E2 ubiquitin conjugase Bendless is essential for PINK1-Park activity to regulate Mitofusin under mitochondrial stress

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Abstract

Cells under mitochondrial stress often co-opt mechanisms to maintain energy homeostasis, mitochondrial quality control and cell survival. A mechanistic understanding of such responses is crucial for further insight into mitochondrial biology and diseases. Through an unbiased genetic screen in *Drosophila*, we identify that mutations in *lrpprc2*, a homolog of the human *LRPPRC* gene that is linked to the French-Canadian Leigh syndrome, results in PINK1-Park activation. While the PINK1-Park pathway is well known to induce mitophagy, we show that in the case of *lrpprc2* mutants, PINK1-Park regulates mitochondrial dynamics by inducing degradation of the mitochondrial fusion protein Mitofusin/Marf. We also discover that Bendless, a K63-linked E2 conjugase, is a regulator of Marf, as loss of *bendless* results in increased Marf levels. We show that Bendless is required for PINK1 stability, and subsequently for PINK1-Park mediated Marf degradation under physiological conditions, and in response to mitochondrial stress as seen in *lrpprc2*. Additionally, we show that loss of Bendless in *lrpprc2* mutant eye results in photoreceptor degeneration, indicating a neuroprotective role for Bendless-PINK1-Park mediated Marf degradation. Based on our observations, we propose that certain forms of mitochondrial stress activate Bendless-PINK1-Park to limit mitochondrial fusion, which is a cell-protective response.
Introduction

Mitochondria are dynamic organelles and their size varies in response to various cellular cues such as developmental signaling (1), metabolic needs (2) or toxin induced stress (2). This change in mitochondrial size is crucial for cellular adaptation under different physiological conditions. For example, upon amino acid deprivation, mitochondria undergo fusion, which results in increased ATP production (3) and protects mitochondria from autophagy (4). Changes in mitochondrial size requires regulation of GTPases essential for mitochondrial dynamics. While the Dynamin 1-like (DNM1/Drp1) protein mediates fission, Mitofusins (Mfn1 and Mfn2 in mammals, Marf in Drosophila) and Optic Atrophy 1 (OPA1) mediate the fusion of mitochondrial outer and inner membranes respectively. Several post-translational modifications, such as phosphorylation, acetylation and ubiquitination are crucial for the activity of these proteins, and thereby play an important role in determining mitochondrial size (5,6). Misregulation of these proteins, and consequently, mitochondrial dynamics, is associated with metabolic and neurodegenerative diseases (7).

The E3 ubiquitin ligase Parkin (Park in Drosophila, PARK2 in humans) and the kinase PINK1, which are linked to autosomal recessive early-onset Parkinsonism, are known to regulate mitochondrial quality control (8). Studies in human cancer cell lines have shown that dissipation of the mitochondrial membrane potential (MMP) can stabilize PINK1 on the outer mitochondrial membrane (OMM) leading to Park recruitment, polyubiquitination of OMM proteins and
mitophagy (9–12). Several in vivo studies have also shown a conserved role for PINK1-Parkin in mitophagy (13–20). While PINK1-Park mediated mitophagy has been extensively studied in cells, how the PINK1-Park pathway is activated under physiological conditions in vivo remains elusive (21). Additionally, in vivo studies suggest a pro-fission role of PINK1-Park (22–26), perhaps through the regulation of mitofusin levels (27). As most of these studies utilize PINK1 and PARK2 mutants to study defects in mitochondrial dynamics, the mechanism by which they are regulated in vivo under various physiological conditions remains unresolved. Additionally, it is unclear as to how the PINK1-Park pathway may activate mitophagy, alter mitochondrial dynamics or selectively target certain OMM proteins in response to mitochondrial stress.

To study the regulation of Mitofusin/Marf in vivo, we undertook an unbiased genetic screen in Drosophila. From this genetic screen, we discovered that mutations in lrpprc2 (ppr), a homolog of human LRPPRC that is required for mitochondrial mRNA stability and translation (28,29), results in activation of the PINK1-Park pathway. This activation then leads to proteasome-mediated Marf degradation, but not mitophagy. We also discovered that mutations in bendless (ben), which encodes a K63-linked E2 ubiquitin conjugase, is essential for Marf degradation in ppr mutants. Further, we demonstrate an essential role for Ben in regulating the stability of PINK1, which in turn is required for maintaining steady state Marf levels in healthy cells. Finally, we show that in ppr mutants, Ben suppresses excessive mitochondrial fusion and prevents neuronal death under mitochondrial stress.

Results:

Loss of ppr results in reduced Marf levels
To identify novel regulators of mitochondrial dynamics, we screened a collection of *Drosophila* X-chromosome lethal mutations (30,31). This collection was generated to identify mutants with neurodegenerative phenotypes and has previously uncovered mutations in *Marf* (32) and several other genes required for mitochondrial function (29,33). We tested these mutants for misregulation of Marf protein using an HA-tagged Marf genomic construct (*Marf::HA*). We used the *FLP*-FRT mediated mitotic recombination strategy to create mutant clones (non-GFP cells) in a heterozygous background (GFP expressing cells) in the developing wing disc epithelium (34). This allowed us to compare Marf levels in mutant and wild type cells within the same tissue (Figure S1A-A’).

