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Silent Information Regulator 2 (Sir2) and Forkhead Box O (FOXO) Complement Mitochondrial Dysfunction and Dopaminergic Neuron Loss in *Drosophila* PTEN-induced Kinase 1 (*PINK1*) Null Mutant*[S]

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Background: *PINK1* loss of function induces mitochondrial dysfunction and dopaminergic neuron loss in *Drosophila*.
Results: Sir2 shows a specific genetic interaction with PINK1 and rescues *PINK1* null mutant phenotypes via FOXO.
Conclusion: The strong genetic and functional interactions suggest that Sir2 and FOXO protect mitochondria and dopaminergic neuron downstream of PINK1.
Significance: Understanding the molecular roles of PINK1 will be helpful for deciphering the molecular pathogenesis of Parkinson disease.

PTEN-induced kinase 1 (*PINK1*), which is associated with early onset Parkinson disease, encodes a serine-threonine kinase that is critical for maintaining mitochondrial function. Moreover, another Parkinson disease-linked gene, *parkin*, functions downstream of *PINK1* in protecting mitochondria and dopaminergic (DA) neuron. In our fly genetic screening, knockdown of *Sir2* blocked PINK1 overexpression-induced phenotypes. Consistently, ectopic expression of Sir2 successfully rescued mitochondrial defects in *PINK1* null mutants, but unexpectedly, failed in *parkin* mutants. In further genetic analyses, deletion of *FOXO* nullified the Sir2-induced mitochondrial restoration in *PINK1* null mutants. Moreover, overexpression of *FOXO* or its downstream target gene such as *SOD2* or *Thor* markedly ameliorated *PINK1* loss-of-function defects, suggesting that FOXO mediates the mitochondrial protecting signal induced by Sir2. Consistent with its mitochondria-protecting role, Sir2 expression prevented the DA neuron loss of *PINK1* null mutants in a *FOXO*-dependent manner. Loss of *Sir2* or *FOXO* induced DA neuron degeneration, which is very similar to that of *PINK1* null mutants. Furthermore, *PINK1* deletion had no deleterious effect on the DA neuron loss in *Sir2* or *FOXO* mutants, supporting the idea that Sir2, FOXO, and PINK1 protect DA neuron in a common pathway. Overall, these results strongly support the role of Sir2 and FOXO in preventing mitochondrial dysfunction and DA neuron loss, further suggesting that Sir2 and FOXO function downstream of PINK1 and independently of Parkin.

Parkinson disease (PD)[3] is one of the most common neurodegenerative diseases characterized by movement disorders such as rigidity, tremor, bradykinesia of the limbs, and postural instability. In addition, selective loss of dopaminergic (DA) neurons in the substantia nigra is the neuropathological hallmark of the disease (1). PD probably occurs sporadically as the result of many different environmental factors, but it could also occur genetically by mutations in a number of genes such as α-synuclein (PARK1), *parkin* (PARK2), PTEN-induced kinase 1 (*PINK1* (PARK6)), *DJ-1* (PARK7), and leucine-rich repeat kinase 2 (*LRRK2* (PARK8)) (2–6). Among them, *PINK1*, *parkin*, and *DJ-1* were found to be associated with early-onset autosomal recessive parkinsonism (3–5).

PINK1 is a serine-threonine kinase mainly localized to the mitochondrial membrane via an N-terminal mitochondrial targeting sequence (4). Cells from patients with a *PINK1* mutation demonstrated reduced mitochondrial function when compared with controls (7). Moreover, a *PINK1* deficiency in *Drosophila* resulted in obvious phenotypes that resemble human PD symptoms, such as locomotive defects and loss of DA neuron cells (8–10). Further examination of *PINK1* mutants showed indirect flight muscle degeneration accompanied with severe reduction in ATP levels and mitochondrial mass. In addition, mitochondrial swelling occurred in indirect flight

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[3] The abbreviations used are: PD, Parkinson disease; Sir2, silent information regulator 2; FOXO, Forkhead box O; PINK1, PTEN-induced kinase 1; PTEN, phosphatase and tensin homolog; DA, dopaminergic; TRAP1, TNF receptor-associated protein 1; TH, tyrosine hydroxylase; SOD, superoxide dismutase; DL1, dorsolateral region 1; ANOVA, analysis of variance; *hs*, heat shock; *arm*, *armadillo*.

muscles and DA neurons. Further genetic analysis with the mitochondrial protein Bcl-2 demonstrated that the mitochondrial defect is the main cause of the defective phenotypes in *PINK1* mutants (8).

Because the fly mutants of *parkin*, the most commonly affected PD gene, which encodes an E3 ubiquitin ligase (11–13), showed phenotypes remarkably similar to *PINK1* mutants (14, 15), the phenotypic analysis of these mutants led to the prediction that PINK1 and Parkin act in a common pathway. Indeed, transgenic expression of Parkin dramatically suppressed all *PINK1* loss-of-function phenotypes, but not *vice versa*, establishing that PINK1 and Parkin are linked in a linear pathway in maintaining mitochondrial integrity and function with Parkin acting downstream of PINK1 (8–10). This result initiated further investigation of the roles of PINK1 and Parkin in mitochondria. Recent fly genetics studies clearly demonstrated that these two genes regulate the mitochondrial remodeling process including mitochondrial fusion and fission (16–19). These findings strongly suggested that mitochondrial dysfunction is the key cause of *PINK1-parkin*-related PD pathogenesis and implied that proper maintenance and reestablishment of mitochondrial function are critical to prevent and cure Parkinson disease.

