Supporting Information

Hao et al. 10.1073/pnas.0911175107

SI Methods

Fly Strains Used. Fly stains included *UAS-DJ-1a*, *UAS-parkinC2*, and *parkin*$^{\Delta 25}$*-24B-GAL4* (L. Pallanck, University of Washington), *UAS-pink1*, *pink1*^{B9}, and *pink1*^{Rev} (J. Chung, Korea Advanced Institute of Science and Technology), *da-GAL4* (Bloomington Stock Center), and *UAS-DJ-1a #5.1* (B. Lu, Stanford University School of Medicine).

Fly genotypes in Fig. 4 are as follows: *pink1B9;;da-GAL4* is *pink1B9;;da-GAL4/+* (in *A*, *B*, *C*, and *D*). *pink1B9;UAS-DJ-1a;da-GAL4* is *pink1B9;UAS-DJ-1a/+;da-GAL4/+* (in *A*, *B*, *C*, and *D*). *pink1Rev* is *pink1 wildtype* (in *A*, *B*, *C*, and *D*). *pink1B9;UAS-parkin; da-GAL4* is *pink1B9;UAS-parkinC2/+,da-GAL4/+* (in *A*, *C*, and *D*). *DJ-1a;parkinΔ25,24B-GAL4* is *UAS-DJ-1a/UAS-DJ-1a;parkinΔ25 24B-GAL4/parkinΔ25 24B-GAL4* (in *E*). *parkinΔ25 24B-GAL4* is *parkinΔ25 24B-GAL4/parkinΔ25 24B-GAL4* (in *E*).

Real-Time PCR. Total DNA was extracted using Gentra DNA Isolation kit (Qiagen). SYBR reactions were set up according to the ABI manual and performed on an ABI 7500 system (Applied Biosystems). For mtDNA determinations, NADH-ubiquinone oxidoreductase chain 5 locus was used (NB1613 AAGAATGAACTAAAGCAGAAACAGG and NB1614 TGCTATAACTAAAAGAGCTCAGATTCC) and normalized to the *let-7* locus (CCGAACCAATGATATCCAGAA and TTTTGTATGGCTGGGGATTG). The reaction conditions were as follows: one cycle of 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 60 s. At the end of each reaction, a dissociation curve was generated to ensure specificity of the reactions. For *parkin*, *drp1*, and *dMfn2* reactions, total RNA was isolated from eight male flies using TRIzol according to the manufacture's protocol (Invitrogen). Total RNA was digested with DNaseI before cDNA synthesis using Invitrogen's First Strand cDNA synthesis kit according to the manufacturer's protocol. Vic labeled eukaryotic 18S was the control with FMN labeled *parkin* taqman probes purchased from Applied Biosystems and with the following reaction conditions: one cycle of 95 °C for 20 s and 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Both *drp1* and *dMfn2* were assayed using SYBR reaction with *rp49* as the endogenous control using the following conditions: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 55°C for 30 s, and 72°C for 60 s. At the end of all of the SYBR runs, the melting curve was performed to ensure that a single product was amplified.

Western Blotting. Male flies (10–15 thoraces or four to eight whole flies) were homogenized in Laemmli sample buffer (Bio-Rad) with 2-mercaptoethanol and boiled at 100 °C, then centrifuged at 12,000 × *g* for 2 min. The supernatant was then kept on ice until use. Antibodies used were β-actin at 1:1,000 (Abcam 8226), complex I subunit NDUFS3 at 1:2,000 (Mitosciences MS112), and DJ-1 (PA636 for DJ-1b at 1:1,000; PA632 for DJ-1a at 1:1,000; PA930 for hDJ-1 at 1:1,000) (1).

