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# Loss of PINK1 function decreases PP2A activity and promotes autophagy in dopaminergic cells and a murine model

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ABSTRACT

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. Mutations in PTEN-induced kinase 1 (*PINK1*) are a frequent cause of recessive PD. Autophagy, a pathway for clearance of protein aggregates or impaired organelles, is a newly identified mechanism for PD development. However, it is still unclear what molecules regulate autophagy in PINK1-silenced cells. Here we report that autophagosome formation is promoted in the early phase in response to *PINK1* gene silencing by lentivirus transfer vectors expressed in mouse striatum. Reduced PP2A activity and increased phosphorylation of PP2A at Y307 (inactive form of PP2A) were observed in PINK1-knockdown dopaminergic cells and striatum tissues. Treatment with C2-ceramide (an agonist of PP2A) reduced autophagy levels in PINK1-silenced MN9D cells, which suggests that PP2A plays an important role in the PINK1-knockdown-induced autophagic pathway. Furthermore, phosphorylation of Bcl-2 at S87 increased in PINK1-silenced cells and was negatively regulated by additional treatment with C2-ceramide, which indicates that Bcl-2 may be downstream of PP2A inactivation in response to PINK1 dysfunction. Immunoprecipitation also revealed dissociation of the Bcl-2/Beclin1 complex in PINK1-silenced cells, which was reversed by additional treatment with C2-ceramide, and correlated with changes in level of autophagy and S87 phosphorylation of Bcl-2. Finally, Western blots for cleaved caspase-9 and flow cytometry results for active caspase-3 revealed that PP2A inactivation is involved in the protective effect of autophagy on PINK1-silenced cells. Our findings show that downregulation of PP2A activity in PINK1-silenced cells promotes the protective effect of autophagy through phosphorylation of Bcl-2 at S87 and blockage of the caspase pathway. These results may have implications for identifying the mechanism of PD.



## 1. Introduction

Parkinson's disease (PD) is the most common movement disorder and results from selective and massive loss of dopaminergic neurons in the substantia nigra pars compacta. Recent studies have reported that mutations of several genes, such as PTEN-induced kinase 1 (*PINK1*), are associated with PD pathogenesis (Nuytemans et al., 2010; Cookson and Bandmann, 2010).

PINK1 is a serine/threonine kinase of 581 amino acids that localizes in both the cytosol and mitochondria. More than 40 *PINK1* mutations have been identified in both familial and sporadic PD (Valente et al., 2004; Mills et al., 2008). Moreover, PINK1 immunoreactivity in the brain of PD patients was observed in a small proportion (5–10%) of neuronal inclusions resembling Lewy bodies (Gandhi et al., 2006). Some studies have indicated that PINK1 can protect against PD by maintaining mitochondrial integrity (Lutz et al., 2009; Poole et al., 2008; Mai et al., 2010) or antagonizing oxidative stress (Gautier et al., 2008; Wang et al., 2006) and neurotoxins (Deng et al., 2005; Haque et al., 2008).

Recent studies in cell models have indicated PINK1 is quite related to autophagy induction, a pathway for degradation of abnormal proteins and impaired organelles. Parkin, a substrate of PINK1, is translocated to impaired mitochondria and is associated with mitochondrial clearance (Narendra et al., 2010; Vives-Bauza et al., 2010; Geisler et al., 2010). Furthermore, PINK1 silencing promotes autophagy in dopaminergic cells through effects on oxidative stress and mitochondrial fission (Dagda et al., 2009), which is consistent with our previous findings that silencing PINK1 induces mitophagy through Beclin1 pathway. However, the exact mechanisms of autophagy triggered by PINK1 dysfunction are still unclear.

*Abbreviations:* 3-MA, 3-methyl adenine; LC3, microtubule-associated protein light chain-3; mTOR, mammalian target of rapamycin; PD, Parkinson's disease; PI3K-III, phosphoinositide 3-kinase III; PINK1, PTEN-induced kinase 1; PP2A, protein phosphatase 2A.