Recently, proteomics analyses found stable complex formation between PINK1 and TNF receptor-associated protein 1 (TRAP1) (20). In *in vitro* kinase assays, PINK1 could directly phosphorylate TRAP1. Subsequent cell biological studies suggested that TRAP1 is a key signaling molecule mediating the cell-protective action of PINK1 under oxidative stress (20). A mitochondrial protease, high temperature requirement A2 (HtrA2/Omi), was also shown to be phosphorylated in a PINK1-dependent manner (21). Further genetic analysis supported that HtrA2 acts downstream of PINK1 independently from Parkin (22). In addition, loss of *Drosophila* phosphoglycerate mutase 5 (PGAM5) gene successfully suppressed mitochondrial degeneration in *PINK1* mutants, but failed to modulate the phenotypes induced by loss of *parkin* (23). Furthermore, up-regulation of DJ-1 can ameliorate *PINK1*, but not *parkin*, *Drosophila* mutants (24). These results suggest that PINK1 can protect mitochondria and cells using other signaling molecules in addition to Parkin.

In the present study, we found that PINK1 genetically interacts with Sir2, the nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylase, which has a neuroprotective action in animal models (25–28). Transgenic expression of *Sir2* successfully rescued mitochondrial defects and ameliorated the PD-related phenotypes in *PINK1* mutants. These effects of *Sir2* transgene were markedly suppressed by the mutations of *FOXO*, the transcription factor downstream of Sirt1, a mammalian homolog of Sir2 (29–31). Further genetic analysis confirmed that the activation of Sir2-FOXO pathway is sufficient to rescue DA neuron loss and indirect flight muscle degeneration induced by *PINK1* deletion, suggesting that Sir2 and FOXO act downstream of PINK1.

EXPERIMENTAL PROCEDURES

Drosophila Strains—The generation of *PINK1*^{B9}, *parkin*^{1}, UAS-*parkin*, and UAS-*PINK1* was described previously (8, 15). FOXO cDNA was subcloned into the pUAST vector and microinjected into w^{1118} embryos. The *FOXO*^{21} and *FOXO*^{25} lines were from E. Hafen. The UAS-*mitoGFP* line was from H. J. Bellen. The UAS-*Sir2* line was from K. T. Min. The tyrosine hydroxylase (*TH*)-GAL4 fly line was a gift from S. Birman. The *armadillo* (*arm*)-GAL4, *eyeless* (*ey*)-GAL4, heat shock (*hs*)-GAL4, UAS-*SOD2*, UAS-*Thor*, *Sir2*^{EP2300}, and *Sir2*$^{2A\text{-}7\text{-}11}$ were obtained from the Bloomington Stock Center. *white*^{GD30033}, *Sir2*^{GD23201}, and *Sir2*^{KK105502} RNAi lines were purchased from the Vienna *Drosophila* RNAi Center (VDRC).

Climbing Assays—Climbing assays were performed as described with some modifications (15). Groups of 15 3-day-old males were transferred into climbing ability test vials and incubated for 1 h at room temperature for environmental acclimatization. After the flies were tapped down to the bottom, the numbers of the climbing flies in 10 s were counted. For each group, 10 trials were performed, and the climbing score (percentage ratio of the number of climbed flies against the total number) was obtained. The average climbing score (± S.D.) was calculated for four independent tests.

Muscle Section and TUNEL Assay—The thoraces from 3-day-old flies were embedded in Spurr's resin and sectioned as described previously (8). The serial sections were then stained with toluidine blue dye and observed with BX-50 microscope (Olympus). For TUNEL assay, apoptosis in the thoraces of 3-day-old flies was detected using the *in situ* cell death detection kit (Roche Applied Science). DAPI (Sigma) was used to visualize the nucleus of muscle. Fluorescence images were obtained by BX-50 microscope (Olympus).

mtDNA PCR and ATP Assay—For mitochondrial DNA (mtDNA) PCR, total DNA from five thoraces of 3-day-old flies was extracted. Then, quantitative real-time PCR was performed as described previously on a Prism 7000 real-time PCR system (Applied Biosystems) (8). Genomic DNA levels of *rp49* were measured for an internal control. Results were expressed as -fold changes when compared with the control. For ATP assay, five thoraces from 3-day-old flies were dissected, and ATP measurement was performed as described previously (8). The relative ATP level was calculated by dividing the measured ATP concentration by the total protein concentration, which was determined by the bicinchoninic acid (BCA) protein assay (Sigma). In mtDNA PCR and ATP assay, average ± S.D. is from three experiments.

Immunostaining—Adult brain was fixed with 4% paraformaldehyde and stained with anti-TH rabbit antibody (1:50, Pel-Freez) as described previously (8). Brains were observed and imaged by LSM 510 confocal microscope (Zeiss) and BX-50 microscope (Olympus). mitoGFP-tagged mitochondria (over 2 μm in diameter) and TH-positive neurons were counted under blinded conditions.

Quantification and Statistical Analyses—For quantification of wing and thorax phenotypes, the percentage of defective thorax and wing phenotypes of 3-day-old males was measured ($n >$ 200). For quantification of DA neurons, dorsolateral region 1 (DL1) clusters from 20 brains of each genotype were observed in a blind fashion to eliminate bias ($n = 40$). To quantify DA cells with enlarged mitochondria, we calculated the percentage of the number of DA cells with mitochondria larger than 2 μm