From this screen, we found that mutant clones of two independent *ppr* alleles (*ppr^A* and *ppr^E*) show reduced *Marf*:HA levels compared to the surrounding wild type cells (Figure 1A-A”, 1E, Figure 1B-B”). To further confirm these results, we used an independent Marf genomic rescue line, Marf::mCherry and found reduced Marf::mCherry staining in *ppr^A* mutant clones (Figure S1C-C’). To test for the possibility that the reduction in *Marf*:HA or Marf::mCherry is caused by reduced mitochondrial content, we checked the levels of an OMM protein Tom20 using an endogenous tagged line (Tom20::mCherry). We did not observe any change in Tom20::mCherry staining in *ppr^A* mutant clones (Figure 1B-B”, 1E). Taken together, these data suggest that downregulation of Marf in *ppr* mutants is not due to reduced mitochondrial content. Additionally, we also looked at other proteins involved in mitochondrial dynamics — Opa1 and Drp1— using genomic tags. While we found the levels of Opa1::HA to be slightly increased in *ppr^A* mutant clones (Figure 1C-C”, 1E), Drp1::HA levels remained unaltered (Figure 1D-D”, 1E). As mutations in *ppr/LRPPRC* result in mitochondrial defects due to reduced stability of
mtRNA (28,29,37), reduced Marf levels in *ppr* mutants appears to be an adaptation to segregate defective mitochondria by suppressing their fusion.

Since reduced Marf is expected to suppress mitochondrial fusion, we sought to test mitochondrial morphology in *ppr* mutant clones. The cells in wing discs are very compact, and hence it is difficult to study mitochondrial morphology. Hence, we created mutant clones in the peripodial membrane, which is a squamous epithelium overlying wing discs. We used anti-Complex-V staining to mark mitochondria. Interestingly, we found that mitochondrial size is increased in these *ppr* mutant clones (Figure S1E-E”, 1F). Similar increase in mitochondrial size has been observed in *LRPPRC* knockdown in mouse liver (35) and in *C.elegans* (36). As many studies have shown that mitochondrial stress can result in increased mitochondrial size (3,4,36,38), we suspect a similar mechanism results in increased mitochondrial size in *ppr* mutant cells, while an independent mitochondrial quality control mechanism may suppress their fusion by inducing Marf turnover.

**UPS dependent Marf degradation in *ppr* mutants**

Reduced Marf levels in *ppr* mutant clones could be increased protein turnover via selective autophagy or ubiquitin-proteasomal system (UPS). We tested the possibility of autophagic degradation of Marf. We fed chloroquine, an inhibitor of autophagosome-lysosome fusion (39), to larvae and found that Marf::HA levels remain reduced in *ppr* mutant clones (Figure 2B-B’, 2E). Moreover, we found that the levels of p62, a protein degraded primarily via autophagy, was not altered in *ppr* mutant clones (Figure S1D-D’). Thus we conclude that autophagy is neither enhanced nor likely the cause of Marf reduction in *ppr* mutant clones.
To investigate the role of UPS in Marf downregulation in ppr mutants, we fed larvae with the proteasomal inhibitor MG132 (40,41). While in DMSO-fed larvae, ppr<sup>4</sup> mutant clones had lower levels of Marf:HA as compared to the neighboring wild type cells, MG132-fed larvae show no change in Marf::HA levels (Figure 2A-A’, 2C-C’, 2E). We further expressed a dominant negative form of Pros<sup>6</sup> to inhibit UPS activity (42) and tested its effect on Marf::HA levels in ppr<sup>4</sup> mutant clones. Similar to MG132 treatment, we found that Marf::HA levels were restored in ppr<sup>4</sup> mutant clones upon Pros<sup>6′</sup> overexpression (Figure 2D-D’, 2E). These results suggest that UPS-mediated degradation of Marf results in Marf downregulation in ppr<sup>4</sup> mutant clones.

**PINK1 and Park dependent Marf regulation in ppr mutants**

Several E3 ubiquitin ligases have been linked to Mitofusin degradation. For example, Mitofusin degradation by HUWE1 occurs under genotoxic stress or on altered fat metabolism (43,44) while Mitofusin degradation by Park occurs upon mitochondrial membrane depolarization (45,46). In *Drosophila* too, HUWE1, MUL1 and Park have been shown to affect Marf levels (27,43,47). Similar to our observation in ppr<sup>4</sup> mutant clones (Fig. 3A-A’, 3D), we found a down regulation of Marf::HA levels in ppr<sup>4</sup> HUWE1<sup>B</sup> double mutant clones (Figure S2A-A’) and ppr<sup>4</sup> mutant clones in MUL1<sup>46</sup> mutant background (Figure S2B-B’). Interestingly, ppr<sup>4</sup> mutant clones in park<sup>121</sup> mutant background did not show Marf::HA down regulation suggesting Park, but not HUWE1 and MUL1, is required for Marf down regulation in ppr (Figure 3B-B’, 3D). Since park is genetically downstream to Pink1 (48,49), we tested whether PINK1 is also required for Marf degradation in ppr mutant clones. We generated ppr<sup>4</sup> Pink1<sup>5</sup> double mutant clones and found that these clones do not show a reduction in Marf::HA levels (Figure 3C-C’, 3D),
suggesting that mitochondrial impairment in \textit{ppr} mutant cells causes PINK1-Park activation, and subsequently, Marf downregulation. Our observations match previous reports of down regulation of Mfn1 and Mfn2 upon CCCP treatment as a mechanism to suppress mitochondrial fusion prior to PINK1-Park mediated mitophagy (45,46).

\textbf{UPR\textsuperscript{mt} is not sufficient to induce Marf downregulation in \textit{ppr} mutants}

The role of the PINK1-Park pathway in mitochondrial quality control is well known. However, the exact mechanism of PINK1-Park activation in \textit{in vivo} contexts remains unclear. In cancer cell lines, dissipation of MMP and increased oxidative stress have been shown to activate PINK1-Park on the OMM leading to mitophagy (10,50). Therefore, oxidative stress or reduced MMP may activate PINK1-Park and subsequent Marf degradation in \textit{ppr} mutants. However, we have shown earlier that \textit{ppr} mutants do not have increased oxidative stress as compared to controls (29). We checked MMP in \textit{ppr} mutant clones using TMRE, a dye that reversibly stains mitochondria in a membrane potential-dependent manner. We observed that TMRE intensity in \textit{ppr} mutant clones is similar to that of wild type cells (Figure S3A-A’). These observations rule out the possibility that PINK1-Park is activated due to oxidative stress or altered MMP in \textit{ppr} mutants.

