DJ-1 is critical for mitochondrial function and rescues PINK1 loss of function

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Mutations or deletions in *PARKIN/PARK2*, *PINK1/PARK6*, and *DJ-1/PARK7* lead to autosomal recessive parkinsonism. In *Drosophila*, deletions in *parkin* and *pink1* result in swollen and dysfunctional mitochondria in energy-demanding tissues. The relationship between *DJ-1* and mitochondria, however, remains unclear. We now report that *Drosophila* and mouse mutants in *DJ-1* show compromised mitochondrial function with age. Flies deleted for *DJ-1* manifest similar defects as *pink1* and *parkin* mutants: male sterility, shortened lifespan, and reduced climbing ability. We further found poorly coupled mitochondria in vitro and reduced ATP levels in fly and mouse *DJ-1* mutants. Surprisingly, up-regulation of *DJ-1* can ameliorate *pink1*, but not *parkin*, mutants in *Drosophila*; cysteine C104 (analogous to C106 in human) is critical for this rescue, implicating the oxidative functions of DJ-1 in this property. These results suggest that *DJ-1* is important for proper mitochondrial function and acts downstream of, or in parallel to, *pink1*. These findings link *DJ-1*, *pink1*, and *parkin* to mitochondrial integrity and provide the foundation for therapeutics that link bioenergetics and parkinsonism.

parkin | Parkinson's disease | oxidative stress | *Drosophila*

Parkinson's disease (PD) is the most common movement disorder and the second most common neurodegenerative disease. Clinically, it is characterized by resting tremor, rigidity, bradykinesia, gait abnormality, and slow movement (1, 2). PD patients show severe dopaminergic neuron loss, resulting in a decrease of striatal dopamine levels responsible for the motor features (3, 4). Age is the most potent risk factor for PD, but other contributing factors include exposure to environmental toxins like paraquat and rotenone. Although predominantly idiopathic, genetic mutations account for ≈10% of cases (5). Studies of genes responsible for familial parkinsonism/PD are yielding critical insight into mechanisms shared by sporadic and familial disease.

Mutations in *DJ-1*/*PARK7*, *PINK1*/*PARK6*, and *PARKIN*/*PARK2* lead to autosomal recessive parkinsonism. Properties of DJ-1 suggest that it may be at a compelling intersection for several risk factors in PD, including genetics, oxidative stress, environmental factors, and age. First, *DJ-1* gene mutations lead to early-onset autosomal recessive parkinsonism (6). Second, the DJ-1 protein is sensitive to oxidative stress and may act as a redox-responsive molecular chaperone that can prevent protein misfolding (7). Third, tolerance toward paraquat in animals is mediated, in part, through modifications of DJ-1 protein (8). Finally, age induces the same modifications of the DJ-1 protein as environmental toxins (8). Mice mutant for *DJ-1* show dopamine reuptake dysfunction (9) and have increased sensitivity to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrindine (MPTP) (10). Two *DJ-1* orthologs (*DJ-1a* and *DJ-1b*) exist in *Drosophila*, and when deleted, flies have decreased climbing ability (11) and increased sensitivity to H_2O_2, paraquat, and rotenone (12). Taken together, these data indicate that the DJ-1 protein responds to key risk factors for PD, including age and toxins.

Parkin is an E3 ubiquitin ligase whose mutations account for the majority of autosomal recessive parkinsonism, and Parkin may be important for mitophagy (13, 14). The second most common cause of early-onset parkinsonism is mutations in PINK1, a kinase localized to mitochondria that may be involved in mitochondrial fission (15). In *Drosophila*, loss of function of either *pink1* or *parkin* leads to male sterility and abnormal wing posture (16–18). Moreover, up-regulation of *parkin* rescues *pink1* mutation, thus placing the two genes in the same genetic pathway, with *parkin* downstream of *pink1* (17, 18). Electron microscopic analysis of *pink1* and *parkin* null mutants shows swollen mitochondria in flight muscle and testes, suggesting that these genes are important for mitochondrial integrity. Interestingly, epidemiological and other studies have linked complex I inhibitors, such as rotenone, paraquat, and 1-methyl-4-phenylpridinium (MPTP metabolite), to parkinsonism in humans (19). Given that DJ-1 is also localized to mitochondria (20–22), and that mutations in DJ-1 lead to parkinsonism, these results raise the question of whether there are links between DJ-1 and mitochondrial function.

Here we demonstrate that flies deleted for *DJ-1* share biological defects in common with flies lacking *pink1* and *parkin*. We further show that up-regulation of the fly *DJ-1* (*DJ-1a* or *DJ-1b*) or of human *DJ-1* (*hDJ-1*) can rescue *pink1* deficiency; moreover, rescue is dependent on C104 of DJ-1, implicating the oxidative functions of DJ-1 in the rescue. These studies suggest that *DJ-1*, *pink1*, and *parkin* function in common biological processes that are critical for mitochondrial function, such that compromise of their activity leads to human disease.

