Pink1 regulates the oxidative phosphorylation machinery via mitochondrial fission

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Mutations in PTEN-induced kinase 1 (PINK1), a mitochondrial Ser/Thr kinase, cause an autosomal recessive form of Parkinson's disease (PD), PARK6. To investigate the mechanism of PINK1 pathogenesis, we used the *Drosophila Pink1 knockout (KO)* model. In mitochondria isolated from *Pink1-KO* flies, mitochondrial respiration driven by the electron transport chain (ETC) is significantly reduced. This reduction is the result of a decrease in ETC complex I and IV enzymatic activity. As a consequence, *Pink1-KO* flies also display a reduced mitochondrial ATP synthesis. Because mitochondrial dynamics is important for mitochondrial function and *Pink1-KO* flies have defects in mitochondrial fission, we explored whether fission machinery deficits underlie the bioenergetic defect in *Pink1-KO* flies. We found that the bioenergetic defects in the *Pink1-KO* can be ameliorated by expression of *Drp1*, a key molecule in mitochondrial fission. Further investigation of the ETC complex integrity in *wild type, Pink1-KO, PInk1-KO/Drp1 transgenic, or Drp1 transgenic* flies indicates that the reduced ETC complex activity is likely derived from a defect in the ETC complex assembly, which can be partially rescued by increasing mitochondrial fission. Taken together, these results suggest a unique pathogenic mechanism of PINK1 PD: The loss of PINK1 impairs mitochondrial fission, which causes defective assembly of the ETC complexes, leading to abnormal bioenergetics.

pathology | mitochondrial movement

Ample evidence indicates that mitochondrial dysfunction plays a pivotal role in the development of Parkinson's disease (PD) (1–6). A 30–40% reduction of mitochondrial electron transport chain (ETC) complex I activity was observed in the postmortem brains of idiopathic PD patients (7–11). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone, inhibitors of ETC complex I, induce clinical and pathological manifestations that recapitulate cardinal PD symptoms in humans and in animal models (4, 12–16), supporting the hypothesis that mitochondrial bioenergetic defects contribute to PD pathogenesis.

The significance of mitochondrial dysfunction in the development of PD was further strengthened by the discovery of *PINK1* as the causal gene of PARK6. PINK1 encodes a mitochondrial kinase (17), but its physiological role remains to be elucidated. Reduction or loss of PINK1 causes bioenergetic deficits that include loss of membrane potential, calcium buffering, ATP synthesis rate, and respiration in cell culture systems (18–21). In *Drosophila* and mouse *PINK1-KO*s, decreased ETC complex I mediated respiration and ATP content have been reported (22–24).

ETC complex assembly depends on inner mitochondrial membrane integrity, which is maintained by fusion and fission processes. Fusion and fission regulate the number, size, and morphology of mitochondria in a dynamic manner, and perturbing these processes could affect membrane stability (25). Several key molecules that regulate these delicate processes have been identified: Dynamin-like GTPase (Drp1) is a key molecule in fission (26), whereas mitofusin (Mfn) and optic atrophy 1 (Opa1) play a major role in fusion. Mutations in Mfn 2 and Opa1 result in Charcot-Marie-Tooth neuropathy (27) and autosomal dominant optical atrophy (28), respectively.

Pink1-KO flies have deficits in mitochondrial fission and abnormal mitochondrial morphology, which can be alleviated by one additional copy of *Drp1* (29–31), indicating that the fission machinery contributes to the mitochondrial pathology in flies. Here, we report that the fission machinery defect contributes to bioenergetic deficits in the *Pink1-KO* flies by impairing ETC complex assembly. This finding raised the possibility that these biochemical and mitochondrial dysfunctions in *Pink1* flies are also the basis of human PD patient pathogenesis. It is therefore important to validate these in human patients.