Mitochondrial unfolded protein response (UPR\textsuperscript{mt}), which is a cellular response to altered mitochondrial proteostasis, has been shown to activate PINK1-Park leading to mitophagy (51). Therefore, we first tested for UPR\textsuperscript{mt} activation in \textit{ppr} mutants. We determined the levels of Hsp60A which is reported to be increased due to elevated UPRmt (52). We found increased Hsp60A levels in \textit{ppr} mutants as compared to controls, suggesting elevated UPRmt in \textit{ppr} mutants (Figure S3B-B’). Activation of UPR\textsuperscript{mt} upon the loss of \textit{LRPPRC} has also been observed
in *C. elegans* and mammalian cells, and hence, it appears to be an evolutionarily conserved phenomenon (53). Increased UPR\textsuperscript{mt} may induce PINK1-Park activity, which in-turn could lead to Marf downregulation. Therefore, we genetically suppressed the UPR\textsuperscript{mt} response pathways and checked its impact on Marf in *ppr* mutants. Transcription factors Crc (homolog of ATF4), Dve and Foxo are known to mediate UPR\textsuperscript{mt} (54–56). We generated *ppr*\textsuperscript{A} mutant clones with either *crc*, *foxo* or *dve* knocked down. None of these interventions affected Marf::HA downregulation in *ppr*\textsuperscript{A} clones, suggesting that the activation of these UPR\textsuperscript{mt} pathways may not be causing PINK1-Park activation (Figure S3C-E’). However, these interventions would not change the altered mitochondrial proteostasis in *ppr* mutants, which can activate PINK1-Park. Since, to the best of our knowledge, there is no reported method to suppress mitochondrial proteostasis defects, we asked whether the induction of mitochondrial proteostasis defects is sufficient to induce Marf degradation. To induce mitochondrial proteostasis defects, we expressed a mutant form of ornithine transcarbamylase (ΔOTC) that accumulates in an unfolded state and is shown to trigger UPR\textsuperscript{mt} in flies (56). We expressed either ΔOTC or wild type OTC in the posterior half of the wing disc using *En-Gal4 (En>Gal4/+; UAS-ΔOTC/+ or En>Gal4/+; UAS-OTC/+)* and tested Marf::HA levels. We found neither OTC nor ΔOTC expression changed Marf::HA levels in the posterior half (marked by RFP) as compared to the anterior half of the wing discs (Figure S3F-F’, S3G-G’). Although these observations do not rule out a role for mitochondrial proteostasis in activating PINK1-Park in *ppr* mutants, our data suggest that UPR\textsuperscript{mt} is not sufficient to cause Marf degradation.

**Bendless, a K63-linked E2 ubiquitin conjugase, is a regulator of Marf**
To gain further insight into PINK1-Park activation and Marf degradation, we screened for a gene whose loss may cause a subtle increase in Marf levels as observed in park^{421} and Pink1\(^{5}\) mutant clones (Figure S4A-A’, S4B-B’). In our genetic screen, we found two independent alleles of bendless (ben\(^{4}\) and ben\(^{8}\)) showing a subtle but consistent increase in Marf::HA levels in mutant clones (Figure 4A-A’, 4E, S4C-C’). This was also confirmed by western blot using whole larval extracts (Figure 4G-G’). Ben is a fly homologue of the K63-linked E2 ubiquitin conjugase UBE2N/UBC13 with a marked similarity from yeast to humans (Figure S4G). We ruled out the possibility that the increase in Marf::HA levels upon the loss of Ben is due to increased mitochondrial content by determining Tom20 levels, as there was no difference in Tom20::mCherry levels between ben mutant clones and controls (Figure 4B-B’, 4E). We also did not find an increase in Marf mRNA levels in ben mutants suggesting that the increase in Marf protein levels is not a consequence of increased transcription (Figure 4F). These data suggest that Ben regulates Marf levels post-transcriptionally.

Next, we asked whether Ben is sufficient to induce Marf degradation. To test this, we generated a C-terminal V5-tagged Ben (UAS-ben::V5) transgenic line for tissue specific expression of ben and confirmed that the fusion protein is biologically functional by complementing the lethality associated with the ben\(^{4}\) mutant allele (Figure S4F, S4H). We then expressed ben::V5 in the posterior half of the wing disc using the En-Gal4 driver and compared the fluorescence intensities of Marf::HA in the posterior and the anterior halves. We found that ben::V5 overexpression did not affect the levels of Marf::HA (Figure 4C-C’, 4E). Additionally, we overexpressed an N-terminal HA-tagged Ben (UAS-HA::ben) using En-Gal4 and found no change in Marf::mCherry levels (Figure S4E-E’). These data suggest that Ben is necessary but
not sufficient for Marf downregulation. Since loss of *ben, Pink1* or *park* results in mild upregulation of Marf, we hypothesize that Ben acts in the PINK1-Park pathway to regulate the steady state levels of Marf.