Results

DJ-1 Double-Mutant Flies Have a Shortened Lifespan and Reduced Climbing Ability. Several functions have been suggested for *DJ-1a* and *DJ-1b* in *Drosophila,* including possible compensatory roles for each other (23). Therefore, to better characterize the effects of *DJ-1* loss, we generated flies mutant for both *DJ-1a* and *DJ-1b* genes (double knockout, DKO) in a homogenous background (*Methods*). We observed reduced lifespan of DKO flies compared with controls, and decreased spontaneous movement of the animals over time (median lifespan of 62 days for controls versus 52 days for DKO; $P < 0.01$) (Fig. 1*A*, Fig. S1). In testing locomotor activity, we found that by 7 weeks of age, DKO flies showed severe climbing defects compared with controls (only 56% of DKO flies compared with 90% of controls were able to climb; $P < 0.01$) (Fig. 1*B*), suggesting an age-dependent decline in motor activity in DKO flies.

Defects in DKO Spermatogenesis. During this characterization, we noted that DKO flies seemed to have reduced fertility. To test

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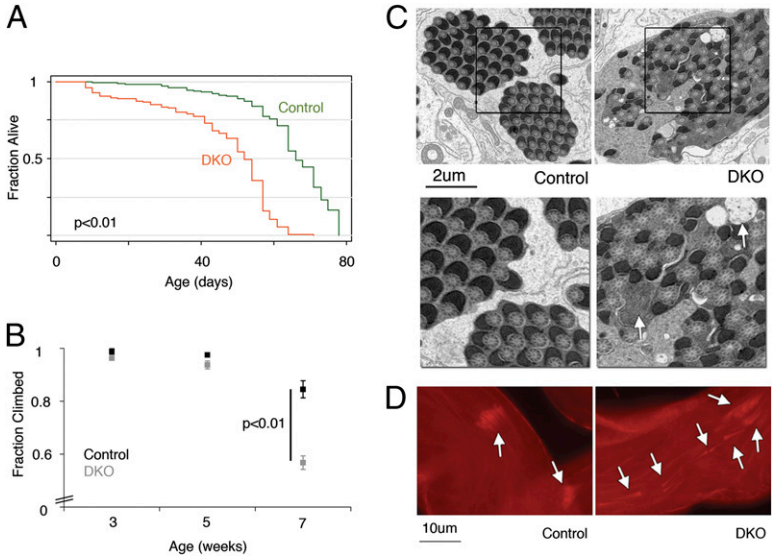

Fig. 1. DKO mutant flies show multiple defects with age. (*A*) Lifespan of control flies compared with DKO flies. More than 200 flies for each genotype were used ($P < 0.001$, log–rank test). (*B*) Climbing ability of control and DKO flies at different ages. The fraction of total flies that climbed out of the required distance in the given time is shown (mean ± SEM; $n > 100$ flies). $P = 0.0004$ (*t* test, two tailed) between control and DKO at 7 weeks. (*C*) Electron micrographic images of control and DKO spermatogenesis. The mitochondrial derivatives are regular and orderly in the control but are aberrant in DKO (white arrows). (*Lower*) Higher magnification of squared areas above. (*D*) Progression of sperm individualization, highlighted with phalloidin, which labels sperm head investment cones (white arrows), is orderly and compact in the control but disorganized in DKO testes (3-day males).

this directly, we set up single-pair matings of control or DKO male flies (<3 days old) with wild-type virgin females and scored successful matings by the presence of progeny pupal casings after 10 days. This assay revealed that whereas 96% of control male flies were fertile, DKO males had high infertility (26 of 27 control males compared with 0 of 26 DKO males tested were fertile; $P = 2.8e\text{-}14$, Fisher's exact test). In contrast, *DJ-1a* and *DJ-1b* single knockout flies were fertile (Table S1). The reverse crosses using virgin DKO mutant females also showed reduced fertility (48.6 ± 3.2 pupal casings per control female compared with 19.5 ± 4.8 for DKO female, mean ± SEM; $P = 1.4e\text{-}5$, Fisher's exact test). The infertility of DKO males prompted further investigation because of the striking similarity to mutants of *pink1* and *parkin*, both of which are male sterile.

To analyze the infertility, we focused on spermatogenesis. DKO sperm appeared morphologically normal but were immotile (Movie S1 and Movie S2). Because dysfunctional mitochondria are the basis of infertility in *pink1* and *parkin* mutants, we assessed the Nebenkern, the structure in the spermatozoa with fused mitochondria, in DKO spermatogenesis. Abnormal vacuoles were present in the Nebenkern of DKO mutants, similar to those observed in *pink1* and *parkin* mutants. We then examined the structure of the mitochondria by electron microscopy and found profound abnormalities in mitochondrial derivatives in DKO sperm compared with control (Fig. 1*C*). The last step in spermatogenesis involves separation of individual spermatozoa from each other and can be visualized by the position of the investment cones. In contrast to the orderly wave of investment cone movement of control testes, DKO mutant testes showed misoriented and asynchronous progression (Fig. 1*D*). Taken together, these results suggest a disruption of mitochondrial-dependent events in DKO flies, akin to the defects of *pink1* and *parkin* mutants.

