Supporting Information

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SI Materials and Methods

Fly Stocks Used. The *UAS-CD8GFP*, *UAS-MitoGFP*, *UAS-GFP*, *TH-GAL4*, *Cha-GAL4*, *Repo-GAL4*, and *Elav-GAL4* transgenic lines were obtained from the Bloomington Drosophila Stock Center. The *UAS-Mfn-RNAi* transgenic line was obtained from the Vienna *Drosophila* RNAi Center. The ParkinC2 transgenic line was generated in-house. The $park^{25}$-null allele (1), $PINK1^{B9}$ null allele (2), the *UAS-ATG8a* (3), *UAS-Drp1* (4), and *UAS-PINK1* (2) transgenic lines have been described previously.

For Fig. 1 *B–D*: w^{1118} = GFP^{-}; UAS-CD8GFP/″; TH-GAL4/″ or UAS-GFP/″; TH-GAL4/″ = dopaminergic (DA)-GFP; UAS-GFP, Cha-GAL4/$^{+}$ = cholinergic (CH)-GFP; UAS-CD8GFP, Elav-GAL4/″ or Y = pan-neuronal GFP; UAS-CD8GFP, Repo-GAL4/″ = Glial-GFP.

For Fig. 1*E*: UAS-CD8GFP/″; TH-GAL4/″ = DA neurons; UAS-GFP, Cha-GAL4/$^{+}$ = CH neurons.

For Fig. S1*A*: UAS-CD8GFP/″; TH-GAL4/″ = DA neurons.

For Fig. S1*C*: UAS-GFP/″; TH-GAL4/″ and UAS-CD8GFP/″; TH-GAL4 and UAS-mitoGFP/″; TH-GAL4/” = DA neurons.

For Fig. 2*A*: UAS-mitoGFP/UAS-CD8GFP or UAS-GFP; $park^{25}$-THGAL4/$park^{25}$, UAS-mitoGFP or MKRS = sibling control and parkin mutant DA neurons.

For Fig. 2*B*: UAS-CD8GFP/CyO or UAS-PINK1; TH-GAL4/$^{+}$ = sibling control and PINK1 overexpressing DA neurons.

For Fig. 2*C*: UAS-GFP, Cha-GAL4/CyO; $park^{25}$/MKRS or $park^{25}$ = sibling control and parkin mutant CH neurons.

For Fig. 2*D*: UAS-GFP, Cha-GAL4/CyO or UAS-PINK1 = sibling control and PINK1 overexpressing CH neurons.

For Fig. 2*E*: Genotypes described above with addition of $PINK1^{rva}$/Y; UAS-CD8GFP/$^{+}$; THGAL4/$^{+}$; and $PINK1^{B9}$/Y; UAS-CD8GFP/$^{+}$; THGAL4/$^{+}$ = PINK1 control and null mutant DA neurons, respectively; UAS-CD8GFP/UAS-$Parkin^{C2}$; $park^{25}$-THGAL4/$park^{25}$ = Parkin overexpression in parkin mutant DA neurons.

For Fig. S2: UAS-CD8GFP/UAS-CD8GFP; TH-GAL4/TH-GAL4 = DA neurons.

For Fig. 3: UAS-mitoGFP or UAS-CD8GFP/CyO; $park^{25}$-THGAL4/MKRS = sibling control; UAS-mitoGFP or UAS-CD8GFP/CyO; $park^{25}$/$park^{25}$-THGAL4 = parkin-null; UAS-mitoGFP/CyO; $park^{25}$, UAS-Drp1/$park^{25}$, THGAL4 = parkin mutant expressing DRP1; UAS-mitoGFP/CyO; $park^{25}$, UAS-MfnRNAi/$park^{25}$, THGAL4 = parkin mutant expressing Mfn-RNAi; UAS-mitoGFP or UAS-CD8GFP/UAS-ATG8a, UAS-GFP; $park^{25}$/$park^{25}$, TH-GAL4 = parkin mutant expressing ATG8a. All expression restricted to DA neurons.