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Protein phosphatase 2A (PP2A) exists as a heterotrimer consisting of a 36-kDa catalytic subunit (PP2A/C), a 65-kDa scaffolding subunit (PP2A/A) and a variable regulatory subunit (PP2A/B). Modifications in the catalytic subunit are quite important for the regulation of phosphatase activity. It has been reported that phosphorylation of Y307 in the catalytic subunit results in 90% inactivation of PP2A in vitro, while demethylation of L309 may lead to significant inactivation of the enzyme (Chen et al., 1992; Sontag et al., 2010). PP2A is an important regulator in diverse signaling pathways and is responsible for regulation of multiple events, including cell growth and proliferation, apoptosis, transcription and translation (Frasca et al., 2010; Lao et al., 2007; Liu et al., 2010; Lu et al., 2009; Zuluaga et al., 2007; Switzer et al., 2009). Maintenance of efficient PP2A plays a critical role in the dephosphorylation state of tau or alpha-synuclein in the pathogenesis of neurodegenerative disease such as Alzheimer's disease and PD (Rudrabhatla et al., 2009; Eroglu et al., 2010). Recent studies indicate that PP2A is involved in the regulation of rapamycin-induced autophagy in yeast (Yorimitsu et al., 2009). However, the function of PP2A in PINK1-dysfunction-induced autophagy in mammals and mammalian cell lines is still unclear.

In this study, we demonstrated for the first time that PINK1 knockdown by RNA interference promotes autophagy in dopaminergic MN9D cells and in striatum of adult mice, in combination with reduced PP2A activity. Moreover, C2-ceramide (a PP2A agonist) reverses elevated autophagy levels in PINK1 knockdown MN9D cells. Furthermore, immunoprecipitation revealed that PP2A inactivation induces autophagy through Bcl-2 phosphorylation at S87 and then dissociation of Beclin1/Bcl-2 complex in PINK1 knockdown cells. Finally, active caspase-9 and -3 assays revealed that the protective effect of autophagy on PINK1-silenced cells occurs via downregulation of PP2A activity and blocking of caspase-dependent cell death. Our findings suggest that PP2A is an important negative regulator of autophagy that diminishes the protective role of autophagy in PINK1-silenced cells and mice. This might provide critical insights into the pathogenesis of PD and other neurodegenerative diseases.

## 2. Methods

### 2.1. Plasmids and lentivirus

ShRNA (sh-1#, 5′-GCA CAC TGT TCC TCG TTA TGA-3′; sh-2#, 5′-GGA GCA GTT ACT TAC AGA AGA-3′) targeting specific region of mouse PINK1 (NM_026880.2) and scrambled negative control (sh-con, 5′-GGA TTG ATT CAA CAC GGA AGA-3′) were cloned into pENTR™/H1/TO (Invitrogen). pcDNA3.1–hPINK1 (mammalian expression vector for human PINK1) and pGFP-LC3 were kindly provided by Dr. Changan Jiang (Sichuan University, China) and Dr. Spencer Gibson (University of Manitoba, Canada), respectively.

Lentivirus (LV) gene transfer vectors encoding GFP-shRNA2# (LV-sh-2#) and negative control RNA (LV-sh-con, 5′-TTC TCC GAA CGT GTC ACG T-3′) were constructed by Genechem (Shanghai, China).

### 2.2. Cell culture and transfection

Murine dopaminergic cell line MN9D (provided by Dr. Bastian Hengerer, Novartis AG) were maintained in D-MEM/F-12 (Gibco) supplemented with 10% fetal bovine serum (PAA Laboratories) and 1% (v/v) penicillin–streptomycin at 37 °C under humidified conditions and 5% $CO_2$/95% air in an incubator.