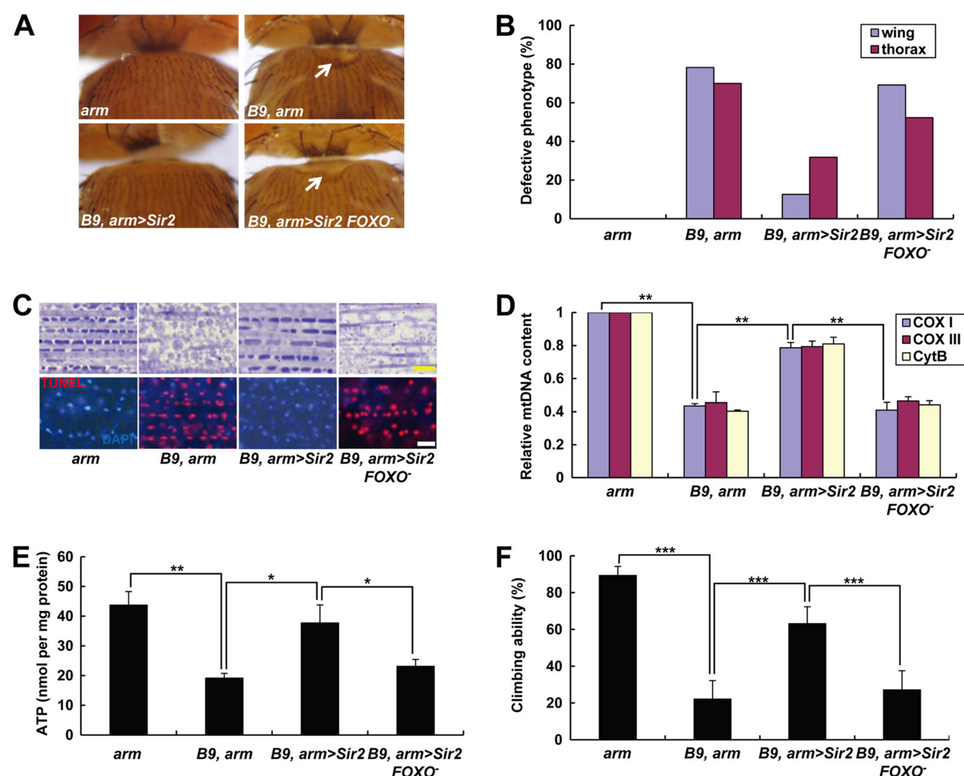

FIGURE 1. **Expression of Sir2 rescues *PINK1* null mutant phenotypes in a *FOXO*-dependent manner.** *A*, light stereo micrographs of the thoraces of *PINK1* null mutants (*B9, arm*), Sir2-expressing *PINK1* null mutants (*B9, arm*>*Sir2*), and Sir2-expressing *PINK1* and *FOXO* double mutants (*B9, arm*>*Sir2, FOXO*$^-$). *arm*-GAL4/+ (*arm*) flies were used as wild type controls. *White arrows* indicate collapsed-thorax phenotypes. *B*, percentage of defective thorax and wing phenotypes. *C*, toluidine blue-stained longitudinal sections (*top panels*) and merged images of TUNEL (*red*) and DAPI (*blue*) staining (*bottom panels*) of indirect flight muscle in the thoraces. *D*, quantification of the mtDNA of thoraces ($n = 3$). *Cox I*, cytochrome *c* oxidase subunit I; *Cox III*, cytochrome *c* oxidase subunit III; *Cyt B*, cytochrome *b*. *E*, comparison of the ATP content of thoraces ($n = 3$). *F*, comparison of climbing ability ($n = 4$). Significance was determined by one-way ANOVA with Bonferroni's correction (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *Error bars* indicate mean ± S.D. *Scale bars*: *yellow*, 5 μm; *white*, 10 μm. Details of all the indicated genotypes in this and other figures are described in the supplemental Experimental Procedures.

in diameter over the total number of DA cells in DL1 clusters from 10 brains of each genotype. To obtain the average percentage of DA cells with enlarged mitochondria, we performed three replicate experiments ($n = 3$). To compare three or more groups, we used a one-way ANOVA with Bonferroni's correction. For two-group comparison, we used two-tailed Student's *t* test. All statistical significance was calculated at $p = 0.05$, using GraphPad Prism 5.

RESULTS

Deletion of *PINK1* induced severe defects in *Drosophila* (8–10). Thoraces were crushed, particularly in the mid-anterior regions (Fig. 1, *A* and *B*). In the indirect flight muscle, mitochondria immensely swelled (Fig. 1*C*), and the levels of mtDNA and ATP were markedly reduced (Fig. 1, *D* and *E*). Locomotor activity was also severely decreased (Fig. 1*F*). In a genetic modifier screening to discover a novel signaling component of the PINK1 pathway, down-regulation of *Sir2* highly suppressed eye size reduction and disrupted ommatidial patterns induced by PINK1 expression using an eye-specific GAL4 driver, *eyeless* (*ey*)-GAL4 (supplemental Fig. 1, *A* and *C*). Moreover, although *PINK1* was deleted, overexpression of Sir2 successfully recapitulated the phenotypes induced by overexpression of PINK1 (supplemental Fig. 1, *B* and *C*). Based on the genetic interactions between these two genes, we tested whether Sir2 expression can ameliorate the various defects caused by loss of *PINK1*. Under the control of heat shock (*hs*)-GAL4, Sir2 expression induced lethality. Thus, we chose *armadillo* (*arm*)-GAL4, which induces weaker gene expression in whole body than *hs*-GAL4, to express Sir2 in *PINK1* null mutants. The crushed thorax and downturned wing phenotypes of *PINK1* null mutants were markedly rescued by Sir2 expression (Fig. 1, *A* and *B*). Muscle sections showed the intact structure of mitochondria in Sir2-expressed *PINK1* null mutants (Fig. 1*C* and supplemental Fig. 2). Moreover, mtDNA content and ATP level in the indirect flight muscle were rescued by Sir2 expression (Fig. 1, *D* and *E*). Sir2-expressing *PINK1* null mutants also showed increased climbing ability (Fig. 1*F*) and no TUNEL signal in the indirect flight muscle (Fig. 1*C*), confirming that Sir2 expression successfully abrogates the muscle degeneration and mitochondrial impairment in *PINK1* null mutants. Collectively, these data demonstrated that Sir2 has an important role in regulating mitochondrial function and integrity downstream of PINK1. In contrast, Sir2 expression could not rescue the defective mitochondrial function and indirect flight muscle structure in *parkin* mutants (supplemental Figs. 2 and 3, *A*–*E*). Moreover, *Sir2* and *parkin* double mutants failed to develop into adult (data not shown), suggesting that two genes downstream of PINK1, Sir2 and Parkin, are involved in different pathways to protect mitochondria.

