**Drosophila** Parkin requires PINK1 for mitochondrial translocation and ubiquitinates Mitofusin

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**Loss of the E3 ubiquitin ligase Parkin causes early onset Parkinson’s disease, a neurodegenerative disorder of unknown etiology.** Parkin has been linked to multiple cellular processes including protein degradation, mitochondrial homeostasis, and autophagy; however, its precise role in pathogenesis is unclear. Recent evidence suggests that Parkin is required for mitochondrial fission and/or fusion, to mediate their autophagic turnover. The precise mechanism of recruitment and the ubiquitination target are unclear. Here we show in *Drosophila* cells that PINK1 is required to recruit Parkin to dysfunctional mitochondria and promote their degradation. Furthermore, PINK1 and Parkin mediate the ubiquitination of the fusion factor Mfn on the outer surface of mitochondria. Loss of *Drosophila* PINK1 or parkin causes an increase in Mfn abundance in vivo and concomitant elongation of mitochondria. These findings provide a molecular mechanism by which the PINK1/Parkin pathway affects mitochondrial fission/fusion as suggested by previous genetic interaction studies. We hypothesize that Mfn ubiquitination may provide a mechanism by which terminally damaged mitochondria are labeled and sequestered for degradation by autophagy.

**Results**

**PINK1 and Parkin Knockdown Cause Mitochondrial Elongation.** Our previous genetic interaction studies in *Drosophila* cells by incubation of long double-stranded RNA molecules complementary to the target gene (26). We performed targeted RNAi against *PINK1*, *parkin*, and the *Drosophila* homologs of Drp1, Fis1, Opal, and Mfn1/2 (here called Mfn). The targets typically caused ~90% knockdown of the endogenous message after 3 days (Fig. S1). We found that sample fixation grossly altered the mitochondrial morphology (Fig. S2A), for this study cells were imaged live using the selective mitochondrial dye rhodamine 123 (Fig. 1A and C). As expected, we found that knockdown of the fusion genes Drp1 and Fis1 resulted in elongated mitochondria whereas knockdown of the fusion genes Opal and Mfn (also known as *Marf*) resulted in fragmented mitochondria. We found that knockdown of *PINK1* or *parkin* caused a significant elongation of mitochondrial lengths. Elongated mitochondria were also seen when transfected mitoGFP was used to label mitochondria (Fig. S2B). Conversely, overexpression of *Opal* and *Mfn* caused a hyperfused network whereas overexpression of *Drp1* and *Fis1* caused...
fragmentation (Fig. 1B and D). Overexpression of parkin also caused fragmentation; however, the effect of PINK1 was not significant, suggesting that PINK1 may not be limiting or may need to be activated itself to ectopically promote fragmentation.

The relative levels of key mitochondrial morphology factor transcripts were unchanged by parkin or PINK1 knockdown (Fig. S3A), indicating that the morphology changes do not occur from a transcriptional response altering the level of fission or fusion gene expression. Also, in contrast to a previous report (8), we find that Parkin protein levels are not changed in a PINK1 mutant (Fig. S3B). Together these observations are consistent with the PINK1/Parkin pathway modulating the action of mitochondrial fission/fusion proteins to alter morphology.

**PINK1 Is Required for Parkin Translocation and Parkin-Mediated Mitophagy.** The recent study by Narendra et al. (18) revealed an important insight into the function of Parkin, reporting that it translocates to dysfunctional mitochondria and promotes mitophagy. We sought to determine whether this was a conserved function. In wild-type Drosophila cells, Parkin-GFP typically showed a diffuse localization throughout the cytoplasm with occasional accumulations at mitochondria (Fig. 2A and Fig. S4). Upon treatment with the mitochondrial uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) or the oxidative stressor paraquat, a large proportion of cells showed Parkin-GFP accumulated at or near mitochondria (Fig. 2A and Fig. S5), whereas overall Parkin-GFP abundance remained constant (Fig. S3C). Prolonged exposure to CCCP also led to the autophagy-dependent, but proteasome-independent, loss of mitochondria (Fig. 2B and Fig. S6). Consistent with previous reports, Parkin was also required for CCCP-induced mitophagy in Drosophila cells (Fig. 2B).