Mitochondrial Respiration and ATP Production Are Defective in DKO Flies. Mitochondrial dysfunction can be rooted in a change in the overall number of mitochondria, mtDNA level or integrity, and/or mitochondrial function. To assess the status of DKO mitochondria, we first determined the ratio of mtDNA to genomic DNA using real-time PCR. Whole-fly analysis revealed similar levels of mtDNA at 3 days but a consistent decrease of ≈40% in DKO at 30 days compared with controls (Fig. 2*A*). The mtDNA decrease, however, did not reflect a change in the protein levels of complex I subunit NDUFS3 (Fig. 2*B*). Because of a lack of antibodies against *Drosophila* mitochondrial subunits, other subunits were not tested. These findings suggest that DKO and control flies have comparable levels of mitochondria but that there is an age-dependent loss of mtDNA in DKO flies.

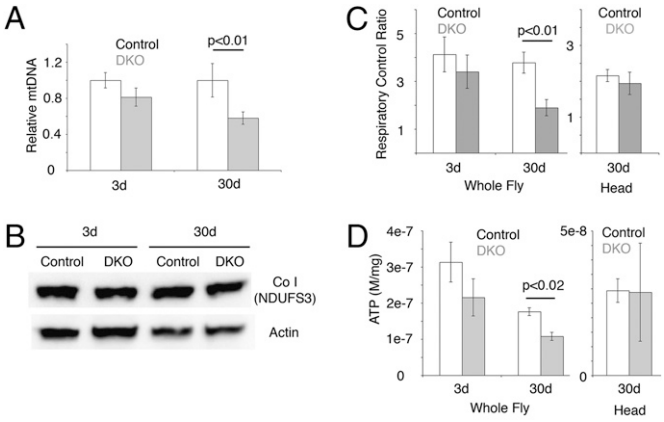

Fig. 2. Mitochondrial dysfunction in DKO mutant flies. (*A*) The relative level of mtDNA in young (3 days) and old (30 days) control (white) and DKO (gray) thoraces (mean ± SEM, three DNA extractions for 3 days, four for 30 days). The *ND5* locus was used to measure mtDNA levels, normalized to the nuclear *let-7* locus. $P = 0.0055$ (*t* test, two tailed). (*B*) Western immunoblot for level of complex I subunit NDUFS3 (*Upper*) and actin shows similar levels in control and DKO with age. (*C*) Mitochondrial RCR of control (white) and DKO (gray). (*Left*) Whole flies (mean ± SEM, three preparations of mitochondria for 3 days, four for 30 days) (two-tailed *t* test, $P = 0.005$ between 30-day control and DKO samples). (*Right*) Heads (mean ± SEM, seven preparations of mitochondria). Mitochondrial RCR showed significance in aged whole fly and a modest but insignificant decrease in aged heads RCR (two-tailed *t* test, $P = 0.096$ between 30-day control and DKO). (*D*) ATP levels (mole/mg) of control (white) and DKO (gray) flies (mean ± SEM, three sample preparations) (two-tailed *t* test, $P = 0.011$ between 30-day control and DKO samples from whole flies).

We then assessed mitochondrial transmembrane potential via tetramethylrhodamine ethylester (TMRE), a membrane-permeate fluorescent probe. When a mitochondrial membrane potential is generated by substrates, TMRE fluorescence decreases owing to sequestration of the probe in the mitochondrial matrix. Conversely, in the presence of carbonyl cyanide m-chlorophenyl hydrazone, an uncoupler, the cross-membrane potential is dissipated and total fluorescence increases. Using this approach, we found that DKO mitochondria were capable of generating and maintaining cross-membrane potential with either complex I or II substrates (Fig. S2).

We next examined mitochondrial respiratory and phosphorylating activities through polarography. Rates of ADP-stimulated and ADP-depleted oxygen consumption (state 3 and 4, respectively) were measured using the complex I substrate pair pyruvate plus malate and substrate II substrate succinate. The respiratory control ratio (RCR; the state 3/state 4 ratio) reflects the degree of coupling between substrate oxidation and ADP phosphorylation and is a way to assess the physical and functional integrity of mitochondria. Mitochondria from 3-day control and DKO flies had comparable RCR. In 30-day flies, state 3 respiratory rates were similar in control and DKO mitochondria; however, state 4 respiration was higher in DKO mitochondria, leading to a significant reduction of RCR in DKO compared with controls (1.9 ± 0.3 compared with 3.8 ± 0.4; $P = 0.0005$) (Fig. 2*C*). These results suggest a decline in mitochondrial function in DKO flies as they age. Consistent with the RCR results, we observed a decrease of ≈40% in total ATP in aged DKO flies (Fig. 2*D*).