**Bendless is essential for Marf downregulation in *ppr* mutants**

Given that Marf undergoes proteolytic degradation in *ppr* mutants, we wanted to look at if Ben is involved in Marf degradation not only basally but under mitochondrial stress as well. We thus created *ppr* and *ben* double mutant clones and found that *ppr<sup>4</sup>* *ben<sup>4</sup>* or *ppr<sup>4</sup>* *ben<sup>8</sup>* double mutant clones showed no reduction in Marf::HA levels, unlike *ppr* mutant clones (Figure 4D-D’, 4E and S4D-D’). This suggests that Ben is essential for Marf degradation in *ppr* mutant cells.

**Bendless is required for PINK1 stability and activity**

To understand the mechanism of Ben mediated Marf degradation, we first looked at subcellular localization of Ben using the Ben::V5 tagged line. We overexpressed Ben::V5 using *Act-Gal4*, and performed cell fractionation from whole larval extracts. We found that in addition to the cytoplasmic fraction (marked by the presence of Tubulin) Ben::V5 is also present in the mitochondria enriched fraction (marked by presence of ComplexV) (Figure 4H). Mitochondria Ben might regulate the activity PINK1-Park pathway to degrade Marf. To study the role of Ben in the PINK1-Park pathway, we tested the functional interaction between *ben* and *Pink1* with respect to Marf degradation. Since PINK1-Park activity is suppressed by PINK1 degradation (57), PINK1 overexpression may activate PINK1-Park mediated Marf downregulation in the wing disc. We overexpressed *Pink1* in the posterior half of the discs (*En-Gal4/UAS-Pink1, UAS-RFP or UAS-GFP*) and checked the levels of Tom20::mCherry, Complex-V and Marf::HA.
We observed no change in Tom20::mCherry and Complex-V levels (Figure 5A-A’, 5D, S5A-A’), but a marked reduction in Marf::HA levels (Figure 5B-B’), suggesting that PINK1 is both necessary and sufficient to downregulate Marf. Pink1 overexpression in the wing discs may not have a significant impact on mitophagy and thus the mitochondrial content (Tom20::mCherry and Complex-V) is not affected. To test the functional interaction between Ben and PINK1, we created benA mutant clones in both wild type and in Pink1 overexpression backgrounds and found that Pink1 overexpression does not induce Marf::HA downregulation in benA mutant clones (Figure 5C-C’, 5D) or in benA mutant wing discs (Figure S5B-B’). These data suggest that Ben is necessary for PINK1 activity to cause Marf downregulation.

To understand how Ben may regulate PINK1 activity, we checked the effect of loss of Ben on PINK1 levels. We performed western blots using whole larval extracts from control and benA mutants containing genomic tagged PINK1::Myc. We found a significant downregulation of full length PINK1::Myc in benA mutants, but an increase in low molecular weight PINK1::Myc bands, suggesting that Ben is required for stability of full length PINK1 (Figure 5E-F’). The low molecular weight bands might be products of PINK1 degradation by mitochondrial proteases as described by Thomas et.al. (58). Taken together, our data suggests that Ben is required for the stability of PINK1 and mediates the homeostatic turnover of Marf.

**Ben regulates mitochondrial dynamics under mitochondrial stress**

An RNAi-based screen in larval fat bodies has shown ben RNAi leads to enlarged mitochondria, similar to when Pink1 or park are knocked down (59). This would be in accordance with our observation wherein loss of ben results in Marf upregulation. To better characterize
mitochondrial morphology we looked at Complex-V antibody staining in mutant larval muscles. In this tissue, loss of ben shows a similar filamentous network as seen in wildtype (Figure 6A-B, S6A-B). However, loss of *ben* in *ppr* mutants exacerbates mitochondrial morphology defects seen in *ppr*. On loss of *ppr* alone larval muscles show distinctive large globular mitochondria along with filamentous and ring-shaped mitochondria (Figure 6C, S6C). *ppr* *ben* double mutants rarely show filamentous mitochondria (Figure 6D). Instead, we observed a significant increase in the size and frequency of large globular and ring-shaped mitochondria as compared to *ppr* (Figure 6D). We also observed that in lesser frequency *ppra* *ben*a double mutants mitochondria form clusters, especially around the nucleus which is not observed in either *ppra* or *ben*a mutants (Figure 6D, S6D). These results suggest that Ben is required to suppress the hyperfusion of defective mitochondria in *ppr* and thereby regulates mitochondrial quality control.

**Loss of Bendless accelerates photoreceptor degeneration in *ppr* mutants**

Mutations in human *LRPPRC* cause Leigh Syndrome, a neurometabolic disease (60). Earlier work has shown that mutations in *ppr* cause activity induced retinal degeneration (29). As mutations in *ben* exacerbates the mitochondrial morphology phenotypes in *ppr* mutants, loss of *ben* may accelerate the degenerative phenotype. To test this hypothesis, we made eye specific *ppr*, *ben* and *ppr* *ben* double mutant clones using the ey-FLP system (30). We found that *ppr* mutant and *ben* mutant eyes show normal morphology but *ppr* *ben* double mutant eyes show severe retinal degeneration in young flies suggesting that loss of *ben* can accelerate retinal degeneration (Figure 6E-H). This result suggests that Marf regulation by Ben is a neuroprotective mechanism.
**Discussion:**

To identify novel regulators of mitochondrial fusion in an *in vivo* system, we screened fly mutants for altered Marf levels. We found that mutations in *ppr*, which result in mitochondrial dysfunction, causes reduction in Marf levels (Figure 1A). We found that in *ppr* mutants, Marf is degraded by the UPS in a PINK1-Park dependent mechanism (Figure 2C-D, 3A-C). In the screen, we also identified mutations in *ben* causing subtle Marf upregulation (Figure 4A, 4G). We found that Ben is essential for PINK1 activity (Figure 5C), regulates Marf levels (Figure 4D) and mitochondrial morphology (Figure 6D) in *ppr* mutants. We also found that a loss-of-function mutation of both *ppr* and *ben* in the eyes results in accelerated retinal degeneration (Figure 6H) indicating that under mitochondrial stress Ben mediated regulation of mitochondrial dynamics is a protective mechanism (Figure 7B).