For Fig. S3: UAS-mitoGFP or UAS-CD8GFP/CyO; $park^{25}$-THGAL4/MKRS = DA parkin sibling control; UAS-mitoGFP or UAS-CD8GFP/CyO; $park^{25}$/$park^{25}$-THGAL4 = DA parkin mutant; UAS-CD8-GFP/″; TH-GAL4/″ = wild-type.

For Fig. 4: CantonS and w^{1118} = WT; +/CyO; $park^{25}$/$park^{25}$, TH-GAL4 = parkin mutant; +/Cyo; $park^{25}$, UAS-Drp1/$park^{25}$, TH-GAL4 = parkin mutant expressing DRP1; +/CyO; UAS-MfnRNAi, $park^{25}$/$park^{25}$, TH-GAL4 = parkin mutant expressing Mfn-RNAi; UAS-ATG8a, UAS-GFP/UAS-mito-GFP; $park^{25}$/$park^{25}$-TH-GAL4 = parkin mutants expressing ATG8a. All expression restricted to DA neurons.

For Fig. S4*A*: UAS-mitoGFP/UAS-CD8GFP or UAS-GFP; $park^{25}$, THGAL4/$park^{25}$, UAS-mito-GFP or MKRS = sibling control and parkin mutant DA neurons.

For Fig. S4 *B* and *C*: UAS-mitoGFP/CyO; $park^{25}$, TH-GAL4/MKRS or $park^{25}$ = sibling control or parkin mutant DA neurons, respectively.

For Fig. S4 *D* and *E*: UAS-mitoGFP/UAS-CD8-GFP; $park^{25}$, TH-GAL4/MKRS or $park^{25}$, UAS-mitoGFP = sibling control and mutant DA neurons, respectively; Cha-GAL4, UAS-GFP/CyO; $park^{25}$/MKRS or $park^{25}$ = sibling control or parkin mutant CH neurons, respectively.

For Fig. S5: UAS-mitoGFP/UAS-CD8GFP or UAS-GFP; $park^{25}$-THGAL4/$park^{25}$, UAS-mito-GFP or MKRS = sibling control and parkin mutant DA neurons.

Analysis of Mitochondrial Morphology. Neural preparations stained with Mitotracker Deep Red (Mt-DR) were plated on 0.1% poly-L-lysine–coated glass cover-slips and maintained in a 5% (vol/vol) CO_2 incubator, at room temperature, for 2.5 h. Cultures were then fixed in 3% (wt/vol) paraformaldehyde on ice for 20 min, followed by the addition of −20 °C methanol and further incubation for 3 min on ice. Cells were then washed and incubated at 4 °C overnight with a mouse anti-GFP antibody (Molecular Probes). Following antibody incubation, cultures were washed in 1% BSA and incubated with a fluorescent secondary antibody (Molecular Probes) for 1 h at room temperature. After washing, the coverslips were incubated for 5 min in buffer containing 4 μg/mL DAPI, then washed thoroughly, and mounted onto glass slides with Fluoromount (Sigma) for subsequent imaging by confocal microscopy. All wash and incubation solutions were prepared in Hanks Buffered Saline Solution (Media Tech). Images were processed using ImageJ software (5) and the fragmentation status of mitochondria assessed by viewing Mt-DR fluorescence in identified cell bodies. Mitochondria were determined as fused if the mitochondrion appeared continuous throughout its entire length and fragmented if mitochondria were present as multiple physically distinct entities. Mitochondrial length was determined using ImageJ software.

Quantification of mtDNA Abundance. Ten DA neurons were pooled by FACS into 5 μL of 10 mM Tris pH 7.9 (Sigma), 1% Triton X-100 (Sigma), and 200 μg/mL Proteinase K (Fisher). Samples were then incubated at 37 °C for 30 min, 55 °C for 30 min, 95 °C for 7.5 min, and stored at −20 °C. Quantitative PCR was performed on a Roche LightCycler 480 using standard cycling protocols. A 5′ hydrolysis probe set was used to determine the Ct value of mtDNA using forward (5′-aatggagctggaacaggatg-3′) and reverse (5′-aagaaatccctgctaaatgtagaga-3′) primers and a 5′ 6-carboxyfluorescein hydrolysis probe (5′-ccacctttatccgctggaattgctca-3′). The Ct value of a nuclear encoded reference gene, *α-tubulin*, was determined using Taqman assay Dm02361072_s1. Experimental samples were normalized to *α-tubulin* Ct values, and the relative fold-change in mtDNA abundance calculated for *parkin* homozygous mutants relative to heterozygous sibling controls using the ΔΔCt method.

