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

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## Abstract

Loss of the E3 ubiquitin ligase Parkin causes early onset Parkinson 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 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 pro-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.

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## Introduction

Parkinson 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) while *parkin* encodes an E3 ubiquitin

ligase (3); a class of enzymes which 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 since 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, 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 pro-fusion factor Mfn. These findings suggest Mfn ubiquitination may provide a mechanism by which terminally damaged mitochondria are recognized for degradation by autophagy.

## Results

### **PINK1 and Parkin knockdown causes 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*, alongside 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,C). As expected we found that knockdown of the pro-fission genes *Drp1* and *Fis1* resulted in elongated mitochondria whereas knockdown of pro-fusion 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 hyper-fused network while overexpression of *Drp1* and *Fis1* caused fragmentation (Fig. 1B,D). Overexpression of *parkin* also caused fragmentation, however, the effect of *PINK1* was not significant, suggesting 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 the morphology changes do not occur from a transcriptional response altering the abundance of fission or fusion genes. Also, in contrast to a previous report (8), we find 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. revealed an important insight into the function of Parkin, reporting it translocates to dysfunctional mitochondria and promotes mitophagy (18). 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), while 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. 2C,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 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, Opa1 and Mfn were co-expressed 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 co-immunoprecipitation 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 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) consistent with this isoform being unmodified. Larger ubiquitinated isoforms of Opa1 are not detected by anti-Flag immunoblotting suggesting these are 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; and Fig. S7A). Interestingly, this appeared to be accompanied by an accumulation of non-ubiquitinated 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 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 co-expression 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 genetic experiments showing PINK1 overexpression cannot compensate for loss of *parkin in vivo*.

Ubiquitination is a common signal for the degradation of proteins and organelles. Since evidence indicates 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*<sup>25</sup> null mutant flies showed a significant increase in Mfn abundance (Fig. 5). Mfn was also significantly more abundant in *PINK1* knockdown or *PINK1*<sup>B9</sup> null mutant flies (Fig. 5). Interestingly, the level of Complex V $\alpha$  did not appreciably increase (Fig. S7B), however, this technique is not sensitive to small changes. These data indicate 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.

Since 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 but 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 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 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 pro-fusion 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 localisation, appears to be necessary for Parkin translocation (23-25). Since 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 Parkin also ubiquitinates VDAC on the outer mitochondrial surface (23), suggesting there may be multiple recruitment substrates. While further work will be required to elucidate these mechanisms, this provides a molecular basis for the genetic hierarchy in which PINK1 acts upstream of Parkin (5-8).

In order to understand the role of Parkin translocation we took a candidate approach to identify putative substrates. Since 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. While it remains to be shown that Parkin directly mediates this ubiquitination, there is evidence that Parkin can mediate mono-ubiquitination (33-37), 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 (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 (41, 42)). While mitochondrial remodeling can contribute to promoting cell death it can also act in a protective manner contributing to a quality control process that likely involves degradation by autophagy/lysosome. 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). Re-fusion 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). Since Mitofusins mediate the tethering and fusion of mitochondrial, 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 their re-fusion 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 re-fusion 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 $\alpha$  levels are not significantly altered by decreased mitophagy. However, this rationale implies that Mfn

accumulates and is selectively ubiquitinated on mitochondria targeted for degradation but 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 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*. While 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% fetal calf serum (Sigma) and 1% penicillin-streptomycin (Invitrogen-Gibco). Cells were transfected using Effectene reagent (Qiagen) following manufacturer's instructions and collected after 24-48hrs. 500 $\mu$ M copper sulfate solution was added to the cells to induce plasmid expression when required. Where indicated cells were treated with 10 $\mu$ M CCCP for 2 hrs or 10mM paraquat for 6 hrs.

### **RNAi treatment and quantification**

Double stranded RNAs were prepared using MEGA script kit (Ambion). Primers used to generate dsRNAs are described in Supplementary material. 1.2 million cells were plated on 6 well plate and treated with 15 $\mu$ 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 RNeasy Mini Kit (Qiagen) following manufacturer's instruction. 1.5 $\mu$ g total RNA 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 Supplementary Information.