Recently, intensive genetic analyses and cell biological studies demonstrated that transcription factor FOXO mediates var-

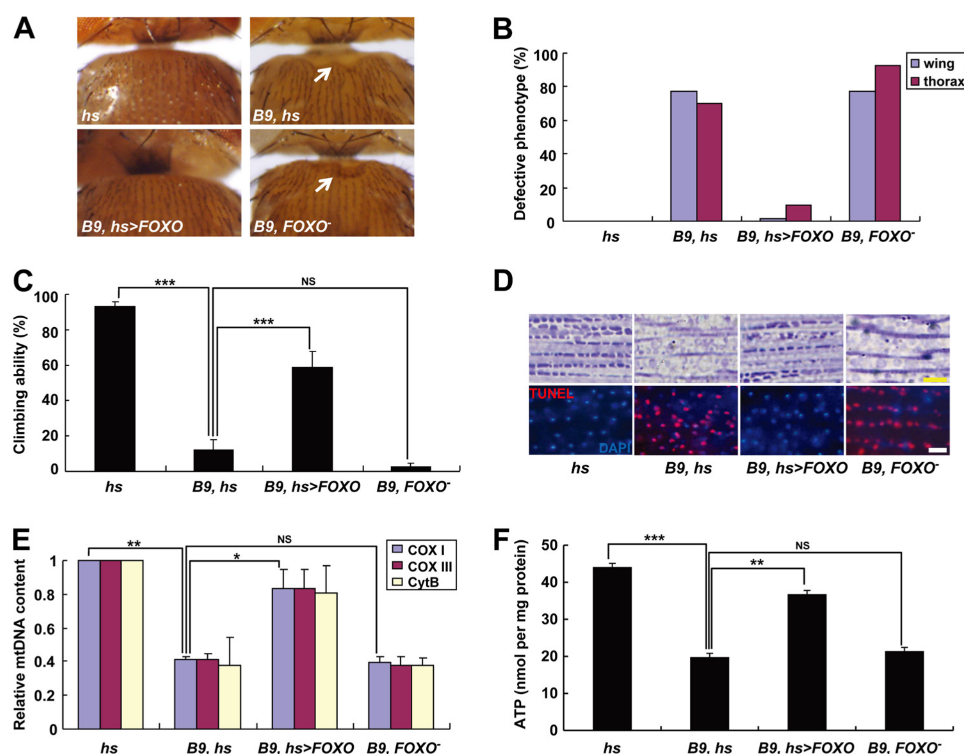

FIGURE 2. **FOXO expression rescues *PINK1* null mutant phenotypes.** *A*, light stereo micrographs of the thoraces of *PINK1* null mutants (*B9, hs*), FOXO-expressing *PINK1* null mutants (*B9, hs>FOXO*), and *PINK1* and *FOXO* double mutants (*B9, FOXO*$^-$). *hs*-GAL4/+ (*hs*) flies were used as wild type controls. *White arrows* indicate collapsed-thorax phenotypes. *B*, percentage of defective thorax and wing phenotypes. *C*, comparison of climbing ability ($n = 4$). *D*, toluidine blue-stained longitudinal sections (*top panels*) and merged images of TUNEL (*red*) and DAPI (*blue*) staining (*bottom panels*) of indirect flight muscle in the thoraces. *E*, quantification of the mtDNA of thoraces ($n = 3$). *Cox I*, cytochrome *c* oxidase subunit I; *Cox III*, cytochrome *c* oxidase subunit III; *Cyt B*, cytochrome *b*. *F*, comparison of the ATP content of thoraces ($n = 3$). Significance was determined by one-way ANOVA with Bonferroni's correction (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *NS*, not significant). *Error bars* indicate mean ± S.D. *Scale bars*: *yellow*, 5 μm; *white*, 10 μm.

ious physiological functions of Sir2. In *Caenorhabditis elegans*, DAF-16, the FOXO homolog in *C. elegans*, is critical in Sir2-induced neuroprotection (26). Sirt1, the mammalian Sir2 homolog, regulates cell survival and stress response signals by deacetylating FOXO (29–31). This close interaction between two genes was also found in genetic analysis using *Drosophila* (32). Therefore, we hypothesized that FOXO mediates the rescue of mitochondrial integrity and function by Sir2 in *PINK1* null mutants. Excitingly, the null mutation of FOXO almost nullified the Sir2-mediated rescue of the defects in *PINK1* null mutants (Fig. 1). Moreover, the phenotypes of *PINK1* null mutants were successfully rescued by expression of FOXO. An almost complete recovery of thorax morphology and wing posture was observed after expression of FOXO in *PINK1* null mutants (Fig. 2, *A* and *B*). In climbing assays, the locomotor activity of *PINK1* null mutants was also rescued by FOXO expression (Fig. 2*C*). Muscle sections showed that FOXO expression ameliorates mitochondria disruption and apoptotic cell death in the indirect flight muscle of *PINK1* null mutants (Fig. 2*D* and supplemental Fig. 2). Further biochemical analysis of the indirect flight muscle showed a marked rescue of the levels of mtDNA and ATP in FOXO-expressed *PINK1* null mutants (Fig. 2, *E* and *F*). Overall, these results clearly demonstrated that FOXO is a critical downstream signaling molecule of Sir2 in regulating mitochondrial integrity and function downstream of PINK1. In addition, we generated and observed *PINK1* and *FOXO* double mutants and found that *FOXO* mutation has no significant detrimental effect on the phenotypes of *PINK1* null mutants (Fig. 2), excluding the possibility that FOXO may play mitochondrial protective roles in PINK1-independent pathways.