Results

Loss of *Pink1* Impairs Mitochondrial Respiration and ATP Synthesis in *Drosophila*. We have identified respiration and ATP synthesis deficits in *PINK1-KD* PC12 cells, and this deficit could be rescued by wild-type PINK1 but not mutant PINK1 (19). Here, we examined *Pink-KO* flies, which display mitochondrial morphological deficits in fly indirect flight muscles and DA neurons (32–34). We measured respiration and ATP synthesis in mitochondria isolated from *Pink1-KO* and *wild-type* (*WT*) control flies. To account for possible variations in mitochondrial content due to isolation procedures, all of the measurements of respiration, ATP synthesis, and individual complex activities were normalized by the activity of citrate synthase, a nuclear-encoded matrix enzyme of the Krebs cycle. Furthermore, in all experiments, mitochondrial quantity was tested by Western blot analysis with pyruvate dehydrogenase E1 subunit α (Fig. 1*F*, PDH).

We measured complex I respiration driven by malate/pyruvate (Fig. 1*A*) and complex IV respiration driven by TMPD/ascorbate (Fig. 1*B*). Both complex I- and complex IV-dependent respiration in *Pink1-KO* flies mitochondria were significantly reduced compared with *WT* flies. We further used spectrophotometry to measure complexes I and IV enzymatic activities, and found that both were severely compromised in *Pink1-KO* fly mitochondria (Fig. 1 *C* and *D*). As a consequence of defective complexes I and IV mediated oxidative phosphorylation (OXPHOS), a severe deficit in mitochondrial ATP synthesis was detected in mitochondria from Pink1 *KO Drosophila* (Fig. 1*E*). Importantly, these results demonstrate that the decline in steady-state ATP levels, previously reported in these flies (32–34), originates from mito-

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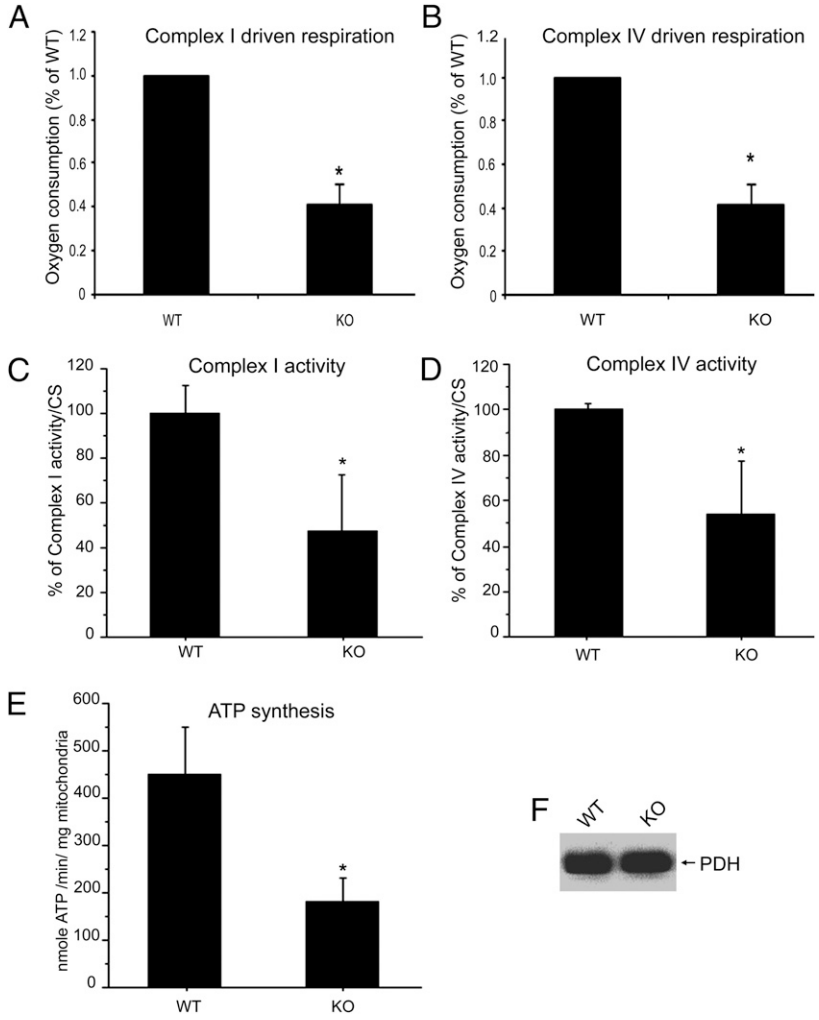