Increased mitochondrial size has been observed in several mitochondrial diseases (61); however, it is not clear as to how these abnormal mitochondria contribute to disease progression. Mitochondrial fusion occurs in a bid to increase oxidative phosphorylation under various cellular and mitochondrial stresses, a response termed as stress induced mitochondrial hyperfusion [SIMH (3)] (62,63). We propose that reduced ETC activity and mitochondrial stress in *ppr* (28,29,36), can induce SIMH (Figure 6D, S1E). Mitochondrial fusion has been observed upon loss of *ppr* homologs in *C.elegans*, mouse and human cell lines (35,64) as well as in other mutants where the ETC is compromised (52,65,66). Since SIMH increases ATP synthesis and inhibits mitophagy (3,4,67,68), increased mitochondrial size appears to be a compensatory adaptation in *ppr* mutants in response to a bioenergetic deficit or mitochondrial stress.
Despite the increased mitochondrial size in ppr mutants (Figure S1E), we observed Marf downregulation. We hypothesize that, while an adaptive mechanism may induce SIMH (cellular response), MQC may induce Marf degradation to suppress fusion of dysfunctional mitochondria (mitochondrial response) (Figure 7B). As proposed earlier (62,63), this condition appears to be a conflict between the bioenergetic adaptations and the MQC mechanisms. Alternatively, increased mitochondrial size in ppr mutant cells may induce PINK1-Park to suppress further mitochondrial fusion by Marf degradation. A similar hypothesis was also proposed by Yamada et al. (69). We found that Marf degradation in ppr mutant clones in developing wing primordium is dependent on Park (Figure 3B). We also observed a subtle increase in Marf levels in park and Pink1 mutants (Figure S4A-B) (27). This suggests that the PINK1-Park plays a homeostatic role in Marf turnover in wild type tissue, while mitochondrial impairments—as in ppr mutants (29)—may further amplify its activity to reduce Marf levels (Figure 1A) possibly to segregate damaged mitochondria (9,45,46). The remarkable discovery by Narendra et al. that CCCP, which dissipates MMP, induces PINK1-Park-dependent mitophagy in cancer cells provided an unparalleled assay to investigate the mechanism further (10,70). However, we observe PINK1-Park activation in the absence of severe mitochondrial depolarisation (Figure S3A) in ppr mutants results in Marf degradation and not mitophagy (Figure 1A-B). We also find the PINK1 overexpression is sufficient to induce Marf degradation without triggering mitophagy (Figure 5A-B, S5A). In vivo studies have shown PINK1-Park to function both in mitophagy (13–20) and mitochondrial dynamics (22–26), but the physiological or cellular contexts that may determine various downstream activities of PINK1-Park are not known (21,71,72). Thus ppr mutants provide a novel and physiologically relevant in vivo system to study PINK1-Park mediated Marf regulation under mitochondrial stress.
In steady state conditions, PINK1 is imported into the mitochondria and cleaved by mitochondrial peptidases, it then retro translocates to the cytoplasm and is degraded by UPS to limit PINK1-Park activity (58,73,74). Loss of MMP, increased oxidative stress or increased UPR\textsuperscript{mt} stabilizes full length PINK1, which then recruits Park leading to ubiquitination of OMM proteins and mitophagy (9,10,51,75,76). Given no change in MMP (Figure S3A) and oxidative stress in \textit{ppr} mutants (28,29,37), we suspected that mitochondrial proteostasis activates PINK1-Park to downregulate Marf. However, activation of UPR\textsuperscript{mt} by ΔOTC expression did not result in Marf degradation suggesting that activation of UPR\textsuperscript{mt} alone may not be sufficient to activate PINK1-Park mediated Marf degradation \textit{in vivo} (Figure S3G). Identification of additional factors leading to PINK1-Park activation for Marf degradation \textit{in vivo} requires further investigation.

In most cells, PINK1 activity is maintained at low levels via its constant turnover by mitochondrial proteases and the UPS (57,77). For example, CHIP-mediated K48-ubiquitination promotes PINK1 turnover (78), while BAG2, a chaperon, prevents ubiquitination and promotes PINK1 stability (79,80). We found that Marf degradation in \textit{ppr} mutants or by \textit{Pink1} overexpression is completely suppressed in the absence of the K63-linked E2 conjugase Ben (Figure 4D, S4D and 5C). Previous studies have observed that the mammalian homolog of Ben, UBE2N, is dispensable for mitophagy but facilitates the clustering of mitochondria during CCCP induced mitophagy (81–83). We also found that the loss of \textit{ben} does not alter developmental mitophagy during larval midgut remodeling (Figure S5C), which has been shown to be dependent on PINK1-Park (13,14).
We hypothesize three possible mechanisms through which Ben regulates Marf degradation. One, given that Park activity is regulated by K63 ubiquitination (84), Ben may ubiquitinate Park. Two, Ubiquitin C-terminal hydrolase L1 (UCHL1), which suppresses Marf degradation (85), is K63 ubiquitinated leading to its autophagic degradation (86). Thus, Ben might mediate K63 ubiquitination and degradation of UCHL1. K63 ubiquitination of PINK1 by the Traf6-SARM1 complex is shown to stabilize PINK1 (87). Therefore, Ben may stabilize PINK1 by K63 ubiquitination. The fact that loss of ben results in reduced PINK1 levels (Figure 5E-E’), suggests Ben is likely to increase the stability of PINK1 by K63 ubiquitination. Indeed human PINK1, in cell culture systems, is known to be ubiquitinated at K137 by both K48 and K63 linkages (88). While K48 chains are linked with PINK1 degradation; the significance of K63 linkage is not obvious. K63 ubiquitination is suggested to protect proteins from proteasomal degradation (89). Overall, Ben-mediated K63 ubiquitination appears to be responsible for PINK1 stability (Fig. 7A).