To extend this observation, we addressed whether PINK1, which acts upstream of Parkin, may affect the recruitment of Parkin to mitochondria and their autophagic degradation. Following PINK1 RNAi knockdown, CCCP- or paraquat-induced Parkin-GFP recruitment to mitochondria was prevented (Fig. 2 of one-way ANOVA with Bonferroni correction (**P < 0.01, ***P < 0.001).
In addition, loss of PINK1 also abrogated mitophagy (Fig. 2B). These data are consistent with PINK1 acting upstream to promote Parkin recruitment to dysfunctional mitochondria, which in turn promotes their degradation.

*M. Drosophila* Mitofusin is Ubiquitinated by PINK1/Parkin and Accumulates in Mutants. From the previous observations, we hypothesized that Parkin translocates to damaged mitochondria to alter their fission/fusion ability and to promote their degradation, presumably via its ubiquitin ligase activity. Thus, we sought to determine if any of the key mitochondrial morphology factors may be modified by ubiquitin and whether such modifications are Parkin dependent.

Flag-tagged forms of Drp1, Opal, and Mfn were coexpressed with hemagglutinin (HA)-tagged ubiquitin (HA-Ub) in *Drosophila* cells. Western blot analysis of these proteins revealed a single major band of the predicted size for Drp1 (Fig. 3A, white arrow). Opal1 was present in a small amount of the full-length form but predominately in the processed form (Fig. 3A, white arrowhead), as previously reported (27). However, Mfn was detected as a number of high-molecular-weight isoforms (asterisks) in addition to the expected full-length form (Fig. 3A, black arrowhead). The smallest Mfn isoform at ∼91 kDa is consistent with the predicted size of the full-length, unmodified form of *Drosophila* Mfn.

To assess whether these isoforms are ubiquitin modified, we performed coimmunoprecipitation experiments against HA-Ub. Immunoprecipitates of Flag-tagged Drp1, Opa1, and Mfn were prepared and Western blots were probed with an antibody against HA. HA-positive bands were detected in the Mfn (asterisks) and Opa1 (white diamonds) samples but not in Drp1 (Fig. 3A). Comparison with Western blots probed against Flag showed bands of equivalent size for Mfn-Flag (Fig. 3A, asterisks), consistent with these isoforms being ubiquitinated. The smallest Mfn isoform was not HA-Ub-positive (Fig. 3A, black arrowhead), which is consistent with this isoform being unmodified. Larger ubiquitinated isoforms of Opa1 are not detected by anti-Flag immunoblotting, suggesting that these are in very low abundance.

We next tested whether this ubiquitination is Parkin dependent by performing the same analysis again following RNAi knockdown of parkin. We also addressed whether attenuating the pathway more generally may affect Mfn ubiquitination, so PINK1 RNAi knockdown was also performed. Western blots of whole-cell lysates from parkin and PINK1 knockdown cells revealed a striking loss of the ubiquitinated forms of Mfn (Mfn-Ub) (Fig. 3B, asterisks; Fig. S7A). Interestingly, this appeared to be accompanied by an accumulation of nonubiquitinated Mfn. Following enrichment by immunoprecipitation, low levels of Mfn-Ub were detected in parkin knockdown cells that were undetectable in whole-cell lysates, likely reflecting incomplete RNAi knockdown. However, in PINK1 knockdown cells, no ubiquitinated Mfn could be detected even upon immunoprecipitation (Fig. 3B). In contrast, however, ubiquitinated forms of Opa1 were unchanged by the loss of parkin or PINK1 (Fig. 3B, white diamonds), suggesting that this modification is not PINK1/Parkin dependent. Again, no modification of Drp1 was observed or its abundance changed by parkin or PINK1 RNAi.

To gain further evidence that Mfn ubiquitination may be mediated by Parkin, we tested whether they may physically interact. Immunoprecipitation assays revealed that upon coexpression Parkin-GFP is detected in precipitates of Mfn-Flag (Fig. 3C). Together these data strongly support that Parkin mediates the ubiquitination of Mfn.

We sought to extend this observation in vivo. Using antibodies against *Drosophila* Mfn, we could detect full-length Mfn and high-molecular-weight Mfn-Ub (Fig. 3D), consistent with that seen in vitro. These bands were absent upon Mfn RNAi knockdown in vivo (Fig. 3D), indicating the specificity of the antisera. Consistent with our in vitro results, Mfn-Ub was greatly reduced in a parkin mutant background (Fig. 3D).

We have shown that PINK1 is required for Parkin recruitment to mitochondria; however, previous genetic experiments in vivo have shown that overexpression of parkin can compensate for loss of PINK1. Therefore, we addressed whether overexpressing parkin may restore Mfn ubiquitination in the absence of PINK1. In PINK1 RNAi knockdown cells only unmodified Mfn was seen (Fig. 4A); however, when Parkin was overexpressed in PINK1 RNAi cells, the Mfn-Ub isoforms were again detected (Fig. 4A). Conversely, if PINK1 was overexpressed in parkin RNAi cells, Mfn-Ub isoforms were not restored (Fig. 4B), consistent with...
accumulate upon loss of sensitive to small changes. These data indicate that Mfn levels are abundant in increase in Mfn abundance (Fig. 5). Mfn was also significantly more abundant in knockdown cells or as shown. Cells were harvested and subjected to Western blot analysis using the specific antibodies indicated.

genetic experiments showing that PINK1 overexpression cannot compensate for loss of parkin in vivo.

Ubiquitination is a common signal for the degradation of proteins and organelles. Because evidence indicates that Parkin and PINK1 promote mitophagy, we next assessed whether Mfn abundance is altered by loss of parkin and PINK1. The steady-state levels of endogenous Drosophila Mfn were determined in vitro and in vivo. Western blot of control treated cells and wild-type flies showed a band of the predicted full-length Mfn, which was absent in Mfn RNAi knockdown cells or flies (Fig. 5). parkin knockout cells or park25 null mutant flies showed a significant increase in Mfn abundance (Fig. 5). Mfn was also significantly more abundant in PINK1 knockdown or PINK1B9 null mutant flies (Fig. 5). Interestingly, the level of Complex Vα did not appreciably increase (Fig. S7B); however, this technique is not sensitive to small changes. These data indicate that Mfn levels accumulate upon loss of PINK1/parkin, consistent with their role in mitophagy, but likely reflects turnover of a small proportion of the entire mitochondrial content.

Because our evidence suggests that Parkin is recruited to mitochondria where it may ubiquitinate Mfn, we addressed whether Mfn is required for Parkin translocation. Parkin-GFP localization upon CCCP and paraquat treatment was monitored as before and compared with Mfn RNAi knockdown cells. Under control conditions we saw Parkin-GFP translocation comparable to previous results; however, in the absence of Mfn, Parkin-GFP translocation was not eliminated although the prevalence was markedly reduced (Fig. 6). As before, the overall abundance of Parkin-GFP was unaffected by treatments in Mfn RNAi cells (Fig. S3C). These results indicate that Mfn is not an absolute requirement for Parkin translocation.

**Discussion**

Maintenance of mitochondrial homeostasis appears to be an important function of the PINK1/Parkin pathway in multiple model systems and is likely a key factor in mediating neurodegeneration. Recent studies have begun to shed light on the potential mechanism by which this pathway maintains a healthy mitochondrial population. Emerging evidence indicates that PINK1 is required to recruit Parkin to damaged or dysfunctional mitochondria, whereupon it promotes mitophagy (23–25). Regulated mitochondrial fission and fusion events are thought to contribute to a quality control mechanism to help “sort out” terminally damaged mitochondria for degradation (19–21). Importantly, PINK1 and parkin have previously been shown to genetically interact with components of the mitochondrial fission/fusion machinery and to affect mitochondrial morphology (9–12); however, the molecular mechanisms are not known. Here we provide further evidence that PINK1 is required for Parkin translocation to damaged mitochondria and that this pathway affects mitochondrial morphology. We also provide evidence that the PINK1/Parkin pathway promotes the ubiquitination and regulates the levels of the profusion protein Mfn, thus providing a potential molecular mechanism by which PINK1/Parkin may modulate mitochondrial dynamics.

Consistent with recent reports (23–25), we find that the translocation of Parkin to damaged mitochondria and their subsequent autophagy is dependent on PINK1. However, the molecular mechanisms that promote Parkin’s recruitment to...
mitochondria are still unclear. PINK1’s kinase activity, but not mitochondrial localization, appears to be necessary for Parkin translocation (23–25). Because PINK1 can be found extramitochondrially (27–30) and may directly phosphorylate Parkin (31, 32), this may be a mechanism to stimulate its translocation. Alternatively, it may phosphorylate a Parkin substrate, e.g., Mfn, and thereby provide a recruitment signal. Interestingly, we find that loss of Mfn reduces but does not eliminate Parkin translocation. Recent evidence indicates that Parkin also ubiquitinates VDAC on the outer mitochondrial surface (23), suggesting that there may be multiple recruitment substrates. Although further work is required to elucidate these mechanisms, these findings suggest a molecular basis for the genetic hierarchy in which PINK1 acts upstream of Parkin (5–8).

To understand the role of Parkin translocation, we took a candidate approach to identifying putative substrates. Because the function of Parkin and PINK1 has been linked with mitochondrial dynamics, we surveyed key components of the mitochondrial fission and fusion machinery for ubiquitin modification. We found that Mfn, which localizes to the outer surface of mitochondria, is ubiquitinated in a PINK1/Parkin-dependent manner and accumulates upon loss of PINK1 or parkin. Interestingly, the ubiquitinated isoforms do not show a typical ubiquitination "ladder" but instead appear to reflect a pattern of one and three or four ubiquitin adducts. Although it remains to be shown that Parkin directly mediates this ubiquitination, there is evidence that Parkin can mediate monoubiquitination (33–37) and K27 (23) and K63 linkages (38, 39). These modes of ubiquitination are not typically linked to proteasome degradation, and there is growing speculation that important pathogenic functions of Parkin may be proteasome independent (reviewed in ref. 40).

Numerous elegant studies have demonstrated that the mitochondrial network is extremely dynamic and responds rapidly and reversibly to many physiological changes including potentially toxic challenges such as oxidative stress and calcium flux (reviewed in refs. 41 and 42). Although mitochondrial remodeling can contribute to promoting cell death, it can also act in a protective manner by contributing to a quality control process that likely involves degradation by autophagy/lysosomes. Recent work has reported observations that, following a fission event, regulated fusion of daughter mitochondria can determine whether they rejoin the network or are sequestered for degradation (19). Refusion appears to be dependent upon the recovery of mitochondrial membrane potential after division and likely represents a mechanism to sort out terminally dysfunctional mitochondria (20). Because Mitofusins mediate the tethering and fusion of mitochondria via homo- and heterotypic interaction of their HR2 domains (43), we hypothesize that Parkin-mediated Mfn ubiquitination may interfere with intermolecular interactions preventing fusion. Alternatively, Mfn ubiquitination may lead to a selective removal of Mfn from damaged mitochondria and thus reduce the refusion capacity of those mitochondria. Consistent with this, we find that loss of parkin or PINK1, and hence loss of ubiquitination, leads to increased Mfn levels and mitochondrial elongation, presumably due to excess fusion. Thus, Mfn ubiquitination may provide a signal that simultaneously prevents the refusion of terminally damaged mitochondria and labels them for safe degradation by autophagy (22).

It is reasonable to suppose that under normal conditions the majority of mitochondria are relatively healthy, and thus mitochondrial turnover is an infrequent event. This is supported by the observation that Complex V0 levels are not significantly altered by decreased mitophagy. However, this rationale implies that Mfn accumulates and is selectively ubiquitinated on mitochondria targeted for degradation although this remains to be shown. Interestingly, our findings provide a molecular mechanism that can explain the previously reported genetic interactions between PINK1 and parkin and the fission/fusion factors—in particular, that promoting mitochondrial fragmentation by overexpression of Drp1 or by reduction of Mfn and Opa1 is able to partially suppress the locomotor deficits, muscle degeneration, and mitochondrial abnormalities (9–12). Together these findings suggest that aberrant accumulation of Mfn may mediate the loss of mitochondrial homeostasis caused by loss of PINK1 or parkin. Although further work will be needed to determine whether this contributes to PD pathogenesis, our results support the emerging hypothesis that the PINK1/Parkin pathway acts to regulate the safe degradation of terminally damaged mitochondria as a quality control mechanism (22, 44).

**Materials and Methods**

**Cell Culture and Transfection.** S2R+ cells were cultured in Schneider’s medium (Invitrogen) supplemented with 5% FCS (Sigma) and 1% penicillin-streptomycin solution (Gibco). Cells were transfected using Effectene reagent (Qiagen) following the manufacturer’s instructions and collected after 24–48 h. Copper sulfate solution (500 μM) was added to the cells to induce plasmid expression when required. Where indicated, cells were treated with 10 μM CCCP for 2 h or 10 mM paraquat for 6 h.

**RNAi Treatment and Quantification.** Double-stranded RNAs (dsRNAs) were prepared using the MEGA script kit (Ambion). Primers used to generate dsRNAs are described in SI Materials and Methods. A total of 1.2 million cells were plated on a six-well plate and treated with 15 μg dsRNA probe in serum-free medium. Two hours after probe treatment, complete medium was added to the wells, and cells were cultured for 2 days before being transfected.
Detection was done using HRP-conjugated secondary antibodies and was raised in rabbit against an N-terminal peptide, DTVDKSGPSPLSRF. Anti-Parkin (1:3,000) has been described before. Anti-Mfn (1:2,000) was constructed by cloning the entire Drosophila Mfn ORF from cDNA (RE04414) into pUAST vector, which was injected into w1118 embryos for germline transformation (BestGene). Multiple independent lines were isolated and assessed.

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Cell Imaging. Cells were plated on imaging dishes and treated as indicated. For mitochondrial morphology analysis, live cells were incubated with 200 μM rhodamine 123 and imaged live in growing medium. Quantification of mitochondria length was performed by using ImageJ software as previously described (45). For transfected cells, cells were fixed with paraformaldehyde and prepared by standard protocols for immunocytochemistry following treatment: Hoechst (2 μg/mL), phallolidin Alexa-Fluor488 (2 units), and anti-Complex V was used to detect mitochondria using a DeltaVision II microscope using standard epifluorescence. Unless stated otherwise, images were deconvolved after acquisition to improve image clarity and sharpness.

Drosophila Stocks and Procedures. Drosophila were raised under standard conditions at 25 °C, park2 and PINK1 mutants and UAS-parkin have been described before (7, 46), w1118 and da-GAL4 strains were obtained from the Bloomington Drosophila Stock Center and UAS-Arth-RNAi from the Vienna Drosophila Research Centre. UAS-Mfn was constructed by cloning the entire Drosophila Mfn ORF from cDNA (RE04414) into pUAST vector, which was injected into w1118 embryos for germline transformation (BestGene). Multiple independent lines were isolated and assessed.

For mRNA quantification, total RNA was extracted using TRI reagent (Sigma) or an RNaseasy mini kit (Qiagen) following the manufacturer’s instructions. Total RNA (1.5 μg) was reverse-transcribed by using a random decamer primer (RETRORscript kit; Ambion). Quantitative real-time PCR was performed using the SYBR Green Master Mix method (Sigma) with a Bio-Rad MyIQ system. Full details are in SI Materials and Methods.
Supporting Information

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SI Materials and Methods

Plasmids. MitoDsRed was subcloned from pDsRed2-Mito vector (Clontech) into the pAct-PPA expression plasmid. EGFP was cloned into pDsRed2-Mito vector in place of the DsRed gene and subcloned into the pAct-PPA expression plasmid. N-terminal Flag tag Drp1 and Fis1 were obtained by amplification from cDNA clones (AT04516 and RE29957, respectively) and cloned into the pAct-PPA expression plasmid. parkin was amplified from a cDNA clone (GM10489) and subcloned into pMK33 copper-inducible expression plasmid. EGFP was cloned at the C-terminal end of the parkin gene. The HA-Ub construct was obtained from Y. Wang (1).

Real-Time Quantitative PCR. Real-time PCR was performed using the SYBR Green Master Mix method (Sigma) with a Bio-Rad MyiQ system. Reactions were performed in a 25 μl reaction mixture containing 12.5 μl Master Mix, 50–90 nM of each primer depending on the primers, 1 μl DNA sample from the reverse-transcription reaction, and nuclease-free water. The PCR protocol used consisted of a 30-s denaturation at 95 °C followed by 30 s annealing at 60 °C and 30 s at 72 °C for 40 cycles. The housekeeping gene GAPDH or Rpl21 was used as the internal control. PCR primers for PINK1, parkin, Mfn, Drp1, Fis1, and Opa1 were designed using GenScript real-time PCR (TaqMan) primer design and are listed below. Primers were optimized to find the concentration that had the lowest CT value. Relative quantitation was performed using the comparative CT method. Data were normalized by subtracting the mean CT value of the housekeeping gene (reference gene) from the mean of the CT value of the gene of interest (ΔCT) for each experimental condition. The ΔCT value at the different times of treatment was compared to that of the control untreated sample (ΔΔCT). The amount of gene expression, normalized to an endogenous reference, was then determined using 2−ΔΔCT. The efficiencies of target and reference amplifications were found to be the same.

Live Imaging. Cells were grown on imaging dishes (Chamber slide Lab-tek II 8; Fisher). After appropriate treatment, cells were treated with the selective mitochondrial dye rhodamine 123 (200 μM; Invitrogen) for 40 s, washed three times with either PBS or Schneider’s medium, and imaged live in growing medium on a DeltaVision DV microscope (100× objective; NA:1.40), using epifluorescence and deconvolved to improve clarity.

Immunocytochemistry. S2R+ cells were cotransfected with parkin-GFP and mitoDsRed constructs as described above. Two days after transfection, cells were plated on imaging dishes (Chamber slide Lab-tek II 8; Fisher) and subjected to appropriate RNA interference (RNAi) probe treatment for 2 days before being treated with 10 nM paraquat (6 h) or 10 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) (2 h). Bafilomycin A1 (Sigma) was used at 20 nM (2 h). After treatment, the medium was removed and the cells were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. Cells were permeabilized with 0.2% TritonX-100 (Sigma) in PBS (10 min at room temperature) and then incubated with blocking solution (1% BSA in PBS, 0.2% TritonX) for 30 min. Cells were incubated in blocking solution for 1 h at room temperature using specific primary antibodies. An appropriate secondary fluorochrome-conjugated antibody was added in blocking solution for 1 h at room temperature (1:400; Molecular Probes). Images were acquired on a DeltaVision DV microscope (100× objective; NA:1.40) by using standard epifluorescence and deconvolved to improve clarity.

Immunoprecipitation. Cells were transfected as described above. Cells were lysed in 50 mM Tris–HCl, pH 8, 150 mM HCl, 1 mM MgCl2, 2 mM EGTA, 1% TritonX, 10% glycerol, 10 mM NEM, 50 μM MG132, and protease inhibitor mixture (Roche) were added fresh before harvesting cells. Protein A agarose beads were incubated with anti-Flag (1:50) or anti-GFP (1:1) antibodies for 1 h at 4 °C. Immunocomplexes were collected by incubating previously prepared Protein A agarose beads with 1 mg of whole-cell extract, rocking at 4 °C overnight. Beads were then resuspended in 4× Laemml buffer and centrifuged at 13,000 g for 10 min. Cleared samples were subjected to Western blot with the indicated primary antibodies.

Primers. Each of the RNAi knockdown primers, listed below, also contained a T7 promoter sequence at the 5’ end:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>PINK1</td>
<td>CAATGTTGACCTTCTCCAGGA</td>
<td>TCGTACGGTTTATCAGGAC</td>
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<tr>
<td>Parkin</td>
<td>CTGTTGGCAATTTGGAGGGA</td>
<td>CTTTGGCAGGGAGCGA</td>
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<tr>
<td>OPA1</td>
<td>GGGAGCGTGGCGTGGAGGA</td>
<td>CTTTGGCAGGGAGCGA</td>
</tr>
<tr>
<td>Drp1</td>
<td>ACATCATGGCCAGCCATT</td>
<td>CCTTGGCAGAGGAGGA</td>
</tr>
<tr>
<td>Fis1</td>
<td>ATGATTTTGGAGGAACGGAG</td>
<td>CCTTGGCAGAGGAGGA</td>
</tr>
<tr>
<td>Mfn</td>
<td>GGAACCTCATTTATCTCTAT</td>
<td>GGTGTGCTTTTGGCCCAAACAT</td>
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dsRNAs for Atg5 and Atg8b were obtained from the Sheffield RNAi screening facility. The following real-time quantitative PCR primers were used:

<table>
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<th>Reverse</th>
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<td>Rpl21</td>
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<td>AGTACATGCCTTTGAGGGGAC</td>
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<td>Mfn</td>
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<td>Fis1</td>
<td>TCGTCTCCCTAAGGGAATATATTTAAA</td>
<td>TAGCATCGCAATGCGCAATGAGGAA</td>
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Fig. S1. Analysis of RNAi knockdown efficiency in Drosophila S2R+ cells. Quantitative real-time PCR was performed for target genes to determine the degree of RNAi-induced knockdown of endogenous message after 2 and 3 days. Knockdown efficiency was preferentially assessed by Western blotting where possible for Parkin and Mfn.
Fig. S2. Sample preparation methods for comparing mitochondrial morphology. (A) S2R+ cells were treated with the indicated dsRNAs [control (Ctrl) dsRNA is targeted against DsRed] and prepared for imaging using standard protocols. One sample were stained with the mitochondria-selective vital dye rhodamine 123 and imaged live. Other samples were fixed with either methanol (MeOH) or paraformaldehyde (PFA) by standard methods. Fixed cells were immunolabeled with anti-Complex Vα antibody to mark mitochondria. Mfn RNAi causes fragmentation that is not changed by fixation, whereas Drp1 RNAi results in long mitochondria in live cells consistent with other reports. PINK1 RNAi also reveals elongated mitochondria. Fixation by either method dramatically reduces the appearance of long mitochondria. (B) S2R+ cells treated with control, PINK1, or parkin RNAi as before. Cells are transfected with plasmids to express mitoGFP to visualize mitochondria. (Scale bar: 5 μm.)
Fig. S3. Analysis of gene expression changes upon PINK1 or parkin RNAi knockdown. (A) Quantitative real-time PCR was performed for key mitochondrial fission and fusion regulators after 3 days of RNAi treatment of PINK1 or parkin compared with control. Statistical significance was determined by one-way ANOVA with Bonferroni correction, but no significant changes were detected. (B) The level of Parkin protein was also assessed in the PINK1<sup>−/−</sup> null mutant. (C) Western blot of S2R+ cells transfected either with empty vector (lane 1) or parkin-GFP–expressing plasmid (lanes 2–8) and treated with the indicated RNAi probe. A total of 10 μM CCCP or vehicle was added 2 h before harvesting cells.
Fig. S4. Effect of CCCP treatment on mitochondrial membrane potential and Parkin-GFP localization over time. S2R+ cells were transfected with (A) mito-GFP or (B) parkin-GFP-expressing plasmids and incubated with the mitochondrial membrane potential sensitive dye MitoTracker Red before treatment with CCCP. Images were taken before CCCP treatment and after 5 min or 1 h. Mito-GFP labels all mitochondria in transfected cells whereas MitoTracker Red intensity reduces over time, indicating that membrane potential is lost. As membrane potential is lost, Parkin-GFP is recruited to a subset of mitochondria. (Scale bar: 5 μm.)
Fig. S5. Comparison of image acquisition and analysis methods for Parkin-GFP localization. S2R+ cells were cotransfected with parkin-GFP and mitoDsRed and imaged on a Deltavision DV epifluorescence microscope. (A) Untreated cells were either fixed or imaged live. Captured images are shown both deconvolved or raw (non-deconvolved). Parkin-GFP is largely cytoplasmic in untreated cells with occasional puncta. Parkin-GFP appears diffuse, although not entirely uniform, in live cells, whereas cytoplasmic Parkin-GFP appears more uneven or dappled in fixed samples. This dappled appearance is further enhanced by deconvolution of the image. (B) Comparison of images shown in Fig. 2 before and after deconvolution. Cells were fixed to maintain precise time exposure to the stressors CCCP or paraquat. (Scale bar: 5 μm.)
Fig. S6. CCCP-induced mitophagy. (A) S2R+ cells were treated with CCCP and fixed and imaged after the indicated time. (B) Cells were treated with dsRNAs against DsRed (Ctrl) and Atg8b or bafilomycin to inhibit autophagy and then treated with CCCP. Hoechst stain marks nuclei, anti-Complex Vα antibody labels all remaining mitochondria, and phalloidin-488 (green) marks the cell membrane. (Scale bar: 5 μm.)

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Fig. S7. Quantification of relative abundance of Mfn-Ub isoforms compared to unmodified. (A) S2R+ cells expressing Mfn-Flag were treated with either DsRed (ctrl), PINK1, or parkin dsRNAs and subjected to Western blot analysis as in Fig. 3 and Fig. 4. (B) S2R+ cells treated with dsRNAs, or (C) wild type or mutant animals were subjected to Western blot analysis for total Complex V α levels normalized to actin levels (Fig. 5). No comparison showed significance $P > 0.05$, one-way ANOVA with Bonferroni correction. WT, wild type.