To determine whether similar changes could be detected in the brain, we performed the same assays on mitochondria from 30-day DKO and control heads. Mitochondria from DKO mutant heads had lower, although not statistically significant, RCR compared with controls (Fig. 2 *C* and *D*). Taken together, these data indicate that mitochondrial function in DKO mutant animals become severely compromised with age.

DJ-1 Mutant Mice Have Mitochondrial Dysfunction. To determine whether mammalian *DJ-1* mutants also exhibited mitochondrial defects, we examined a *DJ-1* knockout mouse line that was generated by exonic trapping of the murine *DJ-1* gene. These studies revealed that *DJ-1* mutant mice showed an age-dependent decline by rotorod assay (Fig. 3*A*). This age-dependent movement impairment parallels the decreased climbing ability of DKO flies (Fig. 1*B*).

We then asked whether *DJ-1* mutant mice have mitochondrial dysfunction; in these studies, we focused on skeletal muscle, given the motility defects. To assess overall skeletal muscle mitochondrial function, mitochondria were isolated from a mixture of quadriceps and calf muscle, including both fast- and slow-twitch fibers. The aged (16 month) *DJ-1* mutant mice had a slightly lower RCR than controls (3.7 ± 0.3 vs. 4.3 ± 0.5, respectively, mean ± SEM). Moreover, we found that ATP levels of skeletal muscle from 13–16-old month *DJ-1* knockout mice were lower compared with aged-matched controls (Fig. 3*B*). Younger mice (4–6 months) showed no significant difference in ATP levels, and we did not observe a change in overall brain ATP levels between control and *DJ-1* mutant mice in either young or aged animals (Fig. 3*B*). These data indicate that age-accentuated mitochondrial defects were observed in both fly and mouse *DJ-1* mutants.

***DJ-1* Can Rescue *pink1 Drosophila* Mutants.** Mitochondrial dysfunction has been well documented in the *pink1*/*parkin* pathway in *Drosophila* (17, 18). Given that the effects in DKO flies were similar to those of *parkin* and *pink1* mutants, we tested whether *DJ-1* genetically interacted with the *pink1*/*parkin* pathway. To do this, we used the GAL4-UAS system to up-regulate *DJ-1a*, *DJ-1b*, or *hDJ-1* to assess whether *DJ-1* can modify *pink1* mutant effects. Wings in *pink* and *parkin* mutants are held up owing to

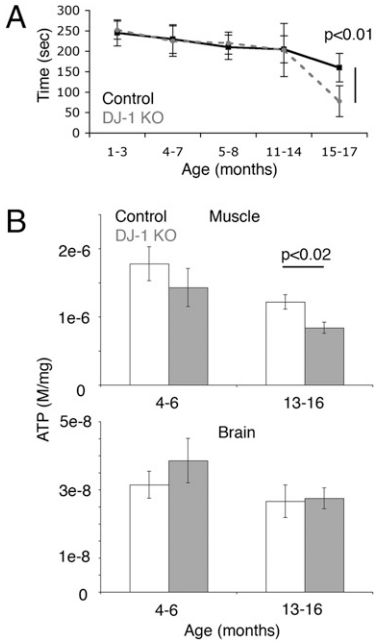

Fig. 3. DJ-1 KO mice show motor and mitochondrial dysfunction. (*A*) Rotorod test of control (solid) and DJ-1 KO (dashed) animals of different ages, showing a significant ($P < 0.01$) difference by 15 months (mean ± SD, n = 6–10 animals per time point). (*B*, *Upper*) ATP levels (moles/mg) in hindlimb skeletal muscle (a mixture of soleus and gastrocnemius muscle) of control (white) and DJ-1 null (gray) mice (mean ± SEM, n = 6 animals) (two-tailed t test, P = 0.017 between 14-month control and KO samples). (*Lower*) ATP levels from brain extracts (mean ± SEM, n = 6 animals).

flight muscle mitochondrial defects, with 6-day *pink1* mutants showing ≈30% normal wing posture at 25 °C; this wing posture defect was rescued by either *pink1* or *parkin* expression, as previously shown (Table 1) (17, 18).