Respiration Analysis. Sixty to eighty flies were anesthesized with CO_2 and homogenized in mitochondrial isolation buffer [MIB; 210 mM mannitol, 70 mM sucrose, 10 mM Mops, and 1 mM EGTA (pH7.2)] with 1% fatty acid–free BSA. The homogenate was centrifuged at 4 °C at 300 × *g* for 3 min and recentrifuged at 4 °C at 6,000 × *g* for 10 min. The pellet was washed with the same isolation buffer and kept on ice until use. Mitochondria were assayed at 25 °C in Clark type electrode (Hansatech or Strathkelvin) in mitochondrial isolation buffer supplemented with 5 mM KH_2PO_4 without BSA. Oligomycin and potassium cyanide were added at the end of each run. For fly heads, 110 heads homogenized in 500 μL of MIB with BSA. Mitochondrial function was assayed using whole homogenate without isolation. For mouse skeletal muscle analysis, muscle was minced into small pieces on ice, then 10 mL of buffer [100 mM KCl, 50 mM Tris, 5 mM MgCl2, and 1 mM EDTA (pH 7.5), plus 100 mM ATP per mL of buffer and 0.5% BSA] was added per 1 g of wet tissue. Muscle was subjected to two strokes of a motor-driven Teflon homogenizer on ice. Four milligrams of diethylprocarbonate-treated trypsin per gram of wet muscle weight was dissolved in 1 mL of buffer and added to the homogenate with stirring for 2 min on ice. The homogenate was diluted 1:1 with buffer without ATP plus soybean trypsin inhibitor (4 mg of soybean trypsin inhibitor was dissolved in 10 mL of buffer per every 4 mg of trypsin used) followed by two more strokes on ice. The homogenate was centrifuged for 10 min at 4 °C at 1,000 × *g*. The low-speed supernatant was centrifuged for 10 min at 4 °C at 14,000 × *g*. The mitochondrial pellet was washed with buffer without ATP and centrifuged at 10,000 × *g* for 10 min at 4 °C. Washed mitochondria were resuspended for assay in a small volume of MIB.

Tetramethylrhodamine Ethylester (TMRE) Staining. Mitochondrial protein concentration was determined using the BCA (Pierce) assay using BSA as standard. Mitochondria (50 μg) were diluted into MIB buffer containing 5 mM KH_2PO_4 and 200 nM TMRE (Invitrogen). Baseline fluorescence was recorded using an excitation wavelength of 549 nm and an emission wavelength of 574 nm. Substrate (10 mM pyruvate plus 2 mM malate or 10 mM succinate) was added to energize mitochondria, followed by addition of 2 μM carbonyl cyanide m-chlorophenyl hydrazone to uncouple mitochondria.

ATP Analysis. ATP levels were determined using the ATP Bioluminescence Assay Kit HT II (Roche) according to the manufacturer's protocol. In brief, five thoraces were homogenized in cell lysis buffer and immediately frozen in dry ice bath with 95% ethanol. Samples were then heated to 99 °C for 3 min and briefly centrifuged at 6,010 × *g*. Two microliters of supernatant were diluted into 1,000 μL of dilution buffer, and 50 μL of the final mixture was used to determine ATP level. ATP standards were used to determine ATP level in the samples and then corrected for protein concentration input determined by BCA protein assay (Pierce). For mouse tissues, half of the mouse brain cortex and ≈0.2 g of hindlimb muscle were dissected out. The muscle biopsy was kept in PBS on ice while the brain was first processed. For all tissue, 2 mL of cell lysis buffer from the kit was used with 10 strokes of homogenization using fretted glass on fretted glass homogenizer. The homogenate was quickly placed into methanol/dry ice bath. The samples were then placed in 100 °C for 5 min and then briefly centrifuged. The supernatant was taken out, and 2 μL was diluted into 1,000 μL of dilution buffer. Fifty microliters of the final solution was used for ATP assay.

Generation of *DJ-1* Null Mice. Murine embryonic stem cells (clone XE726) with a recombination resulting in the disruption of the *DJ-1* gene were obtained from BayGenomics. This cell line was generated by random exon trapping, and it was determined by DNA sequencing that the recombination event occurred after exon 6 in the murine *DJ-1* gene. These embryonic stem cells were injected in blastocytes as a service of the University of Pennsylvania Transgenic and Chimeric Mouse Facility, and chimeric mice were generated. The germ line transmission of the null gene was determined by breeding chimeras with control mice and PCR analyses of tail DNA with primers specific for the *neo* gene (CCCAGGTCCCGAAAACCAAAGAAGAAGAACG and CGGCGGATTGACCGT-

AATGGGATAGGTCACG). Mice homozygous for the null allele were generated by crossing F1 mice, and homozygous null mice were determined by Western blot analysis of mouse tail protein samples (dissolved in 4% SDS, 8 M urea) using an antibody to DJ-1.