Sh-RNAs were transiently transfected into cells using Lipofectamine 2000 (Invitrogen). The inducible PINK1 deficient MN9D cells were generated as previously reported (Cui et al., 2011). Inducible PINK1 deficient MN9D cells that expression sh-2# were transfected with pcDNA3–hPINK1 for 48 h as rescue group (sh-2#+hPINK1).

### 2.3. Preparation of PINK1 knockdown mice by lentivirus infection

The animal protocol was approved by the Animal Care and Use Committee of Capital Medical University and was consistent with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23). C57BL/6 mice (7 weeks old, male, 18–20 g) were anesthetized with 6% chloral hydrate (6 ml/kg, i.p.). Stereotaxic coordinates for injection in the striatum were: bregma anteroposterior (AP) +1 mm; lateral (L) ±1.8 mm; and ventral (V) −2.7 mm from the dura, with the tooth bar set at 0 mm. Suspensions of GFP-labeled lentiviral vector ($10^6$ TU) in a volume of 2 μl were injected (0.2 μl/min) into the striatum. At the end of the injection, the needle was left in place for 15 min before being slowly removed. The skin was sutured and mice were allowed to recover.

### 2.4. PP2A activity assay

Samples from animal or cells were prepared and the PP2A activity was detected according to the instruction of PP2A Activity Assay Kit (GENMED). For mice model, striatum from lateral or contralateral of injection were extracted at 6 or 12 weeks after LV injection. MN9D cells expressing sh-2# for 48 h and cells from rescue group (sh-2#+hPINK1) were collected. The PP2A activity was measured using a spectrophotometer (Bio-Rad) at 660 nm.

### 2.5. Sample preparation and Western blot

Cells or tissues from mice were lysed in RAPI buffer (50 mM Tris–Cl, pH 7.4, 150 mM sodium chloride, 1% NP-40, 0.1% SDS) with phosphate inhibitor cocktail and protease inhibitor cocktail (Roche). Homogenates were centrifuged at 18,000*g* for 30 min at 4 °C. The protein concentration was determined using a BCA kit (Pierce) according to the manufacturer's instructions.

SDS–PAGE and Western blot were carried out as previously reported (Qi et al., 2007). Primary antibodies used in this study were rabbit anti-β-actin (1:5000, Sigma), rabbit anti-Beclin1 antibody (1:1000, ProteinTech Group), rabbit anti-p-Bcl2(S87) antibody (1:50, Abcam), rabbit anti-Bcl2 antibody (1:1000, Cell Signaling Tech.), mouse anti-caspase-9 p35 (1:1000, Santa Cruz), rabbit anti-LC3 (1:1000, Novus Biologicals), rabbit anti-PINK1 (1:1000; Novus Biologicals), rabbit anti-p-PP2A(Y307) (1:1000, Abcam), mouse anti-PP2A (1:10,000, BD Transduction Laboratories), mouse anti-β-tubulin (1:1000, Sigma). HRP-conjugated secondary antibodies (1:5000) were from Chemicon. The expression of β-actin or β-tubulin in the same membrane was simultaneously determined using the same protocol as an internal reference. Signals were visualized using ECL kit (Pierce). Immunoblots were quantitatively analyzed after scanning of X-ray films using a Gel-Doc 2000 imaging system (Bio-Rad).

### 2.6. Immunoprecipitation

Cell extract (100 μg) from each pretreated MN9D groups were incubated with Beclin1 antibody (3 μg, ProteinTech Group) with constant rotation at 4 °C overnight. Protein G-Sepharose beads (30 μl/tube) was prewashed in IP Buffer (10 mM Tris–Cl, pH 7.5, 150 mM sodium chloride, 2 mM EDTA, 0.5% Triton 100) for 15 min at three times and incubated with protein/antibody mixture with constant rotation at 4 °C for 1 h. The precipitant was collected by centrifugation at 10,000*g* for 1 min and washed three times with IP buffer to remove nonspecifically bound proteins.