Strikingly, up-regulation of either *DJ-1* ortholog rescued the *pink1* mutant wing posture to the same degree as up-regulation of *pink1* (Table 1). This result was confirmed with independent transgenic lines of *DJ-1a*, *DJ-1b*, and *hDJ-1*. We did not, however, observe rescue with every transgenic line tested; further analysis suggested an important role of expression level of the *DJ-1* transgene, with too-high expression of *DJ-1* being deleterious (*Discussion*). C104 of DJ-1b (C106 in hDJ-1) is oxidized in aged animals and in animals treated with oxidative stress and has been found to be critical for DJ-1b function in response to oxidative stress (8). Mutating C104 to Ala in DJ-1b abrogated rescue of the $pink1^{B9}$ wing postural defect, whereas mutation of C45 to Ala, a second site capable of being oxidized, retained normal function (Table S2). Moreover, DJ-1b C45A mutants were able to ameliorate both structural defects and TUNEL

Table 1. Rescue of *pink1* loss of function wing posture by *DJ-1*

Genotype*	Rescue % (fraction)†
$pink1^{B9}$;;*da-GAL4/+*	29.4 (20/68)
$pink1^{B9}$;*UAS-pink1/+*;*da-GAL4/+*	64.7 (11/17)
$pink1^{B9}$;*UAS-parkinC2*;*da-GAL4*	97.6 (41/42)
$pink1^{B9}$;*UAS-sDJ-1b/+*;*da-GAL4/+*	52.8 (19/36)
$pink1^{B9}$;*UAS-DJ-1a*;*da-GAL4/+*	57.4 (35/61)
$pink1^{B9}$;*UAS-hDJ-1*;*da-GAL4/+*	57.9 (22/38)

*$pink1^{B9}$ denotes a deletion in *pink1* gene, *parkinC2* is wild-type *parkin*, *sDJ-1b* is wild-type *DJ-1b*.

†Male fly wing posture was scored in 6-day adult flies at 25 °C, with the number of flies rescued/total scored.

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signals seen in the thorax of *pink1*^{B9} flies (Table S2). These data suggest that C104 is critical for DJ-1b function to both rescue and protect against oxidative stress in *pink1* mutants.

To determine whether rescue by *DJ-1* was restricted to the wing posture effect, we examined mtDNA levels and apoptosis in the thorax (17, 18). Consistent with wing posture rescue, up-regulation of *DJ-1a* also restored thoracic muscle morphology in the *pink1* mutant (Fig. 4*A*), rescued muscle apoptosis (Fig. 4*B* and Fig. S3) and mtDNA levels (Fig. 4*C*), comparable to *parkin* rescue. *pink1*^{Rev} is the wild-type revertent of the *pink1*^{B9} mutant in the same genetic background. To address the specificity of *DJ-1* rescue of *pink1*, we determined that *DJ-1* up-regulation failed to rescue polyglutamine toxicity and Hid-induced apoptosis in the fly eye (Fig. S4). These results confirmed that *DJ-1* interacts with the Pink1 pathway and does not generally mitigate deleterious effects in flies. Thus, up-regulation of *DJ-1* can rescue *pink1* mutants in a manner comparable to *parkin*, placing *DJ-1* either downstream of *pink1* or in a parallel pathway that converges with the *pink1*/*parkin* pathway (Fig. S5).

Genetic Pathway of DJ-1. To further address the mechanism of rescue, we tested whether *DJ-1* up-regulation induced a change in the expression level of *parkin*, which rescues *pink1* mutants when up-regulated (17, 18). We measured *parkin* mRNA levels in *pink1* mutants, and in *pink1* mutants with *DJ-1a* up-regulation; these studies showed that *parkin* levels did not increase in *DJ-1* rescued animals (Fig. 4*D*). Changes in expression of genes that modulate mitochondrial fusion/fission (*drp1* and *dMfn2*/*marf*) have also been shown to rescue *pink1* (24, 25). The transcripts level of these genes did not change upon up-regulation of *DJ-1a* in the *pink1* mutant background (Fig. 4*D*). In addition, DKO flies and in DJ-1a up-regulated flies both showed normal mitochondrial morphology, suggesting that DJ-1 is not important for mitochondrial fission or fusion (Fig. S6). These results indicate that the mechanism of *DJ-1* rescue of *pink1* is not through changes in the mRNA levels of *parkin*, *drp1*, or *dMfn2*. We were unable to assess the protein levels owing to a lack of antibodies against these proteins in flies.

These data suggest that *DJ-1* is downstream of *pink1* and does not affect *drp1*, *dMfn2*, or *parkin* gene levels. To further define the placement of *DJ-1* activity relative to that of *pink1*, we tested whether *DJ-1* could also rescue *parkin* mutants. *DJ-1a* up-regulation failed to rescue the wing posture defect, thoracic muscle morphology, and TUNEL-positive signal in *parkin* mutant flies (Fig. 4*E*).