FOXO transcription factors regulate crucial cellular processes by inducing expression of various target genes (33). To find a key FOXO target gene in rescuing the mitochondrial defects in *PINK1* null mutants, we checked mRNA expression of FOXO target genes in our *Drosophila* models using quantitative real-time RT-PCR (Fig. 3, *A* and *B*). When compared with the controls, *PINK1* null mutants showed about a 3-fold reduction in expression of the mitochondrial superoxide dismutase *SOD2*, a FOXO target gene involved in stress resistance (Fig. 3*A*) (34). In addition, expression of *Thor*, another FOXO target gene encoding the *Drosophila* 4E-binding protein (4EBP) (35, 36), was reduced 2-fold in *PINK1* null mutants (Fig. 3*B*). This reduction was completely rescued by expression of FOXO (Fig. 3, *A* and *B*). In FOXO-expressing *PINK1* null mutants, gene expression of these two genes increases even more than that of controls (Fig. 3, *A* and *B*). From these results, we hypothesized that SOD2 and Thor are important mediators of the FOXO-induced mitochondrial protection and tested whether expression of them rescues *PINK1* null mutant phenotypes. Excitingly, the downturned wing position and crushed thorax in *PINK1* null mutants were almost completely rescued by ectopic expression of SOD2 or Thor (Fig. 3, *C* and *D*). We also observed markedly increased locomotor activities in *PINK1* null mutants expressing SOD2 or Thor (Fig. 3*E*). mtDNA content and ATP level in the indirect flight muscle were also successfully rescued

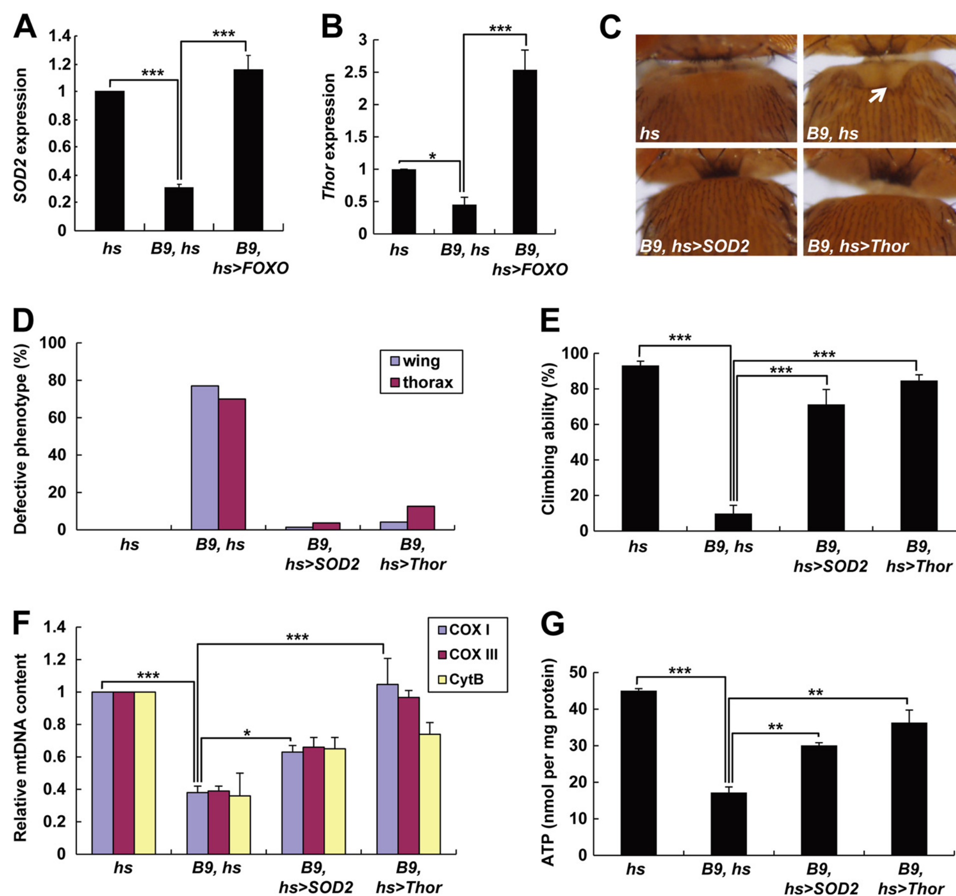

FIGURE 3. **FOXO target genes *SOD2* and *Thor* rescue *PINK1* null mutant phenotypes.** *A*, comparison of SOD2 mRNA level in the thoraces from wild type controls (*hs*), *PINK1* null mutants (*B9, hs*), and FOXO-expressing *PINK1* null mutants (*B9, hs>FOXO*) ($n = 3$). *B*, comparison of Thor mRNA level in the thoraces ($n = 3$). *C*, light stereo micrographs of the thoraces of wild type controls (*hs*), *PINK1* null mutants (*B9, hs*), SOD2-expressing *PINK1* null mutants (*B9, hs>SOD2*), and Thor-expressing *PINK1* null mutants (*B9, hs>Thor*). A *white arrow* indicates collapsed-thorax phenotypes. *D*, percentage of defective thorax and wing phenotypes. *E*, comparison of climbing ability ($n = 4$). *F*, quantification of the mtDNA of thoraces ($n = 3$). *Cox I*, cytochrome *c* oxidase subunit I; *Cox III*, cytochrome *c* oxidase subunit III; *Cyt B*, cytochrome *b*. *G*, comparison of the ATP content of thoraces ($n = 3$). Significance was determined by one-way ANOVA with Bonferroni's correction (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *Error bars* indicate mean ± S.D.

by SOD2 or Thor (Fig. 3, *F* and *G*). These data supported our hypothesis that *SOD2* and *Thor* are critical FOXO target genes in rescuing mitochondrial dysfunction of *PINK1* null mutants.