Fig. 1. Loss of *Pink1* impairs OXPHOS function in *Drosophila*. *Pink1*-KO flies have defects in complexes I and IV driven respiration, and reduced complexes I and IV specific activities. (*A*) Complex I-dependent respiration with pyruvate/malate as substrates. Compared with *the WT* control, a statistically significant deficit in O_2 consumption was detected in the *Pink1-KO* fly (58.6% reduction, $n = 4$, $P = 0.007$, Student's *t* test). (*B*) Complex IV dependent respiration with TMPD/ascorbate as substrates. Compared with the *WT* control, a statistically significant deficit in O_2 consumption was detected in the *Pink1-KO* fly (58.3% reduction, $n = 4$, $P = 0.007$, Student's *t* test). In the spectrophotometric measurement of complex I (*C*) and complex IV (*D*) activities, the *Pink1-KO* fly had a 53% reduction in complex I ($n = 8$; $P = 0.0001$; ANOVA) and 47% reduction in complex IV ($n = 8$; $P = 0.0001$; ANOVA). (*E*) Measurement of ATP synthesis with pyruvate/malate as substrates revealed a significant reduction in ATP synthesis (60% reduction; $n = 19$; $P = 0.001$; ANOVA) in the *Pink1-KO* fly. All of the measurements were normalized by citrate synthase activity. (*F*) PDH was used as a mitochondrial quantity control. Equal amounts of mitochondria were used for all experiments. Asterisks indicate statistical significance.

chondria, and is due to defective ATP synthesis rather than increased ATP consumption.

Bioenergetic Deficits in *Pink1-KO* Flies Can Be Partially Rescued by Drp1. Because *Drp1* can rescue mitochondrial fission and morphological abnormalities in *Pink1-KO* flies (29–31), we examined whether the ATP synthesis deficit (Fig. 1*E*) could also be alleviated by *Drp1*. We compared mitochondrial ATP synthesis rates by using the complex I substrates malate and pyruvate in mitochondria isolated from *WT, Pink1-KO,* and *Pink1-KO* with one extra copy of *Drp1 (Pink1-KO/Drp1*; ref. 29), and transgenic flies with one extra copy *of Drp1 (Drp1-Tg*; ref. 29). *Pink1-KO/Drp1* restored the ATP synthesis rate to ≈80% of *WT* (Fig. 2*A*), whereas *Drp1-Tg* itself had no effect on ATP synthesis. Next, we examined if the enzymatic defects of complexes I and IV in *Pink1-KO* flies could be rescued by *Drp1*. We measured the specific activities of complex I and IV by spectrophotometry in mitochondria. Consistent with the changes in ATP synthesis, *Drp1* expression rescued complex I and IV activity deficits in *Pink1-KO* flies (Fig. 2 *B* and *C*). Western blots of the soluble matrix enzyme complex PDH demonstrated that similar amounts of mitochondrion were used for the measurements in Fig. 2 *A–C* (Fig. 2*D*).

Assembly of ETC Complex Is Compromised in *Pink1-KO* Flies due to Defective Fission. Faulty mitochondrial fission in *Pink1-KO* flies could disrupt the assembly of ETC complexes in the inner mitochondrial membrane. Therefore, we investigated whether the assembly of ETC complex I is compromised in *Pink1-KO*, and whether this assembly deficit can be rescued by enhancing fission machinery activity. We performed blue native gel electrophoresis (BN-PAGE), followed by Western blot analysis with an antibody that recognized complex I NDUFS3 subunit, to examine the integrity of complex I in *WT*, *Pink1-KO, Pink1-KO/Drp1,* and *Drp1-Tg* flies. The amount of assembled complex I, normalized by PDH, in the *Pink1-KO* was only 40% of *WT* (Fig. 3 *A* and *B*). This reduction was significantly improved to 60% of *WT* by the additional copy of *Drp1*. We also examined the amount of the complex I by BN-PAGE with Coomassie blue staining. The results of these analyses (Fig. 4 *A* and *B*) are consistent with the one shown in Fig. 3 *A* and *B*. In addition, we examined the amount of individual