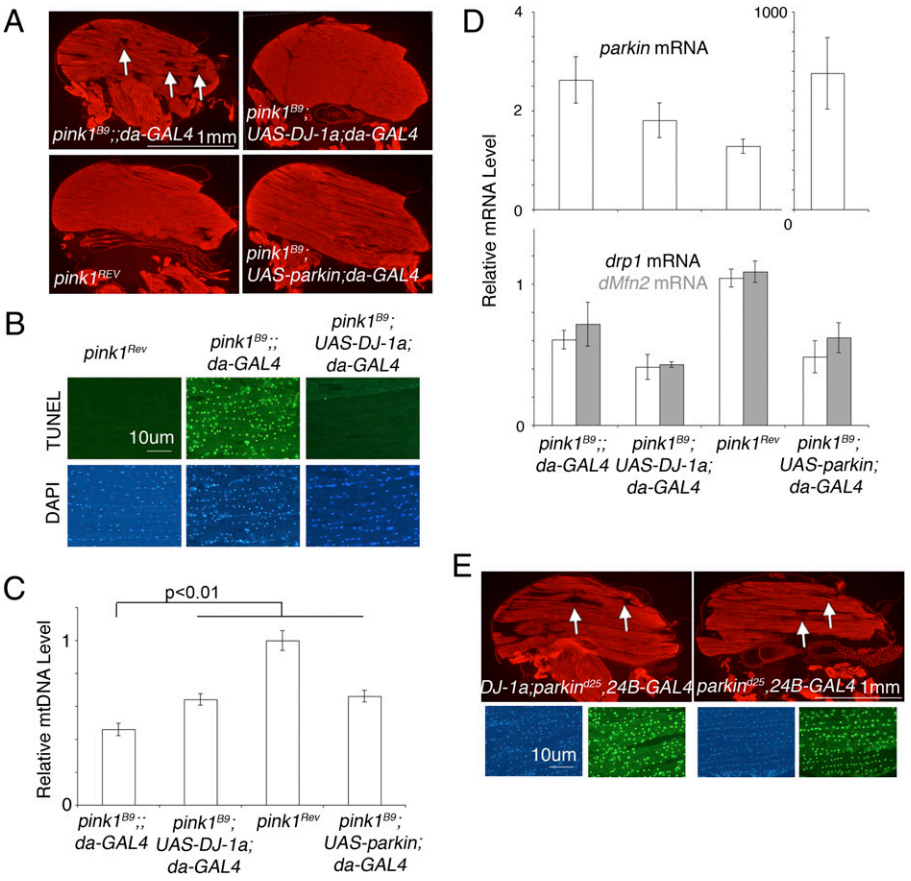

Fig. 4. Up-regulation of DJ-1 rescues Pink1 muscle defects. (*A*) Cryosections of the thorax stained with phalloidin to highlight muscle structure of young (<3 days) male flies. Visible vacuoles (arrows) are observed in the *pink1* deleted mutant, *pink1*^{B9}. Both *DJ-1a* and *parkin* up-regulation ameliorate the defects; compare with the *pink1*^{Rev} control, a normal revertant of the original *pink1*^{B9} mutation. (*B*) Widespread apoptosis revealed by TUNEL staining (*Upper*) in thorax sections of *pink1*^{B9} flies is rescued by *DJ-1a* up-regulation. (*C*) Relative mtDNA levels from male thoraces was assayed by real-time PCR and adjusted to the levels in the control *pink1*^{Rev} (mean ± SEM, four to six independent experiments). The mtDNA level was rescued by *DJ-1a* and *parkin* up-regulation (two-tailed *t* test, $P = 0.004$ between *pink1* with driver and *pink1* with *parkin* up-regulated samples, $P = 0.006$ between *pink1* with driver and *pink1* with *DJ-1a* up-regulated samples. A *p* value of <0.0167 was considered significant after correction for multiple tests). (*D*) Real-time PCR assays for levels of *parkin* (*Upper*), *drp1* (white, *Lower*), and *dMfn2* (gray, *Lower*) mRNA in young (<5 days) male flies (mean ± SEM, three to four independent experiments). The RNA levels were adjusted to *pink1*^{Rev} levels. Flies overexpressing *parkin* had dramatically higher (≈650×) *parkin* mRNA levels, whereas up-regulation of *DJ-1a* did not change *parkin* mRNA levels. *DJ-1a* up-regulation did not change the mRNA levels of *drp1* or *dMfn2*. (*E*) Up-regulation of *DJ-1* does not rescue *parkin* mutation. Cryosections of thoraces stained with phalloidin (red) for muscle structure (white arrows, vacuoles highlighted deteriorated structure). TUNEL for apoptosis (green) and DAPI (blue). Vacuoles and TUNEL signal of *parkin* deleted mutant, *parkin*^{d25}, was unchanged with up-regulation of *DJ-1a*.

Thus, although *DJ-1* can rescue *pink1* loss of function, *DJ-1* cannot rescue *parkin* mutation. We then determined whether *parkin* up-regulation could rescue *DJ-1* loss of function. Strikingly, we found that up-regulation of either *parkin* or *pink1* in *DJ-1* DKO background was lethal (Table 2). Taken together, these data place *DJ-1* in a pathway parallel to that of *pink1*/*parkin* and show that both of these pathways, and their carefully balanced activity, are critical for mitochondrial function (Fig. S5).