### **Immunoblotting and immunoprecipitation**

Standard protocols were used for Western blotting. The following commercial antibodies were used: anti-Complex V $\alpha$  (1:5000, MitoSciences), anti-Flag (1:1000, Cell Signaling), anti-HA (1:1000, Cell Signaling), anti-Actin(1:10000, Chemicon), anti- $\alpha$ -Tubulin (1:10000, Sigma), and anti-GFP (1:2000, Abcam), and anti-Myc (1:1000, Cell Signaling). Anti-Parkin (1:3000) has been described before. Anti-Mfn (1:2000) 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 standard lysis buffer (see SI for details). Anti-Flag (1:50) or anti-GFP (1:200) antibodies were conjugated to Protein A agarose beads and incubated with 1mg of whole-cell extract overnight and extracted with 4X Laemmli buffer.

### **Cell imaging**

Cells were plated on imaging dishes and treated as indicated. For mitochondrial morphology analysis; live cells were incubated with 200 $\mu$ 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;

following treatment, cells were fixed with paraformaldehyde and prepared by standard protocols for immunocytochemistry: Hoechst (2 $\mu$ g/ml), phalloidin Alexa-Fluor488 (2units), anti-Complex V $\alpha$  (1:1000). All images were acquired on a DeltaVision DV microscope using standard epifluorescence. Unless stated 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*<sup>25</sup> and *PINK1*<sup>B9</sup> mutants and *UAS-parkin* have been described before (46), (7). *w*<sup>1118</sup> and *da-GAL4* strains were obtained from the Bloomington *Drosophila* Stock Center, and *UAS-Mfn-RNAi* from the Vienna *Drosophila* Research Centre. *UAS-Mfn*<sup>3</sup> was constructed by cloning the entire *Drosophila Mfn* open reading frame from cDNA (RE04414) into pUAST vector, which was injected into *w*<sup>1118</sup> embryos for germline transformation (BestGene Inc.). Multiple independent lines were isolated and assessed.

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## Figure Legends

### Figure 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 co-transfected with *mitoGFP* in order to visualize mitochondria. (C,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 is 5  $\mu\text{m}$ . The bar graphs represent the mean and s.e.m. for at least three independent experiments. Significance was determined by one-way ANOVA with Bonferroni correction (\*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ).

### Figure 2. *PINK1* is required for Parkin translocation and mitophagy.

(A) S2R+ cells co-transfected with *parkin-GFP* and *mitoDsRed*, treated with control dsRNA then treated with CCCP or paraquat, and fixed for imaging. Most Parkin-GFP puncta co-localize with (arrow) or abut (arrowhead) mitochondria. (B) Cells were treated with indicated dsRNAs and exposed to CCCP for 24 hours. Samples were fixed and stained to label mitochondria (anti-Complex V $\alpha$ , 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 is 5  $\mu\text{m}$ . The bar graphs represent the mean and s.e.m. of at least four independent tests. Significance was determined by one-way ANOVA with Bonferroni correction (\*\*\*  $P < 0.001$ ).

### Figure 3. Mfn is ubiquitinated in a *PINK1*/*Parkin* dependent manner *in vitro* and *in vivo*.

(A) S2R+ cells co-transfected 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 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 non-specific band seen in all samples is denoted 'ns'. Complex V $\alpha$  and Actin are used as loading controls. Genotypes:  $w^{1118}$ , wild type; Mfn RNAi, *da-GAL4,UAS-Mfn-RNAi*; Mfn overexpression, *da-GAL4,UAS-Mfn*; Mfn overexpression *park<sup>25</sup>*, *da-GAL4,UAS-Mfn,park<sup>25</sup>/park<sup>25</sup>*; *park<sup>25</sup>*, *park<sup>25</sup>/park<sup>25</sup>*. 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.

**Figure 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 specific antibodies indicated.

**Figure 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 $\alpha$  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<sup>25</sup>*, *park<sup>25</sup>/park<sup>25</sup>*; *PINK1<sup>B9</sup>*, *PINK1<sup>B9</sup>/Y*.

Quantification of Mfn levels relative to Complex V $\alpha$  loading control in S2R+ cells (C) and wild type and mutant animals (D). Charts represent mean and s.e.m. for three independent experiments. Significance was determined by one-way ANOVA with Bonferroni correction (\*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ).

**Figure 6. Loss of Mfn partially impairs Parkin translocation to mitochondria.**

S2R+ cells were co-transfected 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 analysed 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 is 5  $\mu\text{m}$ . (B) Percentage of cells with green puncta. The chart shows the mean and s.e.m. for counting of at least four independent well fields containing 70-90 cells/field. Significance was determined by one-way ANOVA with Bonferroni correction (\*  $P < 0.05$ , \*\*\*  $P < 0.001$ ).

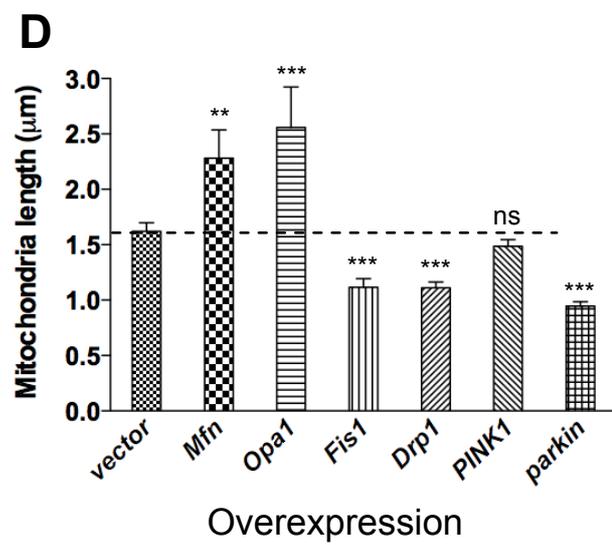
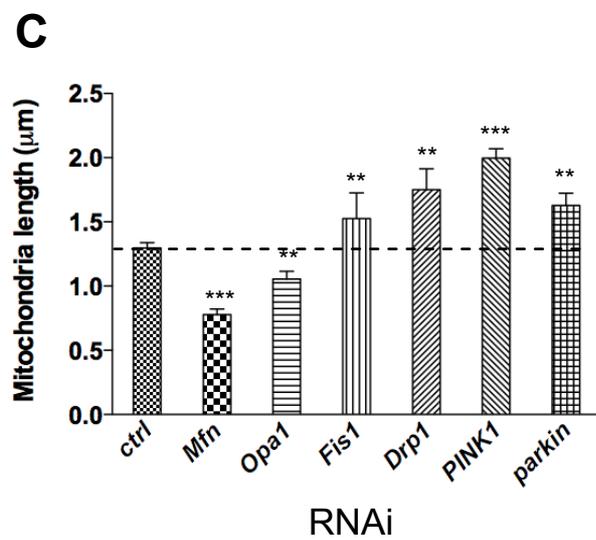
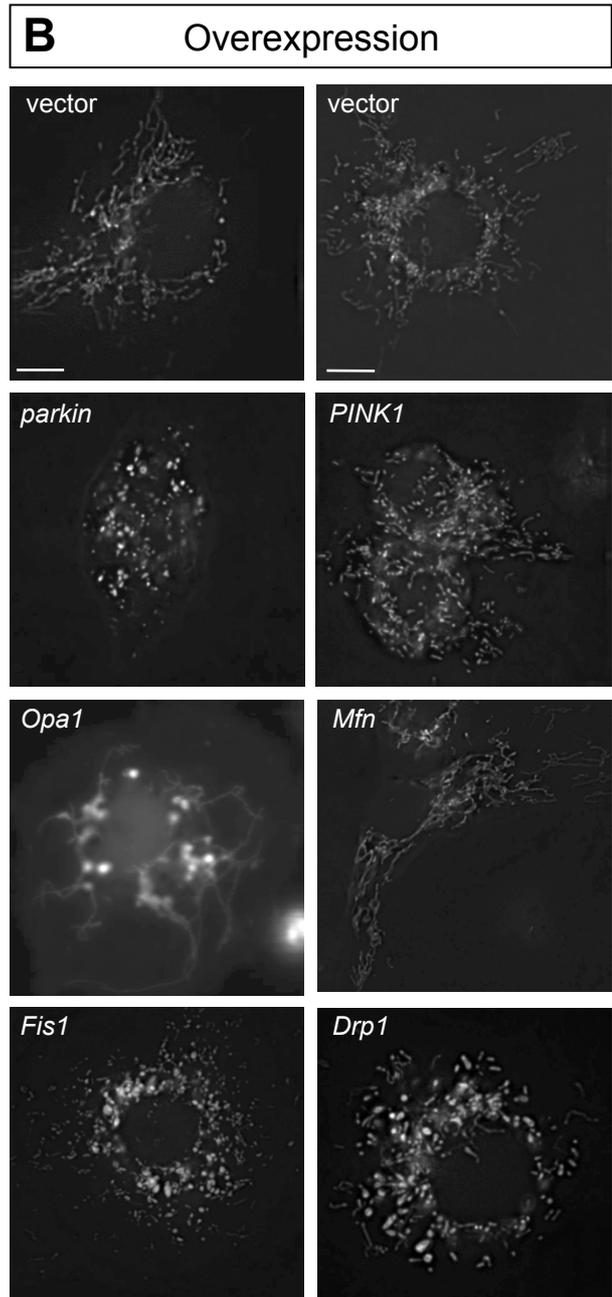
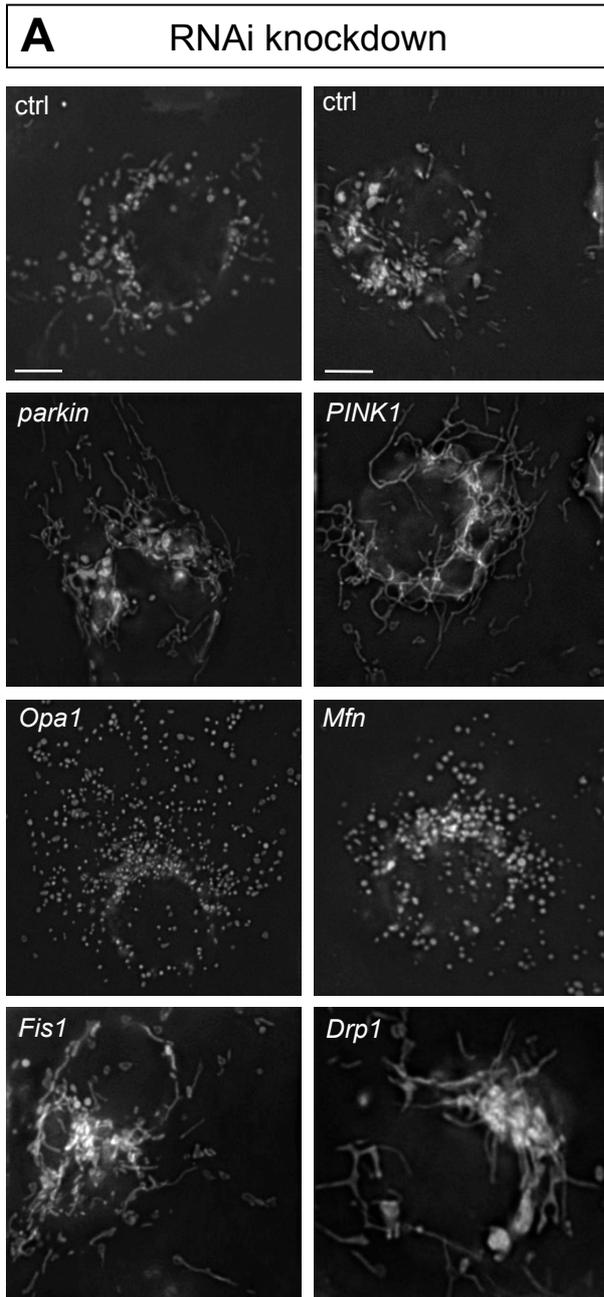