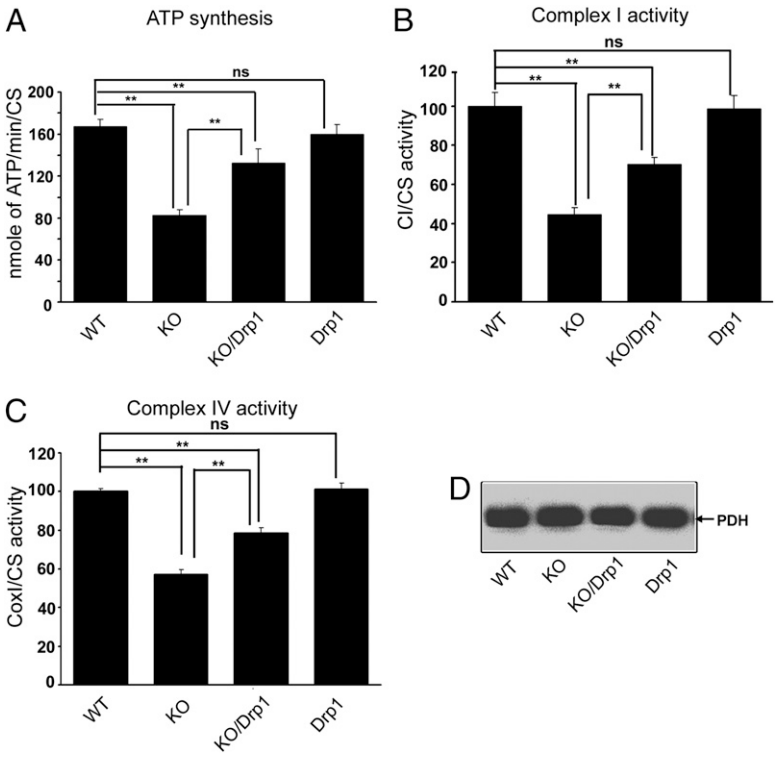

Fig. 2. *Drp1* ameliorates bioenergetics defects in the *Pink1-KO*. An extra copy of *Drp*1 partially rescued ATP synthesis and ETC complex activity deficits in *Pink1-KO* fly. Mitochondria isolated from *WT*, *Pink1-KO (KO)*, *Pink1-KO/Drp1(KO/Drp1)*, and *Drp1-Tg* (*Drp1*) flies were subjected to the following analyses: (*A*) Pyruvate/malate driven ATP synthesis by luciferase assay. ATP synthesis deficits in the *Pink1-KO* can be rescued by *Drp1* (n = 6, P = 0.0011; ANOVA). ATP synthesis in the *Drp 1-Tg* did not change compared with the *WT* (n = 6, P = 0.856; ANOVA). *WT*: 167.12 ± 6.82 (SEM); *Pink1-KO*: 82.34 ± 5.99 (SEM); *Pink1-KO/Drp1*: 132.74 ± 13.21 (SEM); *Drp1-Tg*: 159.78 ± 9.63 (SEM). (*B*) Complex I activity measured by spectrophotometry. Complex I activity deficits in the *Pink1-KO* flies were alleviated by *Drp1* (n = 6, P = 0.003; ANOVA). Complex I activity in *Drp1*-transgenic flies was not different from that of *WT* control flies (n = 6, P = 0.459; ANOVA). *WT*: 99.99 ± 6.67 (SEM); *Pink-KO*: 44.50 ± 3.56 (SEM); *Pink-KO /Drp1*: 70.27 ± 3.48 (SEM); *Drp1-Tg*: 98.59 ± 6.66 (SEM). (*C*) Complex IV activity measured by spectrophotometry. The results indicate that the reduced complex IV activity observed in *Pink1-KO* flies was rescued by one extra copy of *Drp1* (n = 6, P = 0.0001; ANOVA). The *Drp1-Tg* fly showed similar complex IV activities as *WT* fly (n = 6, P = 0.406; ANOVA). *WT*: 100 ± 1.41 (SEM); *Pink1-KO*: 57.06 ± 2.59 (SEM); *Pink1-KO/Drp1*: 78.46 ± 2.72 (SEM); *Drp1-Tg*: 101.22 ± 3.28 (SEM). (*D*) Equal amounts of mitochondria were used for the experiments shown in *A*–*C* as demonstrated by identical PDH content in all samples (SEM). Asterisks indicate statistical significance.