We previously reported that dopaminergic neurodegeneration, one of the major characteristics of early-onset autosomal recessive parkinsonism patients, is also observed in the brains of *Drosophila PINK1* null mutants (8). Mitochondria-targeted green fluorescent protein showed enlarged mitochondria in the DA neurons of 3-day-old *PINK1* null mutants (Fig. 4*A*). In 30-day-old flies, *PINK1* null mutants exhibited a significant decrease in the number of DA neurons (Fig. 5*A*). In particular, the DA neurons in DL1 showed the most severe mitochondrial enlargement (8). To test the protective role of Sir2 in DA neurons, we expressed Sir2 using DA neuron-specific *TH* GAL4 driver (*TH*-GAL4) (Figs. 4 and 5). In *PINK1* null mutants, about 30% of the DA neuron cells in DL1 cluster contained enlarged mitochondria similar to our previous data (Fig. 4*B*). After Sir2 expression, a 3-fold reduction was observed in the percentage of the DA neurons containing enlarged mitochondria (Fig. 4*B*). These results demonstrated that Sir2 rescues damaged mitochondria in DA neurons as well as the indirect fight muscle as shown in Fig. 1*C*. Moreover, reduction of FOXO gene dosage significantly suppressed the rescue activity of Sir2 in DA neuron, and expression of FOXO target genes also substantially rescued the enlarged mitochondria in DA neurons (Fig. 4). These data indicated that FOXO transcription factor is a critical downstream target of Sir2 in rescuing the damaged mitochondria of the DA neurons in *PINK1* null mutants. In addition, overexpression of Sir2 also rescued the DA neuron loss in a FOXO-dependent manner (Fig. 5). Moreover, *SOD2* or *Thor* transgene successfully prevented DA neuron loss similar to Sir2 (Fig. 5). These results demonstrated that Sir2 and FOXO are critical signaling molecules in rescuing the DA neuron degeneration and mitochondrial defects caused by *PINK1* deficiency.

To further confirm the role of Sir2 and FOXO in DA neuron protection, we examined the number of DA neurons in the DL1 cluster of their *Drosophila* mutants. Interestingly, loss of *Sir2* or *FOXO* induced DA neuron loss similar to that of *PINK1* null mutants (Fig. 6), demonstrating their critical roles in protecting DA neuron. Because these three mutants were very similar in terms of the loss of DA neuron, we performed genetic analysis between them. Notably, deletion of *PINK1* had no detrimental effect on the loss of DA neuron in *Sir2* or *FOXO* mutants (Fig. 6), further supporting the idea that Sir2, FOXO, and PINK1 act in the same pathway in preventing DA neuronal degeneration.

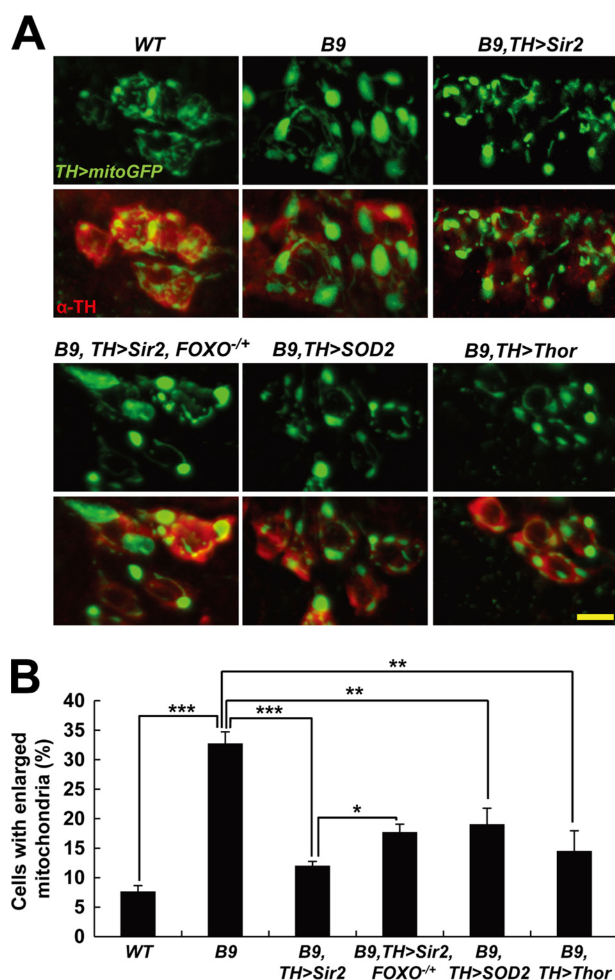

FIGURE 4. **Sir2 and FOXO suppress mitochondrial enlargement in DA neurons of *PINK1* null mutants.** *A*, examination of the mitochondria in DA neurons within the DL1 cluster of adult brain from wild type control (*WT*), *PINK1* null mutants (*B9*), Sir2-expressing *PINK1* null mutants (*B9, TH>Sir2*), Sir2-expressing *PINK1* null mutants with a heterozygous *FOXO* mutation (*B9, TH>Sir2,* FOXO$^{-/+}$), SOD2-expressing *PINK1* null mutants (*B9, TH>SOD2*), and Thor-expressing *PINK1* null mutants (*B9, TH>Thor*). *TH*-GAL4-drived expression of mitochondria-targeted green fluorescent protein (*TH>mitoGFP, green*) showed mitochondrial shape and size in the DA neurons stained with anti-TH antibody (*red*). *B*, graph showing the percentage of the number of DA cells with mitochondria larger than 2 μm in diameter over the total number of DA cells in DL1 clusters ($n = 3$). Significance was determined by one-way ANOVA with Bonferroni's correction (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *Error bars* indicate mean ± S.D. *Scale bars*: *yellow*, 5 μm.

