the anti-apoptotic activity of HDAC3 and prevents c-terminal cleavage of HDAC3. DNA damage of MEFs was determined by Tunnel assay. Arrow indicates cleaved-HDAC3. (D) Phosphomimetic HDAC3S424E mutant further overcame H2O2-induced DA neuronal cell death compared with HDAC3WT. Error bars, SD (n = 3).
HDAC3 KO mice showed that HDAC3 is essential for proliferation of neural stem or progenitor cells, supporting our notion that activation of HDAC3 may prove to be of therapeutic value neurodegenerative diseases (48). Thus, greater understanding of the role of individual HDAC enzymes in the control of biological events should permit the use HDACi as therapeutic agents.

MATERIALS AND METHODS

Yeast two-hybrid screening
HDAC3 bait plasmids [pGBK7-HD3DO1 (1–371 amino acids of HDAC3) and pGBK7-HD3DO2 (372–424 amino acids of HDAC3)] were transformed into the yeast strain AH109. Transformants containing each bait plasmid were mated with the pre-transformed human HeLa and Testis MATCHMAKER cDNA library (Clontech, Shiga, Japan). Two-hybrid screening was performed according to the manufacturer’s protocol. Plasmids were harvested from the positive clones that grew in minimal media lacking tryptophan, leucine, adenosine, histidine, and β-galactosidase expression. Plasmids were identified by DNA sequencing.

Cell culture, transfection and electroporation
SH-SY5Y cells (ATCC) were cultured at 37°C with 5% CO2 in minimum essential medium (MEM; Welgene, Dalseogu, Daegu, Korea) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. For the electroporation experiments with SH-SY5Y cells (ATCC), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. SH-SY5Y cells (ATCC) were cultured at 37°C with 5% CO2 in minimum essential medium (MEM; Welgene, Dalseogu, Daegu, Korea) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. MEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 15% FBS (GIBCO BRL, Gaithersburg, MD, USA) and subcultured 1:3 upon reaching confluence. For the electroporation experiments into MEF cells, 5 µg of plasmids were electroporated into a suspension of SH-SY5Y cells (1 × 10⁶ cells) using a MicroPorator MP-100 (Invitrogen, Carlsbad, CA, USA) using a single pulse with a voltage of 1100 mV and a width of 50 ms. MEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 15% FBS (GIBCO BRL, Gaithersburg, MD, USA) and subcultured 1:3 upon reaching confluence. For the electroporation experiments into MEF cells, 5 µg of plasmids were electroporated into a suspension of MEF cells (5 × 10⁵ cells) using a MicroPorator MP-100 (pulse voltage = 1650; pulse width (ms) = 20; pulse number = 1). Both HCT116/p53⁺/+ and HCT116/p53⁻/⁻ cells were maintained in DMEM containing 10% FBS. Transfections were performed using Lipofectamine2000 (Invitrogen). The CK2 inhibitor TBB was prepared as a 50 mM stock solution in DMSO (Sigma). PINK1⁺/+ and PINK1⁻/⁻ MEFS were a gift from Dr Hansruedi Bueler, University of Kentucky.

Plasmids
Wild-type, full-length PINK1, HDAC1, HDAC2, HDAC3, HDAC8, HDAC3-N and HDAC3-C constructs were generated by PCR and cloned into the plasmid vectors pSG5-KF2M1-FLAG (Sigma, St. Louis, MO) and pGEX4T-1 (Amersham Biosciences, Amersham, Buckinghamshire, UK). Full-length PP4c clone were acquired from the 21C Human Frontier Human Gene Bank (Daejeon, Korea) and subcloned by inserting the PCR amplification product into pSG5-KF2M1. All plasmid constructs were verified by DNA sequencing.

Production of recombinant lentivirus
Lentiviral production was carried out by transecting 293FT cells, human embryonic kidney cells transformed with SV40 large T antigen. For each 100 mm dish gal/pol plasmid pMDLg/pRRE, envelope pRSV-REV and PMD2.G were transfected with various pLECE3-HDAC3 expressing vectors by using Lipofectamine 2000 (Invitrogen). The medium was changed after 6 h with normal media. The resulting supernatant was collected after 48 h from transfection, filtered through syringe driven filter Unit 0.45 um (Sartorius Stedim Biotech GmbH, Goettingen, Germany, Cat No. 16555) and concentrated by Lenti-X Concentrator (Clontech, Cat No. 6312431).