Figure 1. Ziviani et al

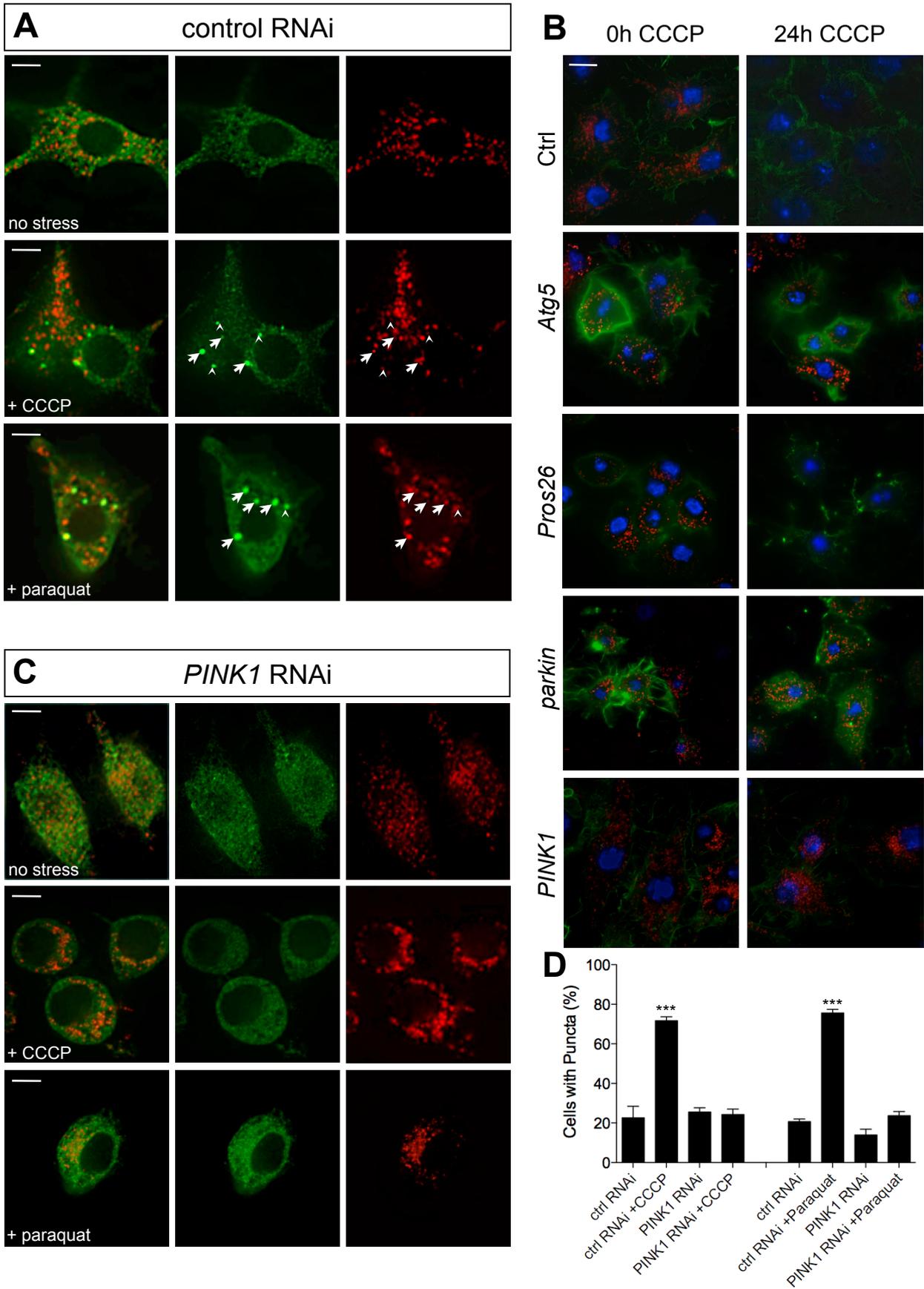


Figure 2. Ziviani et al

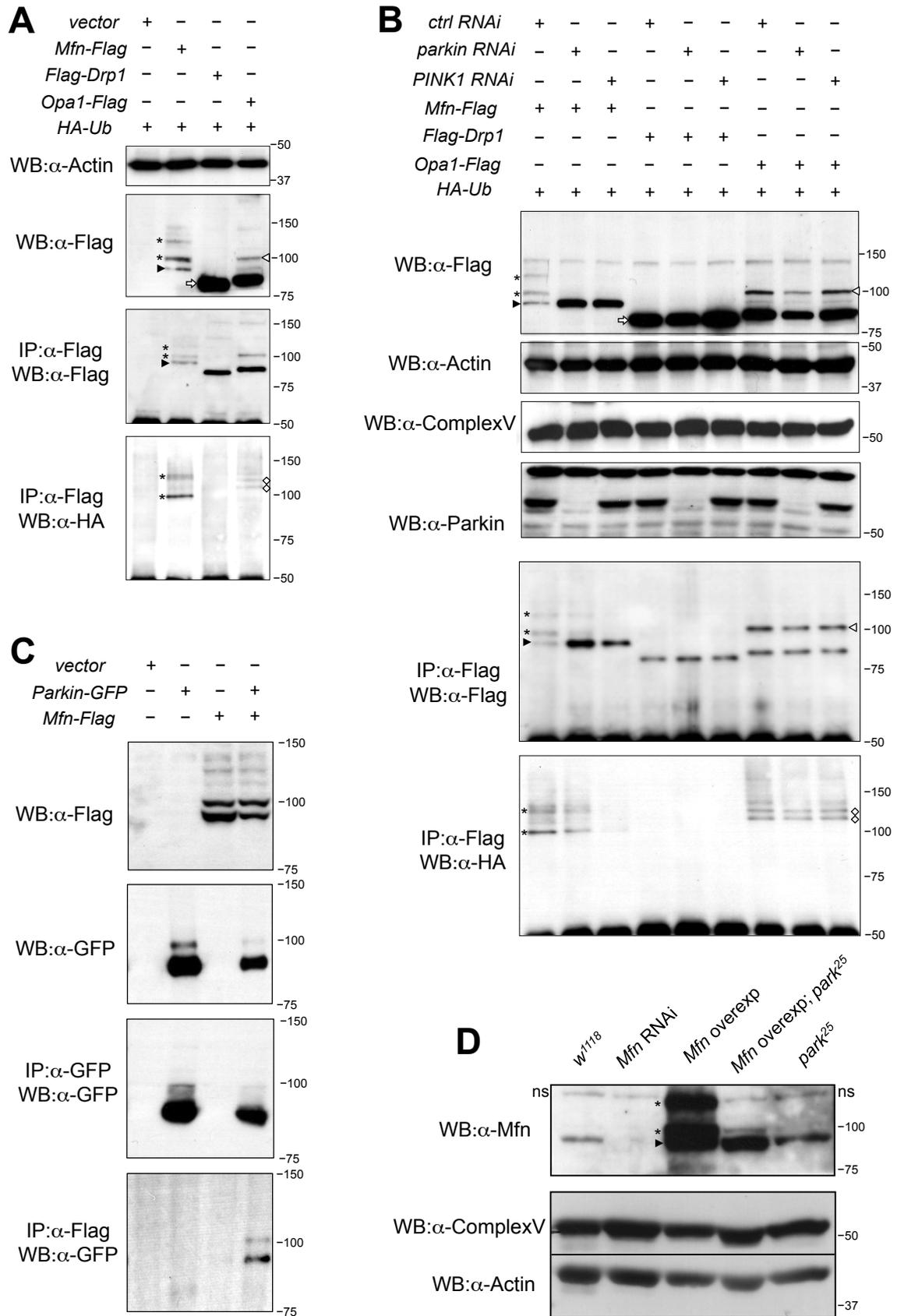
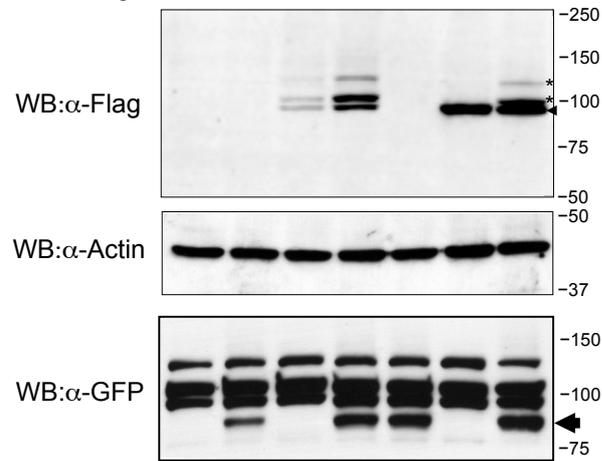