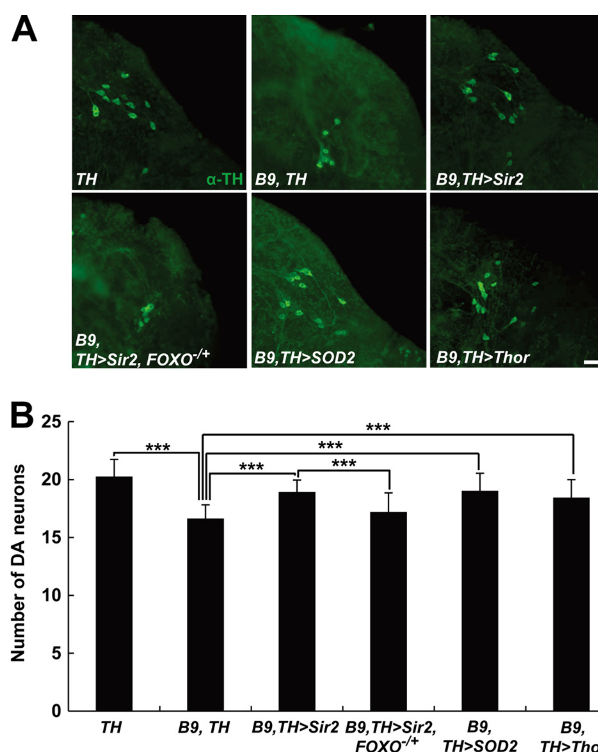

FIGURE 5. **Sir2 and FOXO ameliorate DA neuronal degeneration in *PINK1* null mutants.** *A*, images of the DA neurons within the DL1 cluster of adult brain from wild type control (*TH*), *PINK1* mutants (*B9, TH*), Sir2-expressing *PINK1* null mutants (*B9, TH>Sir2*), Sir2-expressing *PINK1* null mutants with a heterozygous *FOXO* mutation (*B9, TH>Sir2,* FOXO$^{-/+}$), SOD2-expressing *PINK1* null mutants (*B9, TH>SOD2*), and Thor-expressing *PINK1* null mutants (*B9, TH>Thor*). DA neurons were stained with anti-TH antibody (*green*) *B*, graph showing the average number of DA neurons in DL1 clusters ($n = 40$). Significance was determined by one-way ANOVA with Bonferroni's correction (***, $p < 0.001$). *Error bars* indicate mean ± S.D. *Scale bars*: *white*, 20 μm.

DISCUSSION

In the genetic modifier screening and following genetic analyses, we found that Sir2 is a critical mediator of *Drosophila* eye phenotypes induced by PINK1 overexpression (supplemental Fig. 1). Interestingly, in contrast to PINK1, overexpression of Parkin showed normal eye phenotypes (supplemental Fig. 1). Moreover, deletion of *Sir2* had no deleterious effect on *PINK1* null mutants (Fig. 6), but *Sir2* and *parkin* double mutants could not develop into adults (data not shown). These genetic interaction data suggested that Sir2 acts downstream of PINK1 in a Parkin-independent manner. Furthermore, expression of Sir2 successfully rescued the defects in the indirect flight muscle of *PINK1* null mutants (Fig. 1), but failed to rescue defects in *parkin* mutants (supplemental Fig. 3, *A–E*), demonstrating specific interactions between Sir2 and PINK1.

Although Sir2 and Sirt1 shuttle between the nucleus and the cytosol, they have profound effects on mitochondrial functions (37). The specific genetic interaction between *Sir2* and *PINK1* in this study may provide a clue to resolve the cross-talk between mitochondria and Sir2. Also, it raises a question; how does PINK1 signal to Sir2? Because PINK1 localizes in the outer membrane of mitochondria facing the cytoplasm or in the cytoplasm, PINK1 can directly access its cytosolic targets (38). A recent proteomics study reported 13 *in vivo* phosphorylation sites on Sirt1 (39). For example, c-Jun N-terminal kinase 1 (JNK1), casein kinase 2 (CK2), and dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) can phosphorylate Sirt1 and regulate its activity (40–42). Therefore, we suspected that PINK1 may directly phosphorylate and regulate Sir2 in the cytoplasm. However, we have not observed direct evidence for that, such as the protein-protein interaction between two molecules (data not shown). Otherwise, PINK1 may also indirectly signal to Sir2 through its upstream regulators including active regulator of Sirt1 (AROS) or deleted in breast cancer 1 (DBC1) (43–45).

Further genetic analysis identified that FOXO is a critical mediator of Sir2 in rescuing the *PINK1* loss-of-function phenotypes. Deletion of *FOXO* almost completely abrogated the Sir2-induced rescue of mitochondrial defects in *PINK1* null mutants (Fig. 1), and expression of FOXO markedly ameliorated the *PINK1* null mutant phenotypes previously rescued by Sir2 (Fig.

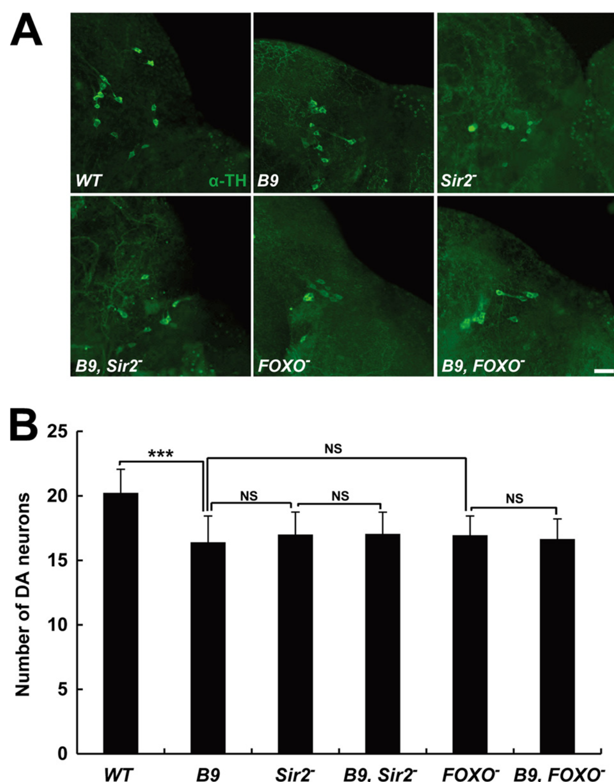

FIGURE 6. **PINK1, Sir2, and FOXO act in same pathway in preventing DA neuron loss.** *A*, images of the DA neurons within the DL1 cluster of adult brain from wild type control (*WT*), *PINK1* null mutants (*B9*), *Sir2* mutants (*Sir2*$^{-}$), *PINK1* and *Sir2* double mutants (*B9, Sir2*$^{-}$), *FOXO* mutants (*FOXO*$^{-}$), and *PINK1* and *FOXO* double mutants (*B9, FOXO*$^{-}$). DA neurons were stained with anti-TH antibody (*green*). *B*, graph showing the average number of DA neurons in DL1 clusters ($n = 40$). Significance was determined by one-way ANOVA with Bonferroni's correction (***, $p < 0.001$; *NS*, not significant). *Error bars* indicate mean ± S.D. *Scale bar*: *white*, 20 μm.