1. Greene JC, et al. (2003) Mitochondrial pathology and apoptotic muscle degeneration in *Drosophila parkin* mutants. *Proc Natl Acad Sci USA* 100:4078–4083.

2. Park J, et al. (2006) Mitochondrial dysfunction in *Drosophila* PINK1 mutants is complemented by parkin. *Nature* 441:1157–1161.

3. Simonsen A, et al. (2008) Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult *Drosophila*. *Autophagy* 4:176–184.
4. Deng H, Dodson MW, Huang H, Guo M (2008) The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in *Drosophila*. *Proc Natl Acad Sci USA* 105:14503–14508.
5. Abramoff MD, Magalhaes PJ, Ram SJ (2004) Image processing with ImageJ. *Biophotonics International* 11:36–42.

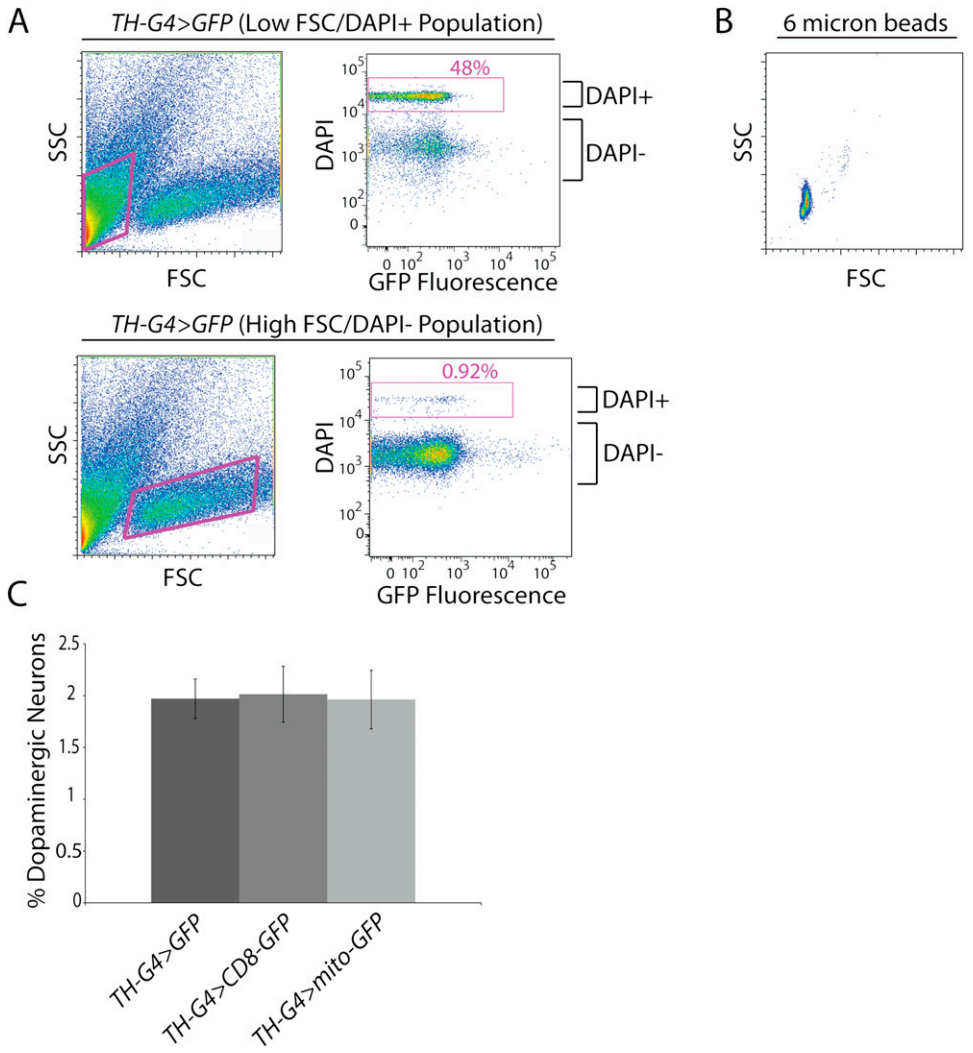

Fig. S1. Purification and characterization of DA neurons from the adult *Drosophila* brain. (*A*) A neural suspension was prepared from flies expressing GFP in DA neurons and examined by flow cytometry. A total of 123,560 particles were analyzed for forward scatter (FSC), which provides an indication of particle size, and side scatter (SSC), which provides an indication of internal cellular complexity. The purple boxes in the *Upper* and *Lower Left* define a subpopulation of 66,642 cells with low FSC values or small particle size, and a subpopulation of 20,438 cells from the same preparation with high FSC values, respectively. These two cell populations were subjected to DAPI and GFP fluorescence intensity analysis depicted in the *Upper* and *Lower Right*. The percentage of cells