Mouse Rotorod Assay. Mice were tested for motor endurance with a rotorod treadmill (model 7650, Ugo Basile) set with accelerating revolution (4–40 revolutions per minute) over a 5-min period. Mice were given three trials with 45-min intertrial intervals on each of 2 consecutive days for 3 weeks. The time that each mouse remained on the beam was recorded in seconds, and the average endurance time was calculated for the third week trial.

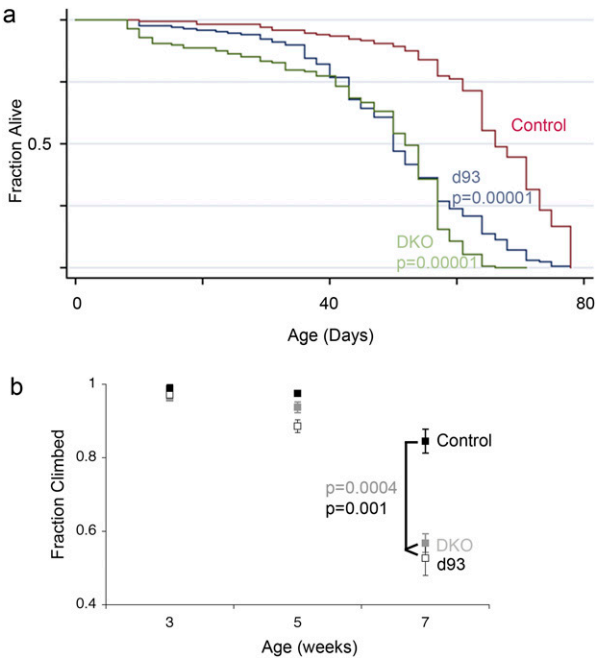


Fig. S1. Double knockout (DKO) and DJ-1b single knockout flies have reduced lifespan and climbing ability. DJ-1b single knockout flies, *d93*, show a similar lifespan curve compared with DKO flies (n = 214 for DKO, 337 for *DJ-1b[d93]*, and 299 for control flies). The control and DKO survival curve and climbing graphs are the same as those in Fig. 1 (main text).

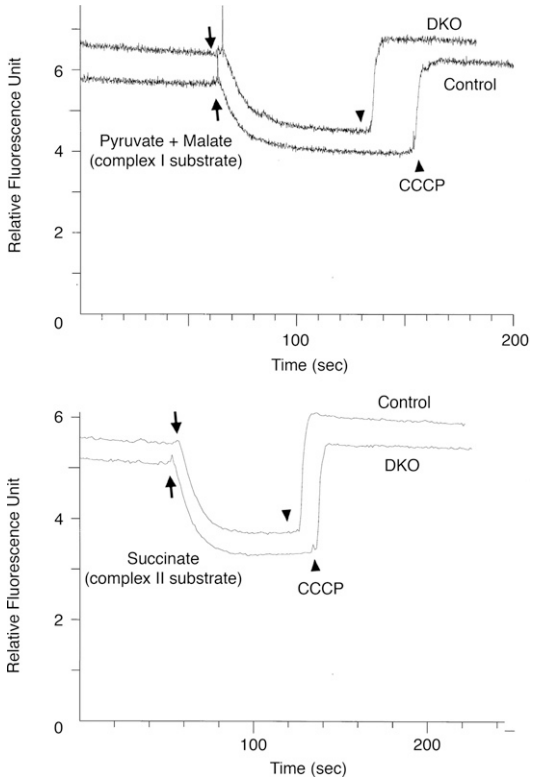
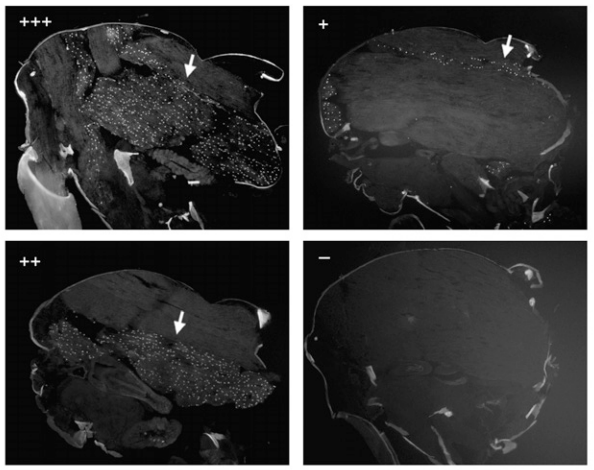