complex I NDUFS3 subunit in *WT*, *Pink1-KO, Pink1-KO/Drp1,* and *Drp1-Tg* fly by SDS/PAGE. The amount of the NDUFS3 subunit was significantly reduced in *Pink-KO* and was not rescued in *Pink1-KO/Drp1* (Fig. 3 *C* and *D*). Therefore, the increased amount of assembled complex I in *Pink1-KO/Drp1* (Fig. 3 *A* and *B*) is likely a result of a higher complex I assembly efficiency, and not of increased subunit biosynthesis.

To further test the impact of defective fission on mitochondrial respiration, we investigated whether other OXPHOS complexes were affected in *Pink1-KO*. Because of the lack of available antibodies, we were unable to examine *Drosophila* complex IV integrity. However, we were able to use the F1 α subunit antibody to analyze the complex V assembly. Typically, incomplete assembly of complex V is reflected by a decrease of fully assembled complex V accompanied by an increase of unassembled F1 sub-complex or F1 α monomers (35). BN-PAGE blots containing mitochondrial proteins from *WT*, *Pink1-KO, Pink1-KO/Drp1,* and *Drp1-Tg* were probed with F1 α subunit antibody. The α subunit was found in fully assembled complex V (≈660 kDa), F1 sub-complex (≈400 kDa), and as monomers (55 kDa; Fig. 5*A*). We verified that the 660-, 400-, and 55-kDa bands are associated with α subunit, rather than spurious bands on the BN-PAGE/Western by 2D BN-PAGE/SDS/PAGE analyses (Fig. S1). Total amount of F1 α subunit was similar in all genotypes (Fig. 5*B*). The amount of fully assembled complex V was reduced to 84.2% of WT in the *Pink1-KO*, and this reduction can be rescued to the level of WT by the expression of Drp1 (Fig. 5 *A* and *C*). The reduction of fully assembly complex V observed by Western blot with anti-Vα antibody is consistent with that of BN-Native/Coomassie blue analysis (Fig. 4 *A* and *C*). Interestingly, the amount of α subunit monomer was significantly increased in the *Pink1-KO* compared with *WT*, and this increase was reversed by Drp1 (Fig. 5*C*). These results indicate that complex V assembly in the *Pink1-KO* is less efficient than in the *WT* and that this deficit can be partially rescued by Drp1.

Discussion

Here, we describe two unique PINK1 pathogenic mechanisms: (*i*) Loss of PINK1 causes a defective assembly of the OXPHOS complexes, which leads to impaired mitochondrial OXPHOS. (*ii*) Enhancing mitochondrial fission ameliorates OXPHOS machinery assembly and mitochondrial functional defects.

We observed in vivo that loss of Pink1 leads to impaired ETC complex IV function, and confirmed previous observations by us and other groups that Pink1-*KO*s also have ETC complex I defects (19, 22–24). These data provide strong support to the notion that mitochondrial OXPHOS defects are integral parts of PD pathogenesis. It is important to note that deficits in ETC complex I were reported in human PD patients, as well as in pharmacological mouse models of PD (4, 12–16), and deficits in ETC complex IV were found in an α-synuclein transgenic mouse model of PD (36).