Discussion

Mutations in *DJ-1*/*PARK7*, *Parkin*/*PARK2*, and *PINK1*/*PARK6* lead to early-onset, autosomal recessive parkinsonism. Studies in *Drosophila* indicate that *pink1* and *parkin* are genetically in the same pathway, which impacts mitochondrial function (17, 18). The link between *DJ-1* and mitochondria, however, has been circumstantial and limited to cell culture studies (20, 21). Here we demonstrate that *DJ-1* loss of function leads to mitochondrial dysfunction in an age-dependent manner in both fly and mouse. We further provide genetic evidence that *DJ-1* interacts with the *pink1*/*parkin* pathway, because *DJ-1* up-regulation can compensate for reduction of *pink1* activity. Consistent with important interactions between *DJ-1* and *pink1*/*parkin*, up-regulation of either *pink1* or *parkin* is deleterious in a *DJ-1* mutant background. These findings indicate that *DJ-1* and *pink1*/*parkin* fall into two parallel pathways whose function critically impacts mitochondrial activity (Fig. S5).

Age-Dependent Mitochondrial Dysfunction in DJ-1 DKO Flies. DKO mutant flies showed several classic defects that reflect mitochondrial dysfunction. First, DKO flies have an overall reduced fitness (shortened lifespan), consistent with mitochondrial involvement in reduced lifespan and accelerated aging in worms, flies, and mice (26). Second, DKO flies have defects in spermatogenesis reflective of disrupted mitochondrial function. In the fly, mitochondrial mutants are often infertile because germ cell maturation requires both energy and mitochondrial morphological changes (27). Similarly, DJ-1 protein levels correlate with infertility in pharmacologically treated male rats that leads to reduced glycolytic enzyme activities in the sperm (28–31). Third, DKO flies showed age-dependent declines in both climbing activity and ATP level. The age-dependent onset of these deficiencies is consistent with the notion that nonlethal mitochondrial mutants can compensate for mitochondrial inefficiency until older ages, when more pressure is exerted on the system. Our data show that *DJ-1* DKO mitochondria have a lower RCR, suggesting that the mitochondria can function, albeit at lower capacity and with lower reserve capacity. Over time, DKO cells may no longer effectively compensate, and defects manifest. These data indicate that DJ-1 activity is important for proper mitochondrial function over time.

Mitochondrial Dysfunction in DJ-1 Null Mice. As in fly *DJ-1* mutants, we observed mitochondrial dysfunction in *DJ-1* knockout mice skeletal muscle. Although we did not see dysfunction in mitochondria isolated from mouse brain, muscle is one of the high energy demanding tissues. These data agree with previous findings that *DJ-1* knockout mice do not have a significant change in lifespan or reduction in dopamine levels with age (32). The age-dependent reduction in rotorod endurance in *DJ-1* knockout mice is consistent with findings showing specific age-dependent impairments in endurance in *DJ-1* knockout mice (33). Interestingly, Chandran et al. (33) did not report a change in neuromuscular junction or muscle by histology in *DJ-1* knockout mice. The changes in mitochondrial function and efficiency we observed can explain the loss of endurance. Alteration in muscle-related activities was not observed in another study of *DJ-1* null mice, suggesting that genetic background may affect the presentation of this impairment (34). It is also intriguing that murine muscle seems to be more affected than brain, in that ATP reductions were detected in mouse muscle but not brain (Fig. 3). It is possible that, unlike humans, the energy demand of murine muscle, like that of *Drosophila*, is higher than that of brain, thus the consequence of mitochondrial dysfunction in the absence of *DJ-1* manifests first in the muscle. Taken together, we suggest that loss of *DJ-1* activity may lead to age-dependent mitochondrial dysfunction in tissues with high energy demands.

Table 2. Up-regulation of *parkin* and *pink1* in the DKO mutant background

Genotype	Adult viability*
UAS-parkin;;da-GAL4[†]/+	Viable
UAS-pink1;;da-GAL4/+	Viable
UAS-parkin;d72[‡]*;da-GAL4 d93*[§]*/d93*	Lethal
UAS-pink1;d72;da-GAL4 d93/d93	Lethal

*Flies were scored for viability 14d after the cross at 25 °C.
[†]*da-GAL4* drives expression ubiquitously.
[‡]d72 denotes *DJ-1a* deletion allele.
[§]d93 denotes *DJ-1b* deletion allele.