Fig. S2. Mitochondrial membrane potentials of double knockout (DKO) and control flies. Mitochondria membrane potential from DKO and control flies was assayed using TMRE dye. *Upper:* Generation of cross-membrane potential upon NADH-linked substrate addition (arrows), and dissipation of the potential upon the addition of uncoupler (arrowheads). (*Lower*) Same assay but with an $FADH_2$-linked substrate.



Genotype	Average TUNEL score ± S.E.M.
pink1^{B9}*;da-GAL4*	3.00 ± 0.00 (n=7)
pink1^{B9}*;UAS-DJ-1a;da-GAL4*	1.30 ± 0.17 (n=9)
pink1^{B9}*;UAS-parkin;da-GAL4*	0.50 ± 0.20 (n=7)
pink1^{Rev}	0.25 ± 0.25 (n=4)

Fig. S3. Up-regulation of DJ-1a protects against cell death in *pink1*^{B9} flies. TUNEL signals (white arrows) on fly thoraces in 6-day-old male flies were divided into four categories, depending on the amount of TUNEL signals. The pictures depict examples of widespread TUNEL signal (+++, score of 3 in the table), limited (++, 2), sparse (+, 1), and absence (-,0). Flies with *DJ-1a* up-regulated significantly reduced the TUNEL signals compared with *pink1*^{B9} flies with driver only ($P = 4.7 \times 10^{-7}$, *t* test).

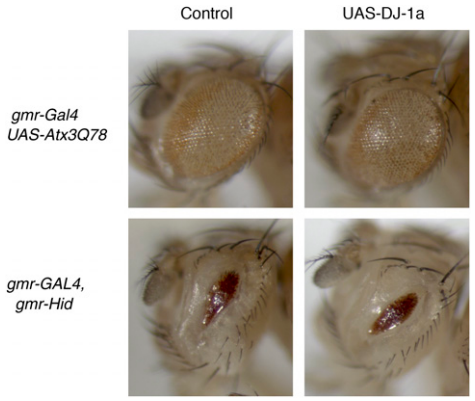


Fig. S4. DJ-1 up-regulation does not rescue all deleterious gene activities. Pictures of eyes of flies expressing pathogenic polyglutamine protein (*Upper*) or Hid-cell death gene (*Lower*) shows that up-regulation of DJ-1 has no effect on these deleterious eye effects. Flies used were *gmr-GAL4;UAS-Atx3Q78*, *UAS-DJ-1a*, and *gmr-GAL4;gmr-Hid*, *UAS-DJ-1a*.

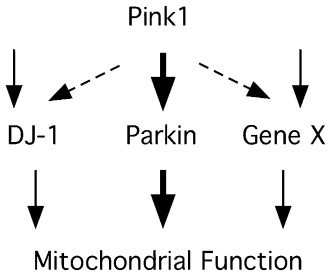


Fig. S5. Model of how *DJ-1* relates to *pink1* genetically. Diagram shows how *pink1*, *parkin*, and *DJ-1* function in relation to mitochondrial function in *Drosophila*. Up-regulation of Parkin ameliorates phenotypes in *pink1* null flies. When *DJ-1* is up-regulated, *pink1* null flies can be partially rescued, specifically in the thorax. The partial rescue of *pink1* by *DJ-1* suggests that *DJ-1* is downstream of *pink1*, thus the dashed arrow. DJ-1 may likely respond to other input independent of *pink1*. Gene X denotes other possible genes that act in a similar manner as *DJ-1*, in that they may partially rescue *pink1* but act independently of *parkin*. *Omi/HtrA2*, a possible PD gene, has been suggested to interact with *pink1/parkin* in this fashion.

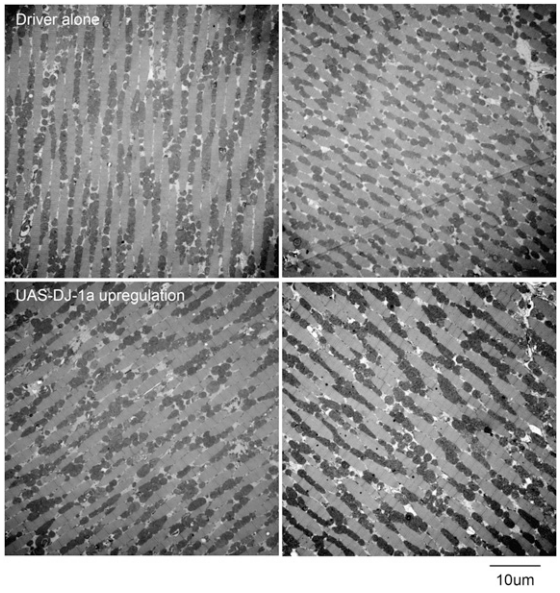


Fig. S6. DJ-1 up-regulation does not alter mitochondrial morphology. Electron microscopy of two young male thoraces (<3 days) showing similar mitochondrial morphology in flies with or without DJ-1a ubiquitous up-regulation. Flies used were *da-GAL4* and *UAS-DJ-1a;da-GAL4.* (Magnification, ×2,500.)

Table S1. Fertility of DJ-1 mutants

Male genotype*	Fertility (%)†
DJ-1a[d72]‡	15/17 (88)
DJ-1b[d93]§	10/10 (100)
DJ-1a[d72];UAS-sDJ-1b DJ-1b[d93]	9/34 (26)
DJ-1a[d72];da-GAL4 DJ-1b[93]/DJ-1b[d93]	10/21 (48)
DJ-1a[d72];UAS-sDJ-1b DJ-1b[d93]/da-GAL4 DJ-1b[d93]	15/16 (94)

Whereas the individual *DJ-1a* and *DJ-1b* mutants have robust fertility, double mutants have reduced fertility. The fertility is rescued robustly upon rescue by *DJ-1b*.

*All crosses were set up using male flies after six backcrosses using Bloomington line 5905.

†Fertility was scored 10 days after single-pair mating of denoted male and Bloomington line 5905 virgins was set up.

‡Allele d72 denotes null allele for *DJ-1a*.

§Allele d93 denotes null allele for *DJ-1b*.

Table S2. Rescue of *pink1* loss of function by *DJ-1*

Genotype	Rescue % (total)	TUNEL score (total)
$pink1^{B9}$;;*da-GAL4/+*	39.7 (60/151)	2.25 ± 0.75 (*n* = 4)
$pink1^{B9}$;*UAS-sDJ-1b.C104A/+;da-GAL4/+*	34.6 (35/101)	1.80 ± 0.48 (*n* = 5)
$pink1^{B9}$;*UAS-sDJ-1b.C45A/+;da-GAL4/+*	85.6 (101/118)	0.60 ± 0.24 (*n* = 5)

A transgene expressing *DJ-1b* with a point mutation at C104 (analogous to C106 in *hDJ-1*) cannot rescue *pink1* wing posture or TUNEL signal in the thorax, whereas DJ-1b with a point mutation at C45 retains rescue activity. DJ-1b with the C104 point mutation also cannot rescue the sensitivity of *DJ-1b* mutants to oxidative toxins, whereas the C45 mutant can rescue this toxicity (1). The number of thoraces scored is reported in parentheses, and the TUNEL score is based on numerical scale of 0 (absence of TUNEL signal), 1 (sparse signal), 2 (limited signal), and 3 (widespread TUNEL signal).

1. Meulener MC, et al. (2006) Mutational analysis of DJ-1 in *Drosophila* implicates functional inactivation by oxidative damage and aging. *Proc Natl Acad Sci USA* 103:12517–12522.

Movie S1. Control sperm are motile. Testes from control flies (3 days) were dissected in Ringer's solution and immediately pressed under a coverslip to release mature sperm from the seminal vesicle and testicular duct. The control sperm released have rotating tails, indicating motility.

Movie S1

Movie S2. Double knockout (DKO) sperm are immotile. Testes from DKO flies (3 days) were prepared as control testes. In contrast to the rotating sperm tails of control sperm, the DKO sperm showed no movement.

Movie S2