In vitro kinase assay
Baculovirus-expressed PINK1 and GST-HDAC3 proteins were incubated in the presence of kinase reaction buffer (10 µl 5X kinase buffer, 10 µl magnesium/ATP cocktail solution 90 µl 75 mM MgCl₂/500 mM ATP plus 10 µl [100 µCi] of [γ³²P]-ATP [3000 Ci/mmol]) in a total volume of 50 µl for 30 min at 30°C. Reactions were terminated by washing twice with 1X kinase buffer. Samples were resuspended in 15 µl 5X Sodium dodecyl sulfate (SDS) sample loading buffer and boiled for 5 min. After electrophoresis, SDS polyacrylamide gels were stained with Coomassie blue and dried, and the phosphorylated proteins were visualized by autoradiography or quantified by PhosphorImager analysis.

In vitro deacetylation assay
Human recombinant HDAC3 enzyme was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Flag-p300 proteins were immunoprecipitated from 0.5 mg of HeLa cells nuclear extracts using anti-flag antibodies plus Protein A/G-agarose (Santacruz Biotechnology, Inc., Santa Cruz, CA). Immunoprecipitates were washed twice in phosphate buffered saline containing 0.1% Tween 20 (PBS-T), and once in acetyltransferase assay buffer [50 mM Tris–Cl pH 8, 10% glycerol, 10 mM butyric acid, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT, 1 mM PMSF]. Individual reactions contained immunoprecipitated proteins from GST-p33 with or without rhHDAC3 in 50 µl of assay buffer with 10 µM acetyl CoA (Sigma–Aldrich). Acetylated reactions were incubated for 60 min at 30°C on a rotating platform. Increased amounts of rhHDAC3 proteins were added to acetylated GST-p33 after washing with deacetylation buffer as described in the section of HDAC activity assay. The reaction was stopped by addition of SDS-PAGE sample buffer and electrophoresis through 10% SDS-PAGE gels. Proteins were detected by immunoblotting assay.

Cell fractionation
Briefly, cells were cultured on 100 mm plates, washed twice with ice-cold PBS. Harvested cells were homogenized using a Teflon pestle (Thomas Scientific) in Buffer A [250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF and 20 mM HEPES (pH7.5) containing protease inhibitor cocktail (Roche)], and centrifuged at 1000 g at 4°C for 10 min. The pellets were used as nuclear fractions, and the
postnuclear supernatant fractions were additionally pelleted by centrifugation at 10,000g at 4°C for 10 min to obtain mitochondria. The pellets containing mitochondria were washed with buffer A and resuspended in TXIP-1 buffer [1% Triton X-100, 150 mM NaCl, 0.5 mM EDTA, 50 mM Tris–HCl (pH 7.4)] containing protease inhibitor cocktail. The post-mitochondrial supernatant was centrifuged further for 30 min at 100,000g to obtain cytosolic protein fractions.

**GST pull-down analysis**

All in vitro translations, and labeling with [35S]-methionine, were performed using the indicated expression constructs and the TNT T7 quick coupled transcription/translation kit from Promega (San Luis Obispo, CA). GST pull-down protocols for analysis of protein–protein interaction were described previously (2).

**Chromatin immunoprecipitation (ChIP) assays**

We isolated chromatin as described previously (49). The ChIP assays were then performed with the indicated antibodies essentially as described, but without SDS in all buffers. Sequences of primers for ChIP assays are listed in Supplementary Material, Table S2. All reactions were normalized relative to input activities and are presented as mean ± standard deviation (SD) of three independent experiments. The results are shown as the percentage of input.