In conclusion, Ben-PINK1-Park regulation of Marf appears to be a homeostatic function which is further activated in response to aberrant mitochondrial function. ppr ben double mutants show aberrant mitochondrial morphology in larval muscles and severe retinal degeneration as compared to ppr mutant eyes indicating a protective role for Ben (Figure 6). Given that mutations in LRPPRC result in Leigh syndrome, it would be crucial to check the activation of Ben-PINK1-Park in Leigh syndrome and other mitochondrial diseases. Indeed, altered mitochondrial dynamics has been reported in many mitochondrial diseases (61,90–92). It is possible an adaptive response in these diseases can modify mitochondrial dynamics in a
Ben-PINK1-Park-dependent mechanism. Thus, further studies on the mechanisms of Ben-PINK1-Park activation will be crucial for understanding mitochondrial quality control in mitochondrial disease.

**Figure Legends**

**Figure 1. ppr mutants show Marf down regulation:** (A-D')ppr<sup>A</sup> mutant clones (non green cells, A-D and dashed white line, A'’-D’’), wing discs immunostained for Marf::HA (red, A-A’’), Tom20::mCh (red, B-B’’), Opa1::HA (red, C-C’’) and Drp1::HA (red, D-D’’) (genomic rescue tags). A’’-D’’ are magnified images of insets shown in A’-D’. (E) Quantification for relative fluorescence intensities of Marf::HA (n=9), Tom20::mCh (n=15), Opa1::HA (n=16) and Drp1::HA (n=19) in ppr<sup>A</sup> mutant clones. Graphs represent average intensity values normalized to that of control cells. Two tailed paired t-test between control and ppr<sup>A</sup> mutant cells. Significance represented by p<0.001***.

**Supplementary Figure 1:** (A) Schematic to illustrate Flp-FRT mediated recombination system. (A’) Green marks wild type and heterozygous cells (solid white line, +/- and +/-) absence of GFP marks mutant clones/cells (dashed white line, -/-). (B-B’)ppr<sup>E</sup> mutant clones (non green cells, B- and dashed white line, B’), wing discs immunostained for Marf::HA (red). (C-C’)ppr<sup>A</sup> mutant clones (non green cells, C and dashed white line, C’), wing discs immunostained for Marf::mCh (red). (D-D’)ppr<sup>A</sup> mutant clones (non green cells, D and dashed white line, D’), wing discs immunostained for endogenous p62 (red). (E-E’)ppr<sup>A</sup> mutant clones (non green cells, E) in peripodial cells of third instar larval wing discs, immunostained for Complex-V (gray). Inset of
control (E’) and ppr^d mutant cell (E’’) from E. (E’-E’’). Binary image of Complex-V staining. Scale bar represents 10µm in (A) and 4µm in (E’ and E’’).

**Figure 2. ppr mutants show UPS mediated Marf degradation:** (A-C’)ppr^d mutant clones (non green cells, A-C and dashed white line, A’-C’), wing discs immunostained for Marf::HA (red) after feeding larvae with DMSO (A-A’), chloroquine (B-B’) or MG132 (C-C’). (D-D’)ppr^d mutant clones (non green cells, D and dashed white line, D’) on overexpression of Prosp61 under Actin>Gal4, wing discs immunostained for Marf::HA (red). (E)Quantification for relative fluorescence intensities of Marf::HA in ppr^d mutant clones on treatment with DMSO (n=24), chloroquine (n=6), MG132 (n=10) and on overexpression of Prosp61 under Actin>Gal4 (n=15). Graphs represent average intensity values normalized to that of control cells. Two tailed paired t-test between control and ppr^d mutant cells. Significance represented by p<0.001***

**Supplementary Figure 2:** (A-A’)ppr^d HUWE1^B double mutant clones (non green cells, A and dashed white line, A’), wing discs immunostained for Marf::HA (red). (B-B’)ppr^d mutant clones (non green cells, B and dashed white line, B’) in MUL1^66 mutant background, wing discs immunostained for Marf::HA (red).

**Figure 3. PINK1-Park are required for Marf degradation in ppr mutants:** (A-A’)ppr^d mutant clones (non green cells, A and dashed white line, A’), wing discs immunostained for Marf::HA (red). (B-B’)ppr^d mutant clones (non green cells, B and dashed white line, B’) in park^121 background, wing discs immunostained for Marf::HA (red). (C-C’)ppr^d Pink1^5 double mutant clones (non green cells, C and dashed white line, C’), wing discs immunostained for Marf::HA
(D) Quantification for relative fluorescence intensities of Marf::HA in ppr\textsuperscript{A} mutant clones (n=14), ppr\textsuperscript{A} mutant clones in park\textsuperscript{A21} background (n=17) and ppr\textsuperscript{A} Pink1\textsuperscript{S} double mutant clones (n=13). Graphs represent average intensity values normalized to that of control cells. Two-tailed paired t-test between control and mutant cells. Significance represented by p<0.01**, p<0.001**

**Supplementary Figure 3:** (A-A') ppr\textsuperscript{A} mutant clones (non green cells, A and dashed white line, A'), wing discs stained for TMRE (red) and live imaged. (A’’) Quantification for relative fluorescence intensity of TMRE in ppr\textsuperscript{A} mutant clones (n=20). Graphs represent average intensity values normalized to that of control cells. Two-tailed paired t-test between control and ppr\textsuperscript{A} mutant cells showed no significant change. (B-B’) ppr\textsuperscript{A} mutant clones (non green cells, B and dashed white line, B’), wing discs immunostained for Hsp60 (red). (C-D’) ppr\textsuperscript{A} mutant clones (non green cells, C, D, E and dashed white line, C’ D’, E’) on KD of crc(C-C’), foxo(D-D’) and dve(E-E’) using En>Gal4, wing discs marked by UAS-RFP (green) and immunostained for Marf::HA (red). (F-G’) Overexpression of OTC(F-F’) and ΔOTC(G-G’) using En>Gal4, wing discs marked by UAS-RFP (green) and immunostained for Marf::HA (red).