Most importantly, our findings reveal that defective OXPHOS complex assembly underlies the impaired OXPHOS function. It will be interesting to investigate whether this mechanism is also true in human patients with *PINK1* mutations.

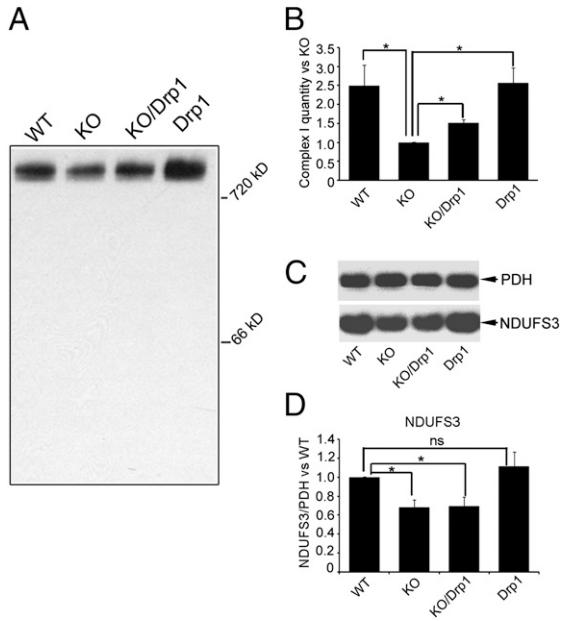

Fig. 3. Drp1 promotes complex I assembly in the *Pink1-KO* fly. Mitochondria isolated from *WT*, *Pink-KO*, *Pink-KO/Drp1*, and *Drp1-Tg* were subjected to BN-PAGE, transferred onto PVDF membrane, and probed with anti-NDUFS3 antibody. (*A*) Comparison of complex I integrity in four genotypes *of* flies. (*B*) The above experiment was repeated three additional times and quantified by densitometry. The total amount of complex I in *Pink1-KO* mitochondria was 40% of that in *WT* ($n = 4$, $P = 0.0097$; ANOVA). The additional copy of *Drp1* increased complex I assembly in *Pink1-KOs* (60% of that in *WT*, $n = 4$, $P = 0.0009$; ANOVA). There was no change in complex I between *WT* and *Drp1* transgenic flies ($n = 4$, $P = 0.87$; ANOVA). The amount of complex I was normalized to PDH. (*C*) NDUFS3 subunit (*Lower*) is degraded in *Pink1-KO* and *Pink-KO/Drp1*. The same blots used for probing with NDUFS3 antibodies were stripped and subsequently probed for PDH E1α subunit antibody for normalization (*Upper*). (*D*) Quantification of nine independent assays of NDUFS3 normalized to PDH. There was a statistically significant reduction of NDUFS3 in the *Pink1-KO* (31.8% reduction, $n = 9$, $P = 0.028$; ANOVA) and in *Pink1-KO/Drp1* (30.6% reduction, $n = 9$, $P = 0.0347$; ANOVA) compared with that of *WT* control. Complex I quantity is the same between *WT* and *Drp1* transgenic flies ($n = 9$, $P = 0.42$; ANOVA). Asterisks indicate statistical significance.