**Immunoprecipitation (IP) and antibodies**

A total of 50 μg of whole-cell lysate was immunoprecipitated using protein A/G agarose (Santa Cruz Biotechnology) or Flag-specific M2 agarose (Sigma). Western analyses were performed using following antibodies. The HA, FLAG, TH (tyrosine hydroxylase) and β-actin antibodies were obtained from Sigma-Aldrich. The Myc, acetyl-p53K379 (#2570) and acetyl-p53K320 (#2525) antibodies were purchased from Cell Signaling (Beverly, MA, USA). The tubulin, GAPDH, p300 (N446), and acetyl-p53K182 (06-1283) antibodies were obtained from Millipore (Billerica, MA, USA). The p53 (sc-126), caspase-3 (sc-7148), caspase-7 (SC-56063), anti-p53 (sc-126), HDAC3(C) (sc-11417), PP4c (sc-6118), Sirt1 (sc-74504), Tom22 (sc-101286), Tom20 (FL-145) and anti-PCAF (sc-13124) antibodies were purchased from Santa Cruz Biotechnology, Inc. PARP-1 (51-6639GR) antibody was purchased from BD Transduction Laboratories (Lexington, KY, USA). The antibody against phospho-HDAC3S424 was generated by LabFrontier (Anyang, South Korea) using the synthetic peptide YDGDHHDNDEKpSVEI. The DJ-1 (2391-1), Parkin (1679-1), HDAC3(C) (2246-1) and Bax (1063-1) antibodies were purchased from Epitomics (Burlingame, CA, USA). The PINK1 (BC100-494) antibody was from Novus Biologicals (Littleton, CO, USA). The acetyl-p53K381 (ab61241) antibody was from Abcam (Cambridge, CB, UK). HDAC1, HDAC2, HDAC3(C), HDAC8, TBL1, TBLR1 and NCoR antibodies were described previously (50). Signals of western blotting were detected by a standard enhanced chemiluminescence (Chromogen) method according to the manufacturer’s protocol (Promega, Madison, Wisconsin, USA).

RNA isolation and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Trizol reagent following the standard protocol (TAKARA, Shigoyō-ku, Kyoto, Japan). Then, cDNA was prepared using random hexamer primers (Chromogen). Sequences of primers for RT-PCR analysis are listed in Supplementary Material, Table S2. The concentration of cDNA was normalized using GAPDH. Q-PCR analyses, and quantitation was performed using SYBR Green PCR master mix reagents and an ABI Prism 7700 sequence detection system (Applied Biosystems, Carlsbad, CA, USA). All reactions were performed in triplicate. Relative expression levels and SD values were calculated using the comparative method.

**Small-interfering RNA and lentiviral shRNAs**

For siRNA transfection, SH-SY5Y cells were incubated for 12 h in serum-free MEM and transfected with 200 nM siRNA using Lipofectamine 2000 following the manufacturer’s protocol (Invitrogen). After 4 h, the media was changed, and cells were incubated for 2 days. Sequences of siRNAs are listed in Supplementary Material, Table S2. For stable knockdown of gene expression, two pairs of oligonucleotides that encoded the shRNA against each target were purchased from MISSION shRNA (Sigma-Aldrich). Next, we prepared lentiviral particles using pLKO.1-PURO HDAC3 or PINK1 shRNA via a three plasmid co-transfection following instructions from Invitrogen. SH-SY5Y cells were then transfected with lentivirus. After incubation for 3 days, the lentivirus was collected from the culture media. Lentivirus PURO shRNA was generated as a control.

**HDAC activity assay**

[3H]-labeled acetylated histone H4 tail peptides were prepared as described (28). Immunoprecipitated HDAC3 samples were incubated with [3H-acetate-labeled GST-H4 histone in 150 μl of reaction buffer (20 mM Tris at pH 8.0, 150 mM NaCl, and 10% glycerol) overnight at room temperature. Reactions were quenched with 1 M HCl and 0.16 M acetic acid (50 μl each sample). Released [3H-acetic acid was extracted with 600 μl ethyl acetate by vortexing and centrifugation (1 min at 10,000 rpm). Ethyl acetate supernatants (300 μl from each sample) were quantified by scintillation counting.

**Annexin V-fluorescein isothiocyanate (FITC) staining**

Cells were stained with both annexin V-FITC and propidium iodide according to the manufacturer’s instructions (BD Transduction Laboratories). Alternatively, the cells were fixed in 70% (v/v) ethanol and stained with a solution containing RNase A (50 μg/ml) and propidium iodide (50 μg/ml).