that fluoresce strongly with DAPI (defined by the purple boxes in the *Upper* and *Lower Right*) is indicated. (*B*) The FSC and SSC distribution for 6-μm beads is shown, illustrating that the size range of the high FSC cell population in *A* is ∼6 μm, and therefore similar to the known size of the neurons of interest. (*C*) The proportion of $DAPI^{-}/GFP^{+}$ DA neurons from flies of the indicated genotypes plotted as a percentage of the total cells observed under fluorescence gating. The number of biological replicates (*n*) and total number of cells analyzed (*N*) were as follows: *TH-G4* > *GFP* (*n* = 3; *N* = 472); *TH-G4* > *CD8-GFP* (*n* = 5; *N* = 1,019); *TH-G4* > *mito-GFP* (*n* = 3; *N* = 255).

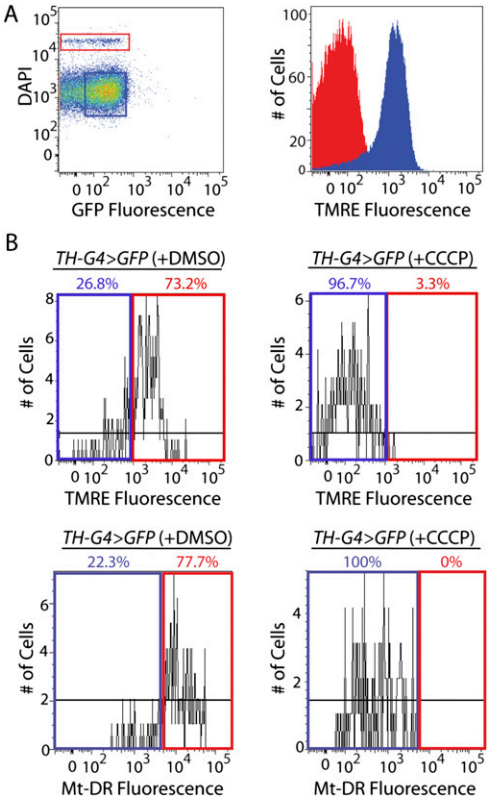

Fig. S2. Characterization of mitochondrial membrane potential (MMP) in isolated DA neurons. (*A*) A neural culture from *TH-G4* > *GFP* flies was labeled with DAPI and TMRE, and analyzed by flow cytometry. The dead ($DAPI^+$) and living ($DAPI^-$) cell populations are demarcated within the red and blue boxes, respectively (*Left*), and the TMRE fluorescence intensity frequency distributions of these two cell populations are shown (*Right*). (*B*) Neural cultures from *TH-G4* > *GFP* flies were treated with either DMSO or CCCP, labeled with either TMRE (*Upper*) or Mt-DR (*Lower*), and analyzed by flow cytometry. The percentage of cells exhibiting TMRE or Mt-DR fluorescence intensities above or below the cut-off values is indicated above each histogram.