Common Pathway for *pink1*, *parkin*, and *DJ-1*. We present evidence that *DJ-1* loss of function causes similar mitochondrial defects in aged *Drosophila*. Moreover, we also show that *DJ-1* can rescue *pink1*, although not *parkin*, loss (Fig. 4). *DJ-1a* does not ameliorate all *pink1* defects: *DJ-1a* could not rescue the *pink1* mutant infertility (0 of 40 male *pink1* with *DJ-1a* up-regulated were fertile). The selective rescue of *pink1* mutants by *DJ-1* places *DJ-1* downstream of *pink1*. The findings that *DJ-1* cannot rescue *parkin* mutants, and that *parkin* cannot rescue DKO mutants, suggest that *DJ-1* may not be directly downstream of *pink1* (neither in between *pink1* and *parkin* nor downstream of *parkin*). We propose that *DJ-1* defines a pathway parallel to that of *pink1*/*parkin*. It is likely that there is partial convergence/overlap downstream between the pathways, given the common effects. We cannot, however, rule out the possibility that *DJ-1* selectively rescues a target downstream of *pink1* pathway that is responsible for the effects on thoracic mitochondria. It is interesting that *Omi*/*HtrA2* has also been suggested to partially rescue *pink1* mutants in *Drosophila* in a pathway that is similarly independent of *parkin* (35).

Our data indicate a critical balance of activities between the *DJ-1* and *pink1*/*parkin* pathways, because up-regulation of either *pink1* or *parkin* leads to lethality in DKO flies. In accord to the finding that up-regulation of *pink1* leads to a deleterious *Drosophila* eye effect in a wild-type background (24), our data further suggest that in DKO, a background sensitized for mitochondrial dysfunction, an increase in *pink1* levels simply causes lethality. The ability of DJ-1 to respond to oxidative stress through C104 modification or as an atypical peroxidase (8, 32) fits nicely with the hypothesis that DJ-1 is important for proper mitochondrial function. However, it should be noted that C104 might sense oxidative stress independent of the proposed peroxidase function. As such, when DJ-1 activity is compromised, cells accumulate more oxidative stress and mitochondrial dysfunction with age. *pink1* deletion may also lead to increased oxidative stress that can be partially reduced through up-regulation of *DJ-1*. In line with this, when we mitigated the ability of DJ-1b to respond to oxidative stress by mutation of C104, but not C45, rescue of $pink1^{B9}$ wing posture was abrogated. Interestingly, DJ-1b with either C104A or C45A also failed to rescue $pink1^{B9}$ fertility phenotype (0 of 15 fertile for DJ-1b C104A and 0 of 10 for DJ-1b C45A). Taken together, these data argue that DJ-1 rescue of $pink1^{B9}$ mutants requires its ability to respond and protect against oxidative stress in specific tissues.

Although previous studies suggest that *DJ-1* cannot rescue *pink1* mutants (36), several important differences exist between those data and our findings: first, the method of *pink1* reduction (RNAi vs. gene deletion in the present study); and second, the method by

which *DJ-1* was up-regulated (muscle-specific vs. ubiquitous driver in the present study). Moreover, when assessing rescue, we used a quantitative measurement because the wing effect was not 100% penetrant in $pink1^{B9}$ mutants. This approach allowed assessment of genes that partially rescue *pink1* mutants. Additionally, we found that the expression level of *DJ-1* transgenes seems to be critical for rescue, with higher levels of expression being less likely to rescue. These findings indicate that *DJ-1* rescues the *pink1* mutant in an expression level–dependent manner. Understanding the detailed mechanism by which *DJ-1* rescue of *pink1* is so critically dose dependent may provide key insight into the intersection of the pathways.

The critical role of mitochondria in neurodegenerative diseases is recognized (37–39). Our findings strongly indicate that mitochondrial dysfunction plays an important role in inherited forms of early-onset parkinsonism, as well as in sporadic disease, which may involve similar genetic players and pathways. These findings highlight the value of defining when mitochondrial function decline occurs relative to disease onset, and the central role of mitochondria as a target in PD therapeutic research.

Methods

***Drosophila* Assays.** *DJ-1a* and *DJ-1b* mutations were generated previously (12). *pink1* and *parkin* mutant flies are described elsewhere (16, 18); the control $pink1^{Rev}$ is a precise excision of the *P*-element insertion that was used to generate the $pink1^{B9}$ allele, and is wild type. For lifespan, flies <3 days old were collected and kept together for 3 days before separation according to sex and transferred every 2 to 3 days, with the number of dead flies recorded. Negative geotaxis was done as previously described (40). To test fertility, the number of pupae casings was counted 10 days after single 3-day male and virgin flies were crossed. For investment cone staining, testes were dissected in PBS and fixed in 4% paraformaldehyde, washed in PBS, and permeabilized with 0.3% Triton/PBS followed by PBS washes. Testes were then stained with phalloidin Alexa633 (Invitrogen) and washed in PBS. For wing posture, 3-day males were place with virgin flies and scored 6 days later.

Statistics. Mean ± SEM are shown on all graphs. Log–rank test was performed on lifespan analysis. Male fertility was performed on a two-tailed Fisher's exact test. For other analysis, Student's *t* test, two tailed, was performed with Bonferroni-corrected α level. *SI Methods* provides complete information on materials and methods.

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