**Supplementary Figure 4:** (A-E’) Wing discs immunostained for Marf::HA (red) in park\textsuperscript{A21} mutant clones (green cells, A-A’) Pink1\textsuperscript{S} mutant clones (non green cells, B-B’), ben\textsuperscript{b} mutant clones (non green cells, C-C’) and ppr\textsuperscript{A} ben\textsuperscript{b} (non green cells, D-D’). Wing discs immunostained for Marf::mCh (red) on overexpression of HA::Ben using En>Gal4 wing discs marked with UAS-GFP (green) (E-E’). (F) Table for Ben mutations and lethal staging. (G) Similarity and identity between Ben and its homologs. (H) Schematic showing point mutations in ben\textsuperscript{A} and ben\textsuperscript{b}
Figure 4: Ben is required for Marf degradation in ppr mutants: (A-B’)ben<sup>4</sup> mutant clones (non green cells, A, B and dashed white line, A’, B’), wing discs immunostained for Marf::HA (red, A-A’), Tom20::mCh (red, B-B’). (C-C’)Overexpression of ben::V5 using En>Gal4, wing discs immunostained for Ben::V5 (green) and Marf::HA (red). (D-D’)ppr<sup>4</sup> ben<sup>4</sup> double mutant clones (non green cells, D and dashed white line, D’), wing discs immunostained for Marf::HA (red). (E)Quantification for relative fluorescence intensities of Marf::HA in ben<sup>4</sup> mutant clones (n=15), Tom20::mCh in ben<sup>4</sup> mutant clones (n=7), Marf::HA on overexpression of ben::V5 (n=6), Marf::HA in ppr<sup>4</sup> ben<sup>4</sup> double mutant clones (n=16). Graphs represent average intensity values normalized to that of control cells. Two-tailed paired t-test between control and mutant cells. (F)Quantification of Marf mRNA levels in third instar ben mutant (y w ben<sup>4</sup> FRT19A) larvae compared to control (y w FRT19A) (n=3). Two tailed unpaired t-test between control and ben<sup>4</sup> mutant larvae. (G-G’)Representative western blot for ben mutant (y w ben<sup>4</sup> FRT19A) and control (y w FRT19A) larval lysate probed for Marf::HA and Actin. (G’)Quantification for intensity of Marf::HA band normalized to Actin band intensity for ben<sup>4</sup> mutant and control larvae (n=5). Two-tailed unpaired t-test between control and ben<sup>4</sup> mutant larvae. (H)Representative western blot for total larval lysate (Total), Cytoplasmic fraction (Cyto), and Mitochondrial enriched fraction (Mito) probed for Ben::V5, Complex V and Tubulin. Ben::V5 is present in Total and Cyto fraction in abundance, while a small amount of Ben::V5 is visible in Mito fraction. Error bars represent S.E.M. Significance represented by p<0.05*, p<0.01**, p<0.0001***
Figure 5. *ben* is required for PINK1 mediated Marf degradation: *(A-A')* Overexpression of Pink1 using En>Gal4, wing discs marked with UAS-RFP (blue, A) and immunostained for Tom20::mCh (red, A-A'). *(B-B')* Overexpression of Pink1 using En>Gal4, wing discs marked with UAS-RFP (blue, B) and immunostained for Marf::HA (red, B-B'). *(C-C')* *ben*^4 mutant clones (non green cells, C and dashed yellow line, C') in background of overexpression of Pink1 using En>Gal4, wing discs marked with UAS-RFP (blue, C) and immunostained for Marf::HA (red, B-B'). *(D)* Quantification for relative fluorescence intensities of Tom20::mCh in UAS-Pink1 cells (n=7), Marf::HA in *ben*^4 mutant clones (n=17), UAS-Pink1 cells (n=8) and *ben*^4 mutant clones in UAS-Pink1 background (n=8). Graphs represent average intensity values normalized to that of control cells. Two-tailed paired t-test between control and mutant cells or cells overexpression of Pink1. *(E-E')* Representative western blot for *ben* mutant (*y w ben*^4 FRT19A) and control (*y w FRT19A*) larval lysate probed for Myc and Actin. *(E')* Quantification for intensity of full length (FL) PINK1::Myc band normalized to actin band intensity for *ben*^4 mutant and control larvae (n=8). Two-tailed unpaired t-test between control and mutant larvae. *(F-F')* Representative western blots for *ben* mutant (*y w ben*^4 FRT19A) and control (*y w FRT19A*) larval lysate probed for Myc and Actin. *(F')* Quantification for intensity of cleaved PINK1::Myc bands normalized to actin band intensity for *ben*^4 mutant and control larvae (n=8). One sample two-tailed t-test of bands of *ben*^4 mutant larvae normalized to its corresponding control band taken as one. Same samples were used to quantify results as in Fig 5E’ and Fig 5F’. Error bars represent S.E.M. Significance represented by p<0.05*, p<0.01**, p<0.0001***, n.s - non significant.