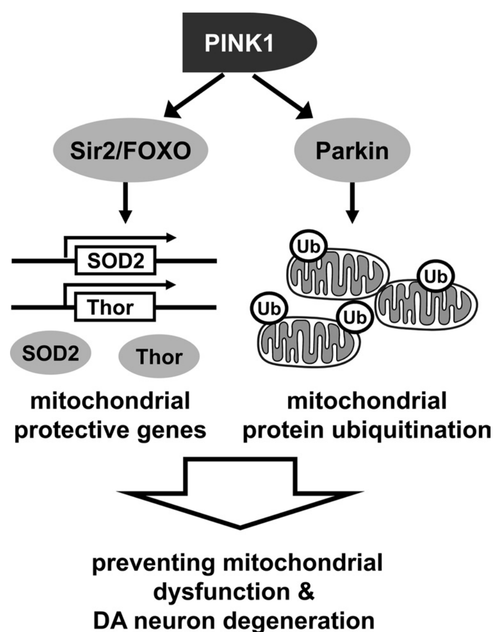

FIGURE 7. **Dual roles of PINK1 in mitochondria protection.** *Ub*, ubiquitin.

2). These strong interactions between Sir2 and FOXO were also observed in recent studies using various animal models and cell lines (26, 29–32). Moreover, *PINK1* null mutants showed a 2- or 3-fold reduction in expression of *SOD2* or *Thor*, key FOXO target genes in mitochondrial protection (Fig. 3). Consistently, Kops *et al.* (34) showed that FOXO3a protects mitochondria through SOD2 during glucose deprivation. In addition, Thor extends *Drosophila* life span by enhancing mitochondrial activity (46), and rapamycin can suppress mitochondrial defects through stimulation of Thor, supporting our finding (47). In contrast, deletion of *parkin* failed to reduce the expression of these two genes (supplemental Fig. 3, *F* and *G*). Furthermore, the decreased expression of *SOD2* or *Thor* was completely recovered by FOXO expression in *PINK1* null mutants (Fig. 3, *A* and *B*), suggesting that FOXO mediates the mitochondria-protective roles of Sir2 in the PINK1 signaling pathway.

Consistent with its role in the indirect flight muscle, Sir2 also can rescue the defective mitochondria in DA neuron and the DA neuron loss in *Drosophila PINK1* null mutants (Figs. 4 and 5). Further genetic analysis revealed that FOXO mediates this DA neuronal role of Sir2 in the mutants (Figs. 4 and 5). Remarkably, loss of *Sir2* or *FOXO* induced DA neuron degeneration very similar to that of *Drosophila PINK1* mutants (Fig. 6). Moreover, deletion of these genes also induced a substantial decrease in climbing ability and ATP level of the indirect flight muscle, especially in 15-day-old flies (supplemental Fig. 4). These data clearly showed that Sir2 and FOXO have protective roles in DA neuron and the indirect flight muscle, tissues with high demand for ATP generated by mitochondria (48). Because these defects were observed in 15- or 30-day-old flies, some may argue that these defects in *Sir2* or *FOXO* mutants result from premature aging induced by loss of two genes. However, Sir2 and FOXO could rescue mitochondrial defects in only 3-day-old flies (Figs. 1 and 2). Moreover, loss of *PINK1* had no additional deleterious effect on the DA neuron loss in *Sir2* or *FOXO* mutants (Fig. 6), strongly suggesting that Sir2 and FOXO act in the same pathway at the downstream PINK1 in preventing mitochondrial dysfunction and DA neuron degeneration.

From these findings, we propose the following model for PINK1-mediated mitochondrial protection (Fig. 7). To protect mitochondria, PINK1 translocates Parkin to mitochondria and activates its E3 ubiquitin ligase activity (49–52). In mitochondria, Parkin ubiquitinates mitochondrial proteins such as voltage-dependent anion channel 1 (VDAC1) and mitofusin (Mfn) to regulate the mitochondrial remodeling process (53–55). In addition to the direct action in mitochondria, PINK1 transduces signals to the cytosol and activates Sir2. Sir2 deacetylates FOXO and induces the FOXO-dependent transcription of mitochondrial protective genes including *SOD2* and *Thor* in the nucleus. The expressed proteins locate to the cytosol or mitochondria and play their roles such as scavenging harmful reactive oxygen species and enhancing production of mitochondrial proteins (34, 46, 47). Through the direct regulation of mitochondrial protein turnover and the induction of mitochondrial protective gene expression, PINK1 can efficiently protect cells from mitochondrial damages.

In summary, we found novel genetic interactions between PINK1 and Sir2-FOXO pathway. In further genetic analyses, Sir2 and FOXO markedly complemented the mitochondrial defects, indirect flight muscle degeneration, and DA neuron loss in *PINK1* null mutants, suggesting that Sir2 and FOXO play

novel mitochondrial protective roles downstream of PINK1. Our findings provide a new perspective to the diverse molecular function of PINK1, which may help the development of more effective treatment strategies for early-onset autosomal recessive parkinsonism and possibly other forms of Parkinson disease.

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