**Immunofluorescence assays and quantification**

Cells were cultured on coverslips. Cells were fixed in 4% paraformaldehyde for 30 min at 4°C and treated with 0.4% Triton X-100 in PBS for 10 min at RT. For detection of endogenous HDAC3, cells were incubated anti-HDAC3 antibody at 37°C for 2 h and stained with a goat anti-rabbit rhodamine (Alexa...
555)-conjugated antibody at 37°C for 1.5 h. The nucleus was detected with Hoechst 33258 staining. Cells were examined using LSM 700 confocal microscope (Carl Zeiss, Munchen-Hallbergmoos, Germany). To quantify the intensity of fluorescence, images were analyzed using the ImageJ® software (http://rsweb.nih.gov/ij/). Briefly, to analyze the individual images for quantification, RGB color images obtained from the confocal microscopy analysis were converted to 8-bit gray-scale images. Mean gray value and integrated density were quantified on each object in images according to the guide instruction of Image J.

Mice experiment
To evaluate the effects of CK2 inhibition, mice were treated with TBB in doses of 10 mg/kg/day by intraperitoneal injection. Each experimental group was tested in a group of five mice. Two weeks after injection, mice were sacrificed, and then tissue lysates were analyzed by immunoblotting. For the measurement of HDAC3 activity, 300 μg of tissue lysates were immunoprecipitated with anti-HDAC3 antibody. HDAC3 activity was measured as described above. Animal studies were performed by obtaining approval according the guidelines of the Institutional Animal Care committee of University of Ulsan College of Medicine.

TUNEL and MTT assay
For the detection of apoptosis in cells, DNA fragmentation was evaluated by TUNEL assay using HT Titer TACS Assay Kit (Trivigen, Gaithersburg, MD, USA, Cat No., 4822-96-K), according to the manufacturer’s instructions. Briefly, the cells were fixed with 3.7% buffered formaldehyde solution for 7 min, washed with PBS, permeabilized with 100% methanol K for 15 min, quenched with 3% hydrogen peroxide, washed with distilled water, labeled with deoxynucleotidyl transferase, and incubated at 37°C for 90 min, and then treated with stop buffer. The cells were incubated with TACS-Sapphire substrate, and the colorimetric reaction was stopped with 0.2 N HCl after 30 min. The colorimetric reaction was measured in a microplate reader at absorbance 450 nm. Cell viability was determined according to the guide instruction of Image J.

Cultures for neural precursor cells (NPCs) and midbrain dopamine (DA) neuronal cells
Primary NPCs prepared from mouse embryonic brains were performed as described previously (29,30). In brief, tissues were obtained from cortex or ventral midbrains of the mouse (C57BL/6, KOATECH, Republic of Korea) embryo at Embryonic day 13.5 (E13.5), mechanically triturated in Ca2+/Mg2+ free Hank’s balanced salt solution (Invitrogen) and seeded at 4–5 dissected tissues on 10 cm culture dishes (Corning, Lowell, MA, USA) pre-coated with poly-l-ornithine (PO, 15 μg/ml, Sigma)/fibronectin (FN, 1 μg/ml, Sigma). NPCs were allowed to proliferate in serum-free N2 + B27 + AA media (DMEM/F12, N2 Supplement (Invitrogen), B27 (Invitrogen) supplemented with basic fibroblast growth factor (bFGF; 20 ng/ml, PeproTech, Minneapolis, MN, USA) and epidermal growth factor (EGF, 20 ng/ml, PeproTech, Rocky Hill, NJ, USA) for 3–5 days. Clusters of expanded precursor cells were dissociated into single cells, plated onto freshly prepared PO/FN-coated 96 wells at 20,000 cells per well, and grown for an additional 1–2 days. Midbrain DA neuronal cell differentiation was induced by withdrawal of mitogens (bFGF and EGF) from the N2+B27+AA media supplemented with dibutyryl cAMP (0.5 mmol/ml, Sigma) and culturing for an additional 8–10 days. Lentiviral transductions were carried out after 8 days from the day of starting differentiation, and the H2O2 (50 μM) was treated.

Statistical analyses
Statistical analyses were performed using a Student’s t-test with Bonferroni correction for multiple comparisons. A P-value of <0.05 was considered statistically significant.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS
We thank Dr Hansruedi Bueler (University of Kentucky, Lexington, KY, USA) for providing the PINK1+/+ and PINK1−/− MEFs.

Conflict of Interest statement. None declared.

FUNDING
This research was supported by Health Fellowship Foundation (K.-C.C.). This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2012R1A6A3A04041010) (H.-K.C.) and by the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MSIP) (no. NRF-2012R1A1A1001862 and NRF-2011-0030086) (H.-G.Y.).

REFERENCES