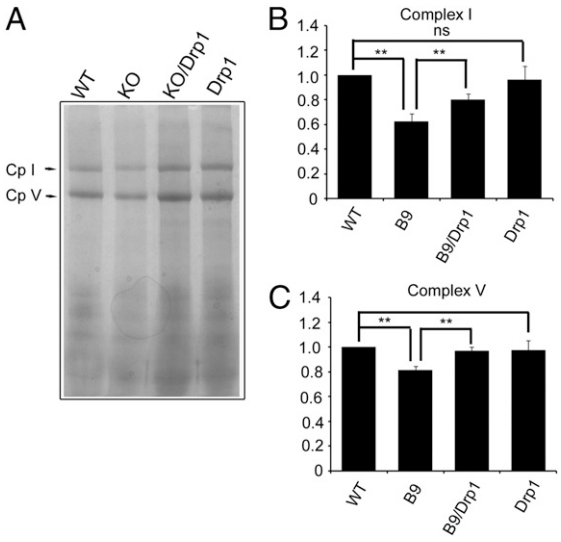

Fig. 4. Drp1 promote complexes I and V assembly. Mitochondrial proteins from four genotypes of flies were subject to blue BNGE, followed by Commassie blue staining as shown in *A*. Complex I (Cp I) and V (Cp V) are as indicated. (*B*) Statistical analysis for complex I assembly shown in *A*. Complex I assembly is reduced in the Pink1 *KO* (38% reduction; $n = 5$; $P = 0.0026$; ANOVA), and this reduction can be partially rescued by *Drp1* ($n = 5$; $P = 0.03$; ANOVA). (*C*) Statistical analysis of complex V in *A*. Complex V is reduced in the Pink1 *KO* ($n = 5$; $P = 0.013$; ANOVA) and can be recovered by *Drp1*(19% reduction; $n = 5$; $P = 0.008$; ANOVA). Asterisks indicate statistical significance.

This study further revealed that OXPHOS complex assembly and function is modulated by mitochondrial dynamics and involves the fission machinery. In this case, Drp1 partially rescued the deficits of OXPHOS assembly and function. It is possible that Drp1 promotes mitochondrial fission, which contributes to "regenerating" functional competent mitochondria through redistribution of mitochondrial DNA, RNA, and proteins (25). OXPHOS complex assembly could be improved by a "rejuvenated" population of mitochondria in the *Pink1-KO/Drp1*. Alternatively, Drp1 could achieve its beneficial effect by promoting the clearance of damaged mitochondria through processes such as autophagy and mitophagy. This possibility is supported by recent evidence that *Pink1* was implicated in the clearance of damaged mitochondria (37–41). It will be interesting to investigate how PINK1 affects mitophagy in vivo, and how mitochondrial dynamics influences mitophagy.

Based on our data, we propose a working hypothesis involving a bifurcated pathway for PINK1 pathogenesis as follows: The direct path may involve reduced or lack of phosphorylation of OXPHOS complex subunits by PINK1, which impairs OXHOS complex assembly and function. The indirect path may involve Drp1 and other intermediates that are important for mitochondrial membrane stability and dynamics, thereby affecting OXPHOS complex assembly and function. With the assays and system we have established, these pathways can now be dissected in greater molecular details, especially with the power of *Drosophila* genetics. Furthermore, if these findings are validated in

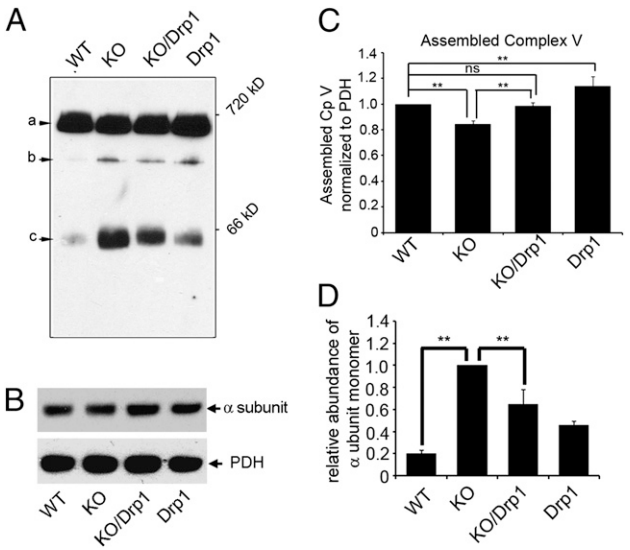

Fig. 5. Complex V assembly in *Pink1-KO* flies. Mitochondria from *WT*, *Pink1-KO*, *Pink1-KO/Drp1*, and *Drp1-Tg* flies were subjected to BN-PAGE (*A*) or SDS/PAGE (*B*), followed by Western blot analysis with anti-complex V α subunit antibody. (*A*) Three bands were detected: assembled complex V (a, ≈600 kDa), F1 subcomplex (b, ≈400 kDa) and α subunit monomer (c, 55 kDa). (*B*) The total amount of α subunit was the same among all of the flies (*Upper*). PDH was used as a mitochondrial quantity control (*Lower*). (*C*) The amount of fully assembled complex V (band *a*, normalized to PDH) was reduced significantly in the Pink1 *KO* (15.8%; $n = 9$; $P = 0.0025$; ANOVA), and this reduction can be recovered by the expression of Drp1 ($n = 9$; $P = 0.009$; ANOVA). (*D*) The amount of α subunit monomers in Fig. 4*A* was normalized by PDH. Compared with *WT, Pink1-KO* flies had significantly increased monomeric α subunit ($n = 3$, $P < 0.001$; ANOVA), which was reversed by additional expression of Drp1 ($n = 3$, $P = 0.0075$; ANOVA). Asterisks indicate statistical significance.

human PD patients, the knowledge of this aspect of pathogenesis is important for designing therapeutic strategies.

Materials and Methods

Fly Stock. *Pink^{B9}* fly stocks, previously identified as a *Pink1* knockout flies, were used in the experiments with a revertant fly stock *Pink1^{RV}* as a *WT* control (33). The *Pink1* knockout alleles are maintained over FM6 balancer. Male *Pink1* knockout flies without FM6 were isolated and used for all of the experiments. All of the flies were maintained at 25 °C. *Drp1-Tg* and *Pink1-KO/Drp1* flies were created and maintained as described (29).

Isolation of Fly Mitochondria. Mitochondria were isolated from 10- to 12-d-old flies as described (42). Briefly, 200 flies were gently homogenized in 1 mL of chilled mitochondrial isolation buffer (250 mM sucrose, 10 mM Tris at pH 7.4, and 0.15 mM $MgCl_2$) with a dounce homogenizer. The homogenate was then centrifuged twice at 1,000 × *g* for 5 min at 4 °C to remove the debris. The supernatant was further centrifuged at 13,000 × *g* for 10 min, and the pellet containing mitochondrial was isolated. The concentrations of mitochondria were determined by DC protein assay kit (Bio-Rad).

Measurements of O_2 Consumption and Complexes Activity. Oxygen consumption from isolated fly mitochondria was measured with a Clark-type polarographic electrode at 27 °C as described (43). Briefly, 250 μg of freshly prepared mitochondria was mixed with 1 mL of respiratory buffer (120 mM KCl, 5 mM K_2HPO_4, 3 mM Hepes, 1 mM EGTA, 1 mM $MgCl_2$, and 0.2% fatty acid free BSA). The oxygen consumption rate was measured in the present of 5 mM ADP with the following sequential administration: 5 mM pyruvate/5 mM malate, 400 nM rotenone, 10 mM succinate, 10 mM ascorbate/400 μM N′-tetrametyl-1,4-phenylenediamine (TMPD), 2 mM KCN. Spectrophotometric measurements of the individual complex and the citrate synthase activities were performed as described (44).

ATP Synthesis Measurement. Ten micrograms of mitochondrial proteins were used to measure ATP synthesis by using a luciferase luminescence-based kinetic assay as described (45).

Electrophoresis and Western Blot Analysis. Blue native gel electrophoresis reagents were purchased from Invitrogen and the electrophoresis was based on the manufacture's protocol. Ten micrograms of mitochondrial protein was added with 0.25% of Coomassie blue G250 in a bis buffer containing 1.5 M HCl and loaded onto a 3–12% native gel. Electrophoresis was carried out in a buffer of 50 mM of Bis, 50 mM Tricine at pH 6.8, and 0.002% of Coomassie blue G250. The second dimensional gel was carried out by using methods described by ref. 46. After electrophoresis, proteins were transferred onto a PVDF membrane, probed with NDUFS3 (complex I), and complex Vα subunit. Pyruvate dehydrogenase (PDH) antibodies were used to normalize the mitochondrial quantity in all of the Western blot analyses (Mitoscience).

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