**Supplementary Figure 5:** *(A-A')* Overexpression of Pink1 using En>Gal4, wing discs marked with UAS-RFP (blue) and immunostained for Complex-V(red). *(B-B')* *ben*^4 mutant on
overexpression of Pink1 using En>Gal4, wing discs marked with UAS-RFP (blue) and immunostained for Marf::HA (red). (C-C’)ben<sup>A</sup> mutant clone (non-green cell, C and dashed white line C’), pupal gut 2h APF expressing Sq>mito-EYFP (red) shows complete clearance of mitochondria.

**Figure 6. Ben is required for maintaining mitochondrial morphology and neuronal health in ppr mutants:** (A-D) Confocal sections of 3<sup>rd</sup> instar larval muscles immunostained for Complex-V (gray) in control(A), ben<sup>A</sup>(B), ppr<sup>A</sup>(C) and ppr<sup>A</sup> ben<sup>A</sup>(D) larvae. Representative individual mitochondrial morphology is marked by different colors: filamentous (red), large globular (yellow) and ring (blue). (E-H) Mutant eye clones from young flies of control(E), ben<sup>A</sup>(F), ppr<sup>A</sup>(G), and ppr<sup>A</sup> ben<sup>A</sup>(H) genotypes.

**Supplementary Figure 6:** (A-D) Confocal sections of third instar larval muscles immunostained for endogenous Complex-V (gray) in control(A), ben<sup>A</sup>(B), ppr<sup>A</sup>(C) and ppr<sup>A</sup> ben<sup>A</sup>(D) larvae.

**Figure 7:** (A) Mitofusin turnover is regulated by Bendless mediated stabilization of PINK1. In presence of Ben, PINK1 is stabilized on the mitochondrial surface and results in Park mediated ubiquitination of Marf. Ubiquitinated Marf is degraded by the proteasome complex. Absence of Ben results in increased proteolytic cleavage of PINK1 resulting in accumulation of Marf. (B) Bendless prevents fusion of impaired mitochondria. In healthy mitochondria Ben-PINK1 mediated Marf turnover keeps adequate Marf protein aiding mitochondrial fusion. Upon mitochondrial stress (as we show in case of mutation in ppr) results in presence of impaired mitochondria, we predict that the increase in Ben-PINK1 mediated Marf degradation
keeps the damaged mitochondria isolated and thus can be repaired or sent for mitophagy. In the absence of Ben however Marf is not removed from impaired mitochondria and results in fusion of impaired mitochondria with the healthy pool.

**Material and Methods**

**Drosophila culture**

Flies were cultured on standard media containing sucrose, malt, yeast and corn flour at room temperature. Crosses were maintained at 25°C. Crosses involving RNAi were maintained at 28°C. *Drosophila* larvae expressing UAS-Proβ6 were maintained at 25°C till 3rd instar stage, and were then transferred to 28°C for 24 hours before dissection, to avoid cell death observed on prolonged inhibition of proteasomal activity. To activate the FLP-FRT system, heat shock was given during first instar larval stages at 37°C for 1hr. Genotypes used are as listed in Table 1. For drug treatments 3rd instar larvae were transferred to food containing 3mM chloroquine, 100µM MG132, or DMSO (vehicle control) for 24 hours prior to dissection. For western blot and qPCR, 3rd instar larvae were used. We observed that development of pprA mutant larvae is substantially delayed. Therefore, we used size matched 3rd instar pprA mutant larvae that are obtained after 14-15 days post hatching.

**Generation of transgenic flies**

*ben* sequence was amplified from genomic DNA. These PCR amplified *ben* ORF sequences were then inserted into a pUAST vector containing attB sites, flanking the insert using EcoRI-XhoI. pUAST vectors containing UAS-ben::V5/UAS-HA::ben were injected into embryos containing attP2 landing site and integrase. Transgenic flies were selected based on presence of
Primers used: P{UAS.ben++;V5.w^{imC}}: Fwd-5’-GGAATTCGCCACCATGTCCAGCC TGCCACGTC-3’ and Rev-5’-CCGCTCGAGTTACGTAGAATCGAGACCGAGGAGA GGGTTAGGGATAGGGCTTACCGTCGTACGACCGCATAT-3’. P{UAS-HA::ben.w^{imC}}:
Fwd-5’-GGAATTCGCCACCATGTACCCATACGACGTCCCAGACTACGCTATGTCCAGCC TGCCACGTC-3’ and Rev-5’-CCGCTCGAGTCAGTCTTCGACGGCATAT-3’.

Opa1::3FLAG-2HA genomic construct was generated using the P(acman) system (93). Briefly, the 3FLAG-2HA tag was amplified from C-terminal tag fusion vector pL452-C-3FLAG-2HA and inserted at the C terminal of Opa1 through recombineering in the P(acman) clone CH322-27B08, which was subsequently injected into y^{1} w^{1118}; PBac{y+;attP-3B}VK00033 flies.

**Immunofluorescence and imaging**

Larvae were dissected in 1X PBS, followed by fixing in 4% paraformaldehyde (Himedia - TCL-119 - 100ml) for 30 minutes at room temperature and three washes in 1X PBS with 0.2% TritonX-100 (Himedia - MB031, 1X PBST). Primary antibodies were incubated overnight at 4°C. Followed by blocking in 5% normal goat serum (Himedia - RM10701) for 1h at room temperature and then secondary antibody incubation followed by washing and dissection. Samples were mounted in Vectashield (VectorLabs - H100) and imaged under 40X or 63X oil immersion Leica Stellaris 5 or Olympus FV3000 confocal microscopes. Images were processed using Fiji. All antibody dilutions and the blocking solution were made in 1X PBST; details of antibodies and their dilutions used are listed in Table 2.

**Eye phenotype imaging**

Mutant eyes were created by crossