

Drosophila Parkin requires PINK1 for mitochondrial translocation and ubiquitinates Mitofusin

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Edited by Barry Ganetzky, University of Wisconsin, Madison, WI, and approved February 2, 2010 (received for review November 20, 2009)

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 recruited to damaged mitochondria, possibly affecting 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 profusion 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.

mitophagy | neurodegeneration | Parkinson's disease | quality control

Parkinson's disease (PD) is a common neurodegenerative disorder principally affecting the degeneration of nigral dopaminergic neurons. The pathogenic mechanisms are unknown, but valuable insight has been gained from identifying gene mutations causative for familial forms of PD (1). Loss-of-function mutations in *PINK1* and *parkin* are the major cause of autosomal recessive, early onset PD. *PINK1* encodes a mitochondria-targeted kinase (2) whereas *parkin* encodes an E3 ubiquitin ligase (3), a class of enzymes that conjugate ubiquitin to target substrates. This modification is usually considered in the context of substrate degradation by the proteasome, but ubiquitination also serves many other cellular functions. Consequently, much emphasis has been put on elucidating a link between Parkin dysfunction and protein aggregation. Despite the identification of numerous putative Parkin substrates, an unequivocal causative link between substrate aggregation and pathogenesis remains debatable.

There is strong evidence, however, that supports an important role for Parkin in regulating mitochondrial homeostasis (4). Studies have revealed a conserved function of Parkin acting downstream of PINK1 to protect mitochondrial integrity and prevent oxidative stress-induced apoptosis (5–8). Recently, we and others have reported that *Drosophila parkin* and *PINK1* genetically interact with components of the mitochondrial fission and fusion machinery (9–12), suggesting that loss of *PINK1/parkin* function may lead to excess mitochondrial fusion. Consistent with this, mitochondrial elongation has been reported in cells derived from PD patients with *parkin* mutations (13). However, the effects of *parkin* or *PINK1* deficiency in mammalian cells remain unresolved because additional reports describe inconsistent phenotypes in *PINK1*- and *parkin*-deficient cells (6, 14–17). The reasons for this discrepancy are unclear but warrant further clarification.

Important insight into the mechanism by which Parkin regulates mitochondrial homeostasis has revealed that upon mitochondrial depolarization Parkin translocates from the cytoplasm to accumulate on a subset of mitochondria and promotes their degradation by autophagy (18). It has also been proposed that regulated mitochondrial fission/fusion helps sort out damaged mitochondria for degradation (19–21). These findings raise the possibility that Parkin receives some signal identifying dysfunctional or damaged mitochondria and translocates and interacts with an unknown factor to effect mitochondrial fission and/or fusion and promote mitophagy (22).

Here we show that PINK1 is required for the recruitment of Parkin to damaged mitochondria and subsequent mitophagy, consistent with recent reports (23–25). Furthermore, we show that PINK1 and Parkin promote the ubiquitination of the profusion factor Mfn. These findings suggest that Mfn ubiquitination may provide a mechanism by which terminally damaged mitochondria are recognized for degradation by autophagy.

Results

PINK1 and Parkin Knockdown Cause Mitochondrial Elongation. Our previous genetic interaction studies in vivo, using both loss- and gain-of-function assays, indicate that *Drosophila* PINK1 and Parkin act to promote fission and/or inhibit fusion of mitochondria. To extend this study, we quantified mitochondrial morphology upon loss or gain of function of *parkin* and *PINK1*, along with key regulators of mitochondrial morphology, in *Drosophila* S2R+ cells.

RNA interference (RNAi) can be robustly achieved 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*, *Opa1*, and *Mfn1/2* (here called *Mfn*). RNAi of these 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), so 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 profusion genes *Drp1* and *Fis1* resulted in elongated mitochondria whereas knockdown of the profusion genes *Opa1* and *Mfn* (also known as *Marf*) resulted in fragmented mitochondria. We found that knockdown of *PINK1* or *parkin* caused a significant elongation of mitochondrial length. Elongated mitochondria were also seen when transfected mitoGFP was used to label mitochondria (Fig. S2B). Conversely, overexpression of *Opa1* and *Mfn* caused a hyperfused network whereas overexpression of *Drp1* and *Fis1* caused

Author contributions: E.Z. and A.J.W. designed research; E.Z., R.N.T., and A.J.W. performed research; E.Z., R.N.T., and A.J.W. analyzed data; and E.Z. and A.J.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0913485107/DCSupplemental.

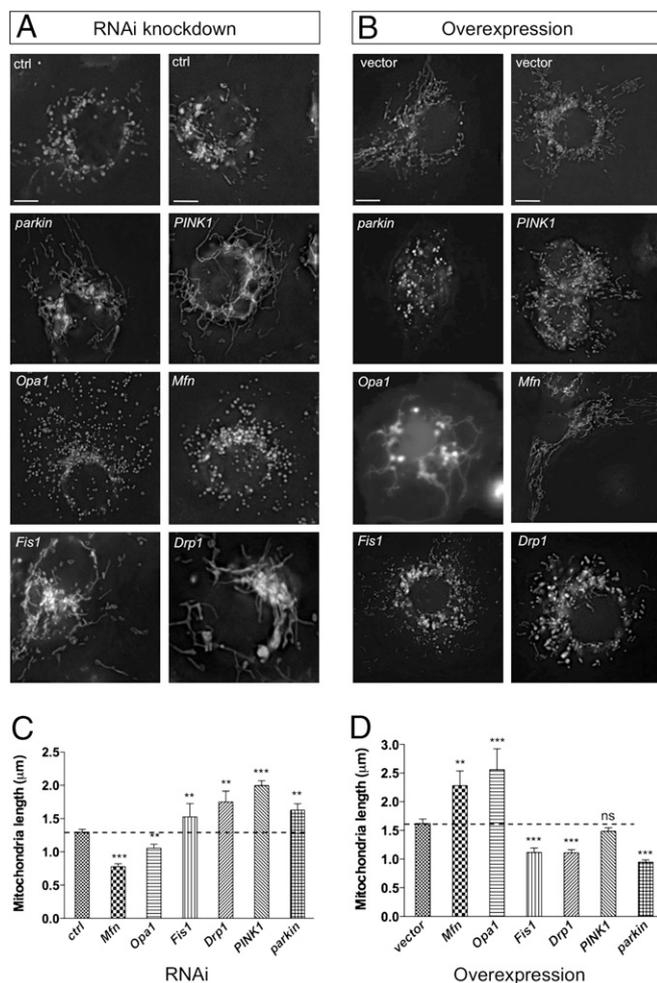


Fig. 1. *Drosophila* *PINK1* and *parkin* activity promotes mitochondrial fission and/or inhibits fusion. (A) S2R+ cells were treated with the indicated RNAi probe for 3 days. Rhodamine 123 was used to visualize mitochondrial morphology in live cells. (B) S2R+ cells were transfected with plasmids to express indicated genes and cotransfected with *mitoGFP* to visualize mitochondria. (C and D) Quantification of mitochondrial length as imaged in A and B, respectively. Two control (ctrl) RNAi and vector-only treatments are shown to demonstrate phenotype variability. (Scale bar: 5 µm.) The bar graphs represent the mean and sem for at least three independent experiments. Significance was determined by one-way ANOVA with Bonferroni correction (** $P < 0.01$, *** $P < 0.001$).

fragmentation (Fig. 1 B 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

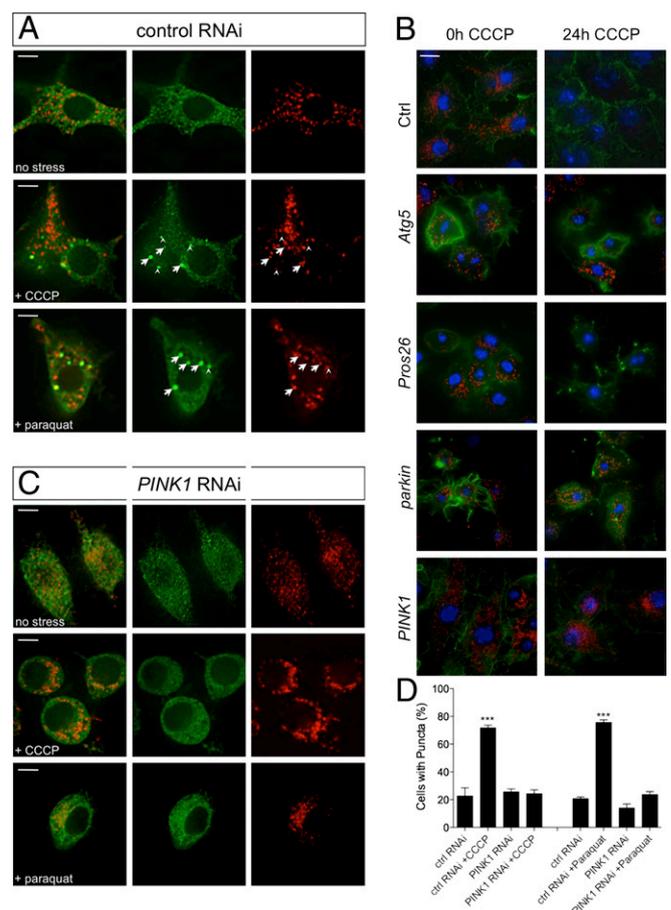


Fig. 2. *PINK1* is required for Parkin translocation and mitophagy. (A) S2R+ cells cotransfected with *parkin-GFP* and *mitoDsRed*, treated with control dsRNA and then treated with CCCP or paraquat and fixed for imaging. Most Parkin-GFP puncta colocalize with (arrow) or abut (arrowhead) mitochondria. (B) Cells were treated with indicated dsRNAs and exposed to CCCP for 24 h. Samples were fixed and stained to label mitochondria (anti-Complex V α , red), actin (phalloidin-488, green) or nuclei (Hoechst, blue). (C) S2R+ cells transfected as in A and treated with *PINK1* dsRNA before exposure to CCCP or paraquat. Comparison of Parkin-GFP distribution under different image analysis methods is shown in Fig. S5. (D) Percentage of cells with Parkin-GFP puncta. (Scale bar: 5 µm.) The bar graphs represent the mean and SEM of at least four independent tests. Significance was determined by one-way ANOVA with Bonferroni correction (** $P < 0.01$, *** $P < 0.001$).

C and D). 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.

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.

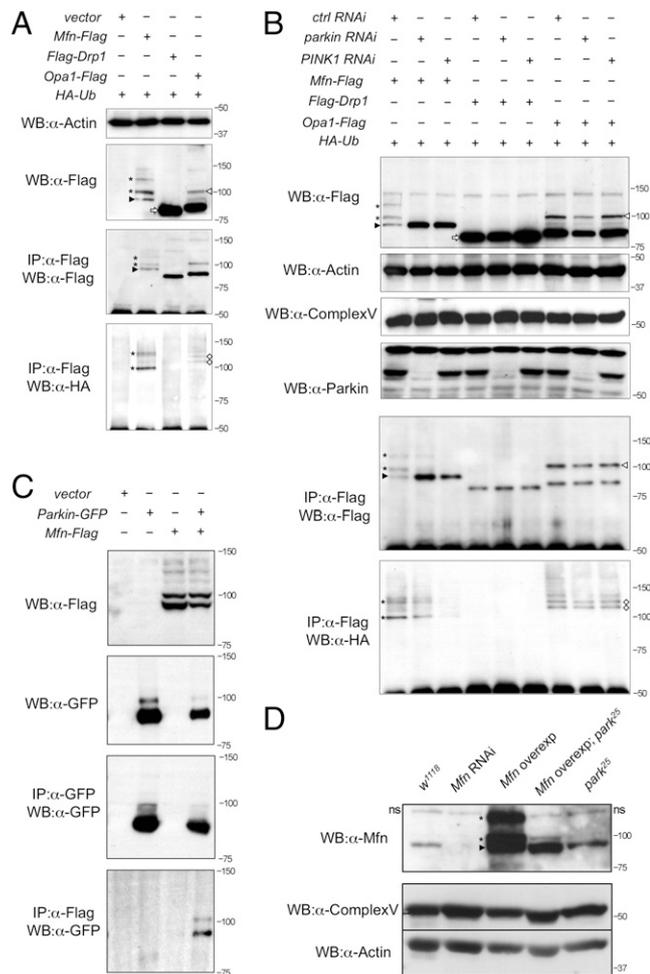


Fig. 3. Mfn is ubiquitinated in a PINK1/Parkin-dependent manner in vitro and in vivo. (A) S2R+ cells cotransfected with HA-Ub plus empty vector or Flag-tagged Mfn, Drp1, or Opa1 plasmids as indicated were immunoprecipitated and subjected to Western blot analysis. (B) S2R+ cells were treated with control, parkin, and PINK1 RNAi before being transfected as indicated. Cells were harvested and subjected to Western blot analysis as shown. Samples were also subjected to immunoprecipitation, and Western blots were probed with antibodies against Flag and HA. (C) Immunoprecipitates of S2R+ cells expressing combinations of empty vector, parkin-GFP, and Mfn-Flag as shown. (D) Western blot analysis of *Drosophila* Mfn levels in vivo. A nonspecific band seen in all samples is denoted "ns." Complex Vα and actin are used as loading controls. Genotypes: *w¹¹⁸*, wild type; Mfn RNAi, *da-GAL4,UAS-Mfn-RNAi*; Mfn overexpression, *da-GAL4,UAS-Mfn*; Mfn overexpression *park²⁵*, *da-GAL4,UAS-Mfn,park²⁵/park²⁵*; *park²⁵*, *park²⁵/park²⁵*. In all panels, black arrowheads indicate full-length Mfn, asterisks are ubiquitinated Mfn, white arrowheads denote full-length Opa1, white diamonds are ubiquitinated Opa1, and white arrows show full-length Drp1.

Flag-tagged forms of Drp1, Opa1, 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). Opa1 was present in a small amount of the full-length form but predominantly 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 antiserum. 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

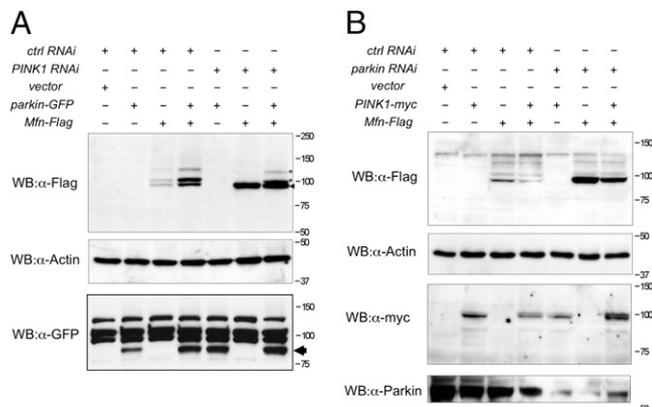


Fig. 4. Parkin overexpression can cause Mfn ubiquitination in the absence of PINK1. (A) S2R+ cells were treated with control or *PINK1* RNAi probe and transfected with combinations of empty vector, *parkin-GFP*, and *Mfn-Flag*, as shown. (B) S2R+ cells were treated with control or *parkin* RNAi probe and transfected with combinations of empty vector, *PINK1-myc*, and *Mfn-Flag*, 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* knockdown cells or *park*²⁵ null mutant flies showed a significant increase in Mfn abundance (Fig. 5). Mfn was also significantly more abundant in *PINK1* knockdown or *PINK1*^{B9} null mutant flies (Fig. 5). Interestingly, the level of Complex V α 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

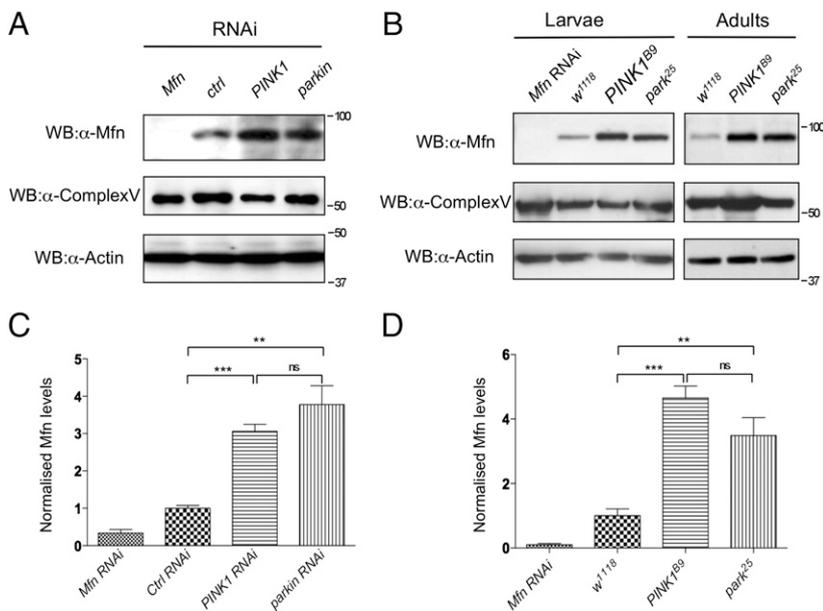


Fig. 5. Mfn accumulates in the absence of PINK1/Parkin. (A) S2R+ cells were treated with RNAi probe as indicated, harvested, and subjected to Western blot analysis by using antibodies against *Drosophila* Mfn. Antibodies against Complex V α and actin are used as loading controls. (B) Wild-type and mutant animals were collected and subjected to Western blot analysis. Genotypes: *w*¹¹¹⁸, wild type; *Mfn* RNAi, *da-GAL4,UAS-Mfn-RNAi*; *park*²⁵, *park*²⁵/*park*²⁵; *PINK1*^{B9}, *PINK1*^{B9}/*Y*. Quantification of Mfn levels relative to the Complex V α loading control in S2R+ cells (C) and wild-type and mutant animals (D). Charts represent mean and SEM for three independent experiments. Significance was determined by one-way ANOVA with Bonferroni correction (***P* < 0.01, ****P* < 0.001).

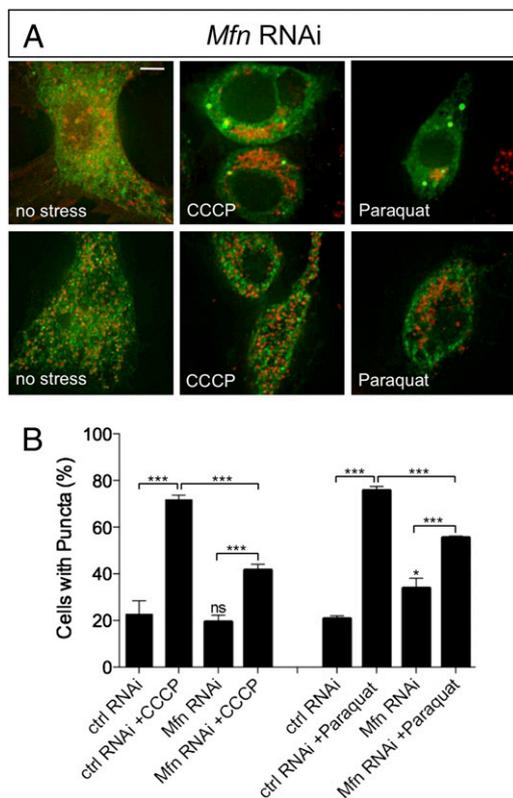


Fig. 6. Loss of Mfn partially impairs Parkin translocation to mitochondria. S2R+ cells were cotransfected with plasmids expressing *parkin-GFP* and *mitoDsRed* and treated with control RNAi probe or *Mfn* RNAi probe before being treated with CCCP or paraquat and analyzed for Parkin-GFP accumulation. Control RNAi-treated cells are represented in Fig. 2. (A) *Mfn* RNAi-treated cells exposed to CCCP, paraquat, or no stress. Two representative images are shown for each treatment to show variability. (Scale bar: 5 μ m.) (B) Percentage of cells with green puncta. The chart shows the mean and SEM for counting of at least four independent well fields containing 70–90 cells/field. Significance was determined by one-way ANOVA with Bonferroni correction (* $P < 0.05$, *** $P < 0.001$).

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 extra-mitochondrially (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 V α 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 (Invitrogen-Gibco). Cells were transfected using Effectene reagent (Qiagen) following the manufacturer's instructions and collected after 24–48 h. Copper sulfate solution (500 μ M) was added to the cells to induce plasmid expression when required. Where indicated, cells were treated with 10 μ M 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 μ g 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.

For mRNA quantification, total RNA was extracted using TRI reagent (Sigma) or an RNeasy mini kit (Qiagen) following the manufacturer's instruction. Total RNA (1.5 μ g) was reverse-transcribed by using a random decamer primer (RETROscript 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*.

Immunoblotting and Immunoprecipitation. Standard protocols were used for Western blotting. The following commercial antibodies were used: anti-Complex V α (1:5,000; MitoSciences), anti-Flag (1:1,000; Cell Signaling), anti-HA (1:1,000; Cell Signaling), anti-actin (1:10,000; Chemicon), anti- α -tubulin (1:10,000; Sigma), anti-GFP (1:2,000; Abcam), and anti-Myc (1:1,000; Cell Signaling). Anti-Parkin (1:3,000) has been described before. Anti-Mfn (1:2,000) was raised in rabbit against an N-terminal peptide, DTVDKSGPGSPLSRF. Detection was done using HRP-conjugated secondary antibodies and ECL chemiluminescence. For immunoprecipitation, cells were lysed in a standard lysis buffer (see *SI Materials and Methods* for details). Anti-Flag (1:50) or anti-GFP (1:200) antibodies were conjugated to Protein A agarose beads, incubated with 1 mg of whole-cell extract overnight, and extracted with 4 \times Laemmli buffer.

Cell Imaging. Cells were plated on imaging dishes and treated as indicated. For mitochondrial morphology analysis, live cells were incubated with 200 μ M 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 μ g/mL), phalloidin Alexa-Fluor488 (2 units), and anti-Complex V α (1:1,000). All images were acquired on a DeltaVision DV 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. *park*²⁵ and *PINK1*^{B9} mutants and *UAS-parkin* have been described before (7, 46). *w*¹¹⁸ and *da-GAL4* strains were obtained from the Bloomington *Drosophila* Stock Center and *UAS-Mfn-RNAi* from the Vienna *Drosophila* Research Centre. *UAS-Mfn*³ was constructed by cloning the entire *Drosophila Mfn* ORF from cDNA (RE04414) into pUAST vector, which was injected into *w*¹¹⁸ embryos for germline transformation (BestGene). Multiple independent lines were isolated and assessed.

ACKNOWLEDGMENTS. We thank D. Strutt, E. Smythe, and L. Pallanck for critical reading of the manuscript. This work is supported by grants from the Wellcome Trust (Grant 081987) and the Parkinson's Disease Society (Grant G-0713) to A.J.W. The Wellcome Trust is acknowledged for support of the Light Microscopy Facility (Grant GR077544AIA). This work is also supported by Grant G070091 from The Medical Research Council Centre for Developmental and Biomedical Genetics.

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