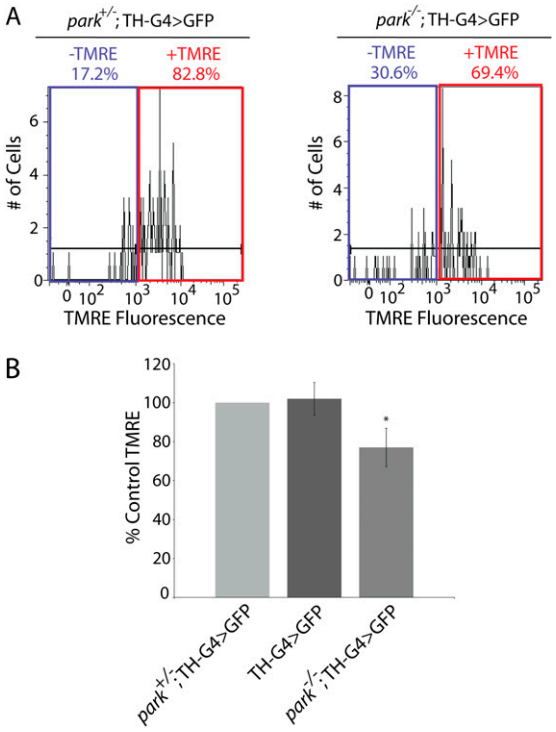

Fig. S3. Characterization of MMP in DA neurons from *parkin* mutants. (*A*) Neural cultures from *parkin*-null heterozygote controls and sibling *parkin*-null homozygotes expressing GFP in DA neurons ($park^{+/-}$; *TH-G4* > *GFP* and $park^{-/-}$; *TH-G4* > *GFP*, respectively) were labeled with TMRE and analyzed by flow cytometry. Representative histograms show the percentage of neurons that exhibit TMRE fluorescence intensities above and below the cut-off value. (*B*) Relative average MMP in DA neurons from animals of the indicated genotypes. The number of biological replicates (*n*) and total number of cells analyzed (*N*) for the following genotypes were as follows: $park^{+/-}$; *TH-G4* > *GFP* (*n* = 4; *N* = 737); *TH-G4* > *GFP* (*n* = 4; *N* = 290); $park^{-/-}$; *TH-G4* > *GFP* (*n* = 4; *N* = 457). $^{*}P \leq 0.05$.

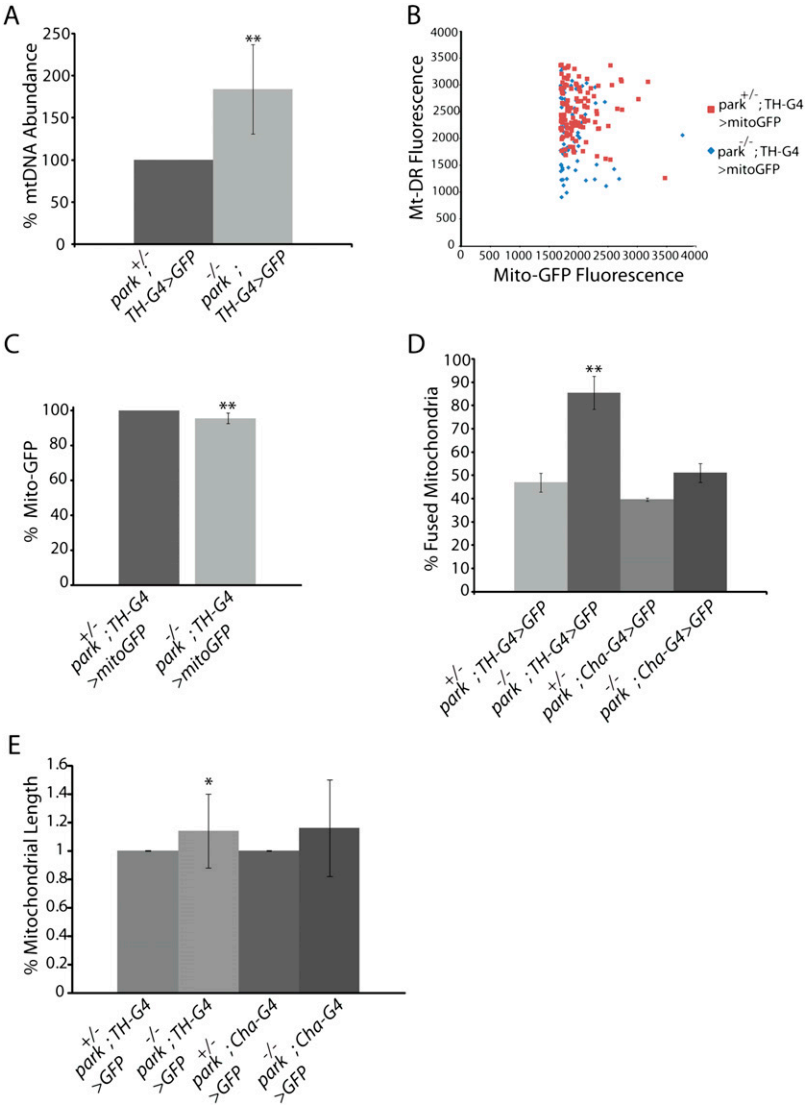

Fig. S4. Effect of null mutations in *parkin* on mitochondrial abundance and morphology. (*A*) FACS isolated DA neurons from *parkin*-null heterozygous (*park*$^{+/-}$; *TH-G4* > *GFP*) and homozygous (*park*$^{-/-}$; *TH-G4* > *GFP*) animals were analyzed by qPCR for mtDNA abundance relative to a reference nuclear gene. The number of biological replicates (*n*) and total number of pooled 10-cell samples analyzed (*N*) were as follows: *park*$^{+/-}$; *TH-G4* > *GFP* (*n* = 3; *N* = 12); *park*$^{-/-}$; *TH-G4* > *GFP* (*n* = 3; *N* = 12). Error bars represent SE. (*B*) Dot plot of Mt-DR fluorescence intensity relative to mito-GFP fluorescence intensity from individual dopaminergic neurons isolated from *parkin* null heterozygotes and sibling *parkin*-null homozygotes expressing mito-GFP in DA neurons (*park*$^{+/-}$; *TH-G4* > *mito-GFP* and *park*$^{-/-}$; *TH-G4* > *mito-GFP*, respectively). Total numbers of cells analyzed (*N*) were as follows: *park*$^{+/-}$; *TH-G4* > *mito-GFP* (*N* = 113; R^2 = 0.0036); *park*$^{-/-}$; *TH-GAL4* > *mito-GFP* (*N* = 76; R^2 = 0.013). (*C*) The mean relative GFP fluorescence intensity in DA neurons from *parkin*-null heterozygotes and sibling *parkin*-null homozygotes expressing mito-GFP in DA neurons (*park*$^{+/-}$; *TH-G4* > *mito-GFP and park*$^{-/-}$; *TH-G4* > *mito-GFP*, respectively). The number of biological replicates (*n*) and total number of cells analyzed (*N*) were as follows: *park*$^{+/-}$; *TH-G4* > *mito-GFP* (*n* = 3; *N* = 177); *park*$^{-/-}$; *TH-G4* > *mito-GFP* (*n* = 3; *N* = 116). (*D*) Neural cultures from *parkin*-null heterozygote controls and sibling *parkin* null homozygotes expressing GFP in DA neurons (*park*$^{+/-}$; *TH-G4* > *GFP and park*$^{-/-}$; *TH-G4* > *GFP*, respectively) or in CH neurons (*park*$^{+/-}$; *Cha-G4* > *GFP* and *park*$^{-/-}$; *Cha-G4* > *GFP*, respectively) were imaged by confocal microscopy. The fraction of cells exhibiting a continuous fused mitochondrion (% Fused Mitochondria) was scored relative to heterozygous controls. The number of biological replicates (*n*) and total number of cells analyzed (*N*) were as follows: *park*$^{+/-}$; *TH-G4* > *GFP* (*n* = 3; *N* = 66); *park*$^{-/-}$; *TH-G4* > *GFP* (*n* = 3; *N* = 62); *park*$^{+/-}$; *Cha-GAL4* > *GFP* (*n* = 3; *N* = 48); *park*$^{-/-}$; *Cha-GAL4* > *GFP* (*n* = 3; *N* = 47). (*E*) Neural cultures, as described in *D*, were imaged by confocal microscopy, and the length of mitochondria within these cells measured using ImageJ software (5). The fractional length of mitochondria in homozygous *parkin* mutant neurons relative to heterozygous controls is indicated (% Mitochondrial Length). The number of biological replicates (*n*) and total number of cells analyzed (*N*) were as follows: *park*$^{+/-}$; *TH-G4* > *GFP* (*n* = 3; *N* = 52); *park*$^{-/-}$; *TH-G4* > *GFP* (*n* = 3; *N* = 47); *park*$^{+/-}$; *Cha-GAL4* > *GFP* (*n* = 3; *N* = 58); *park*$^{-/-}$; *Cha-GAL4* > *GFP* (*n* = 3; *N* = 38). *$P \leq 0.05$; **$P < 0.01$.

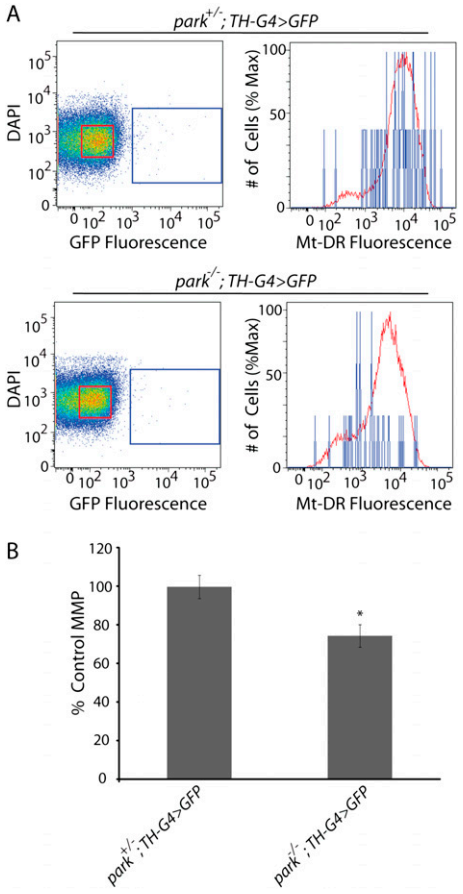

Fig. S5. The MMP of DA neurons relative to other neural cell types in *parkin* mutants. (*A*) Neural cultures from *parkin*-null heterozygotes (*park*$^{+/-}$; *TH-G4 > GFP)* or sibling *parkin*-null homozygotes (*park*$^{-/-}$; *TH-G4 > GFP)* expressing GFP in DA neurons were labeled with DAPI and Mt-DR and analyzed by flow cytometry. The GFP^{-} and GFP^{+} cell populations are demarcated within the red and blue boxes, respectively (*Left*). At right, Mt-DR fluorescence intensity frequency distributions from these two cell populations are shown relative to one another, with the *y* axis scaled as the percentage of the maximum number of cells measured for each neural cell population [# of Cells (% Max)]. (*B*) The mean MMP of DA neurons from the genotypes described in *A* expressed relative to the mean MMP of the non-GFP expressing cells in the same culture tube (% MMP). The number of biological replicates (*n*) and total number of cells analyzed (*N*) were as follows: *park*$^{+/-}$; *TH-G4 > GFP* (*n* = 7; *N* = 330); *park*$^{-/-}$; *TH-G4 > GFP* (*n* = 8; *N* = 444). Error bars represent SE. *$P \leq 0.05$.