Figure 3. Ziviani et al

**A**

<i>ctrl RNAi</i>	+	+	+	+	-	-	-
<i>PINK1 RNAi</i>	-	-	-	-	+	+	+
vector	+	-	-	-	-	-	-
<i>parkin-GFP</i>	-	+	-	+	+	-	+
<i>Mfn-Flag</i>	-	-	+	+	-	+	+

**B**

<i>ctrl RNAi</i>	+	+	+	+	-	-	-
<i>parkin RNAi</i>	-	-	-	-	+	+	+
vector	+	-	-	-	-	-	-
<i>PINK1-myc</i>	-	+	-	+	+	-	+
<i>Mfn-Flag</i>	-	-	+	+	-	+	+

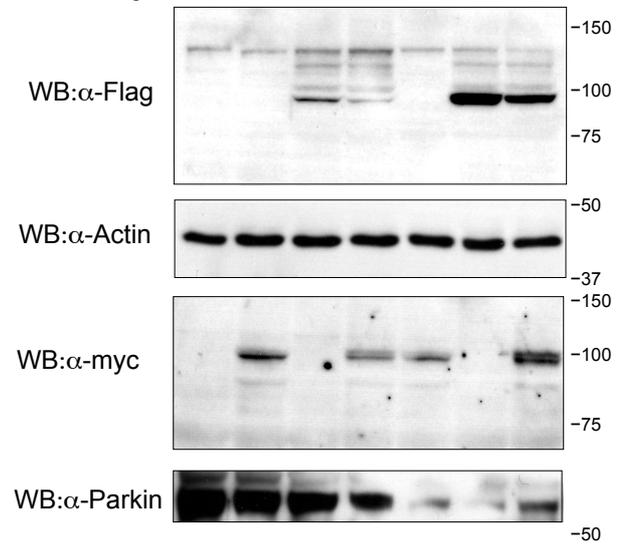


Figure 4. Ziviani et al

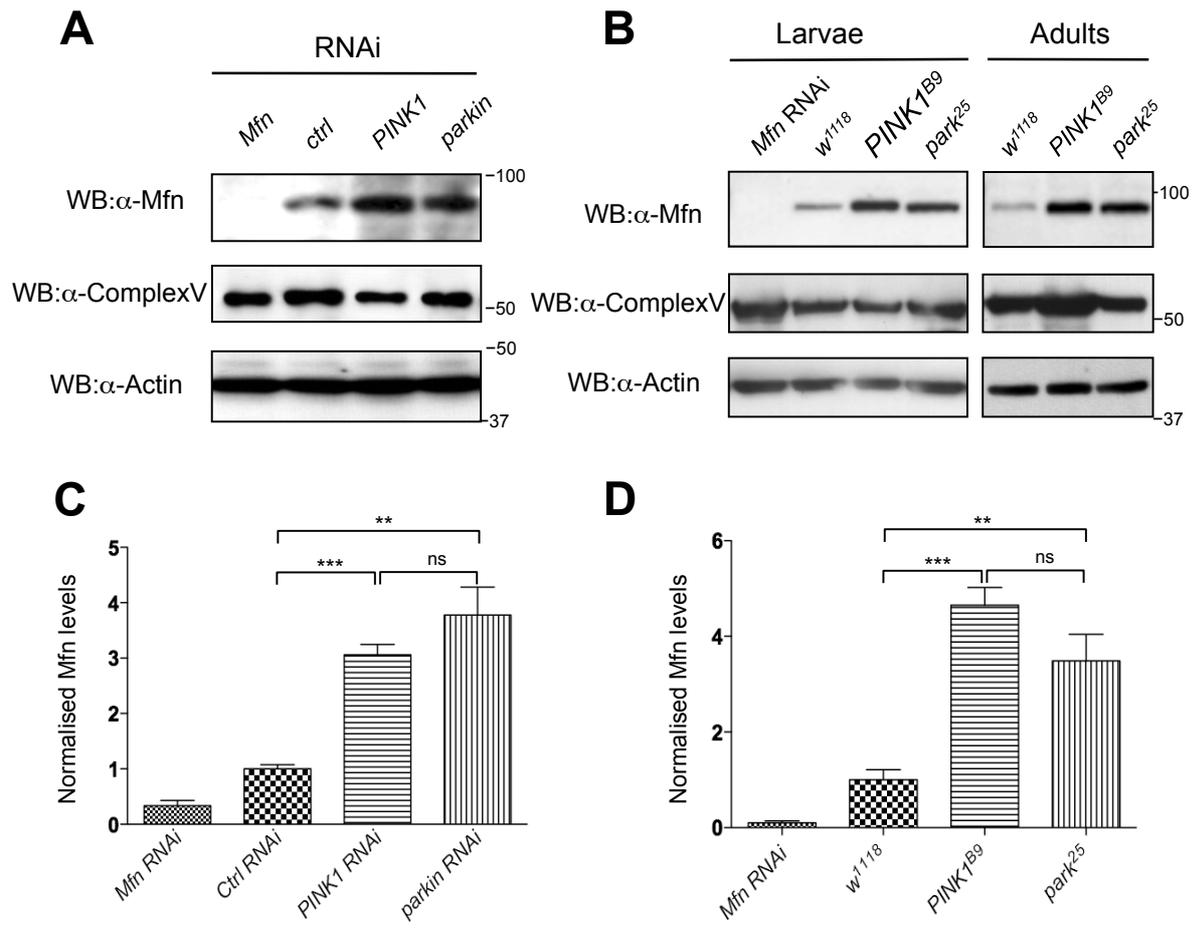


Figure 5. Ziviani et al

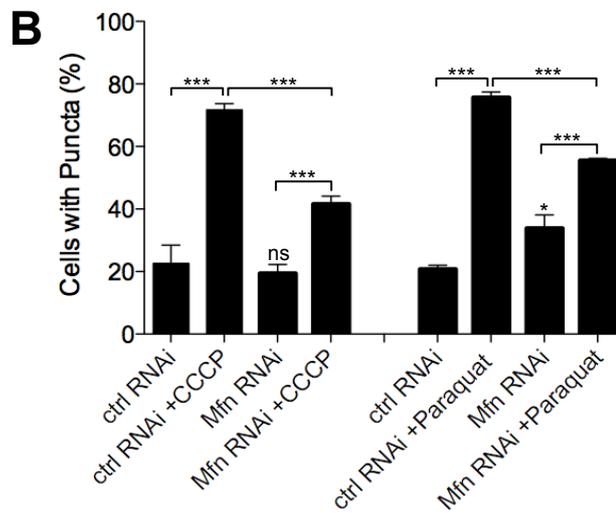
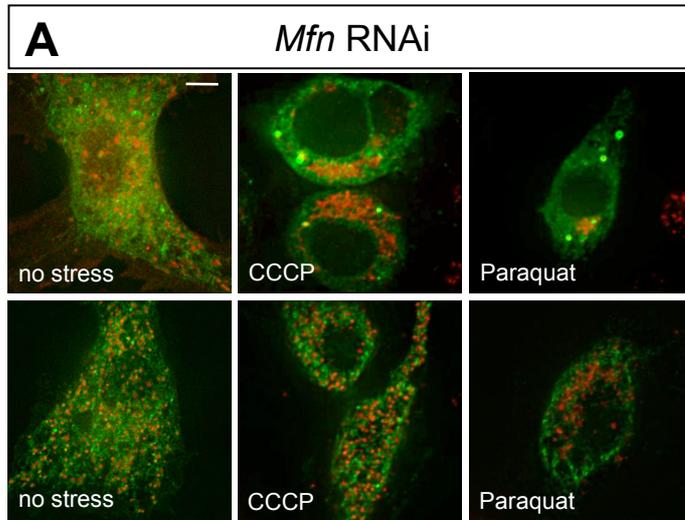


Figure 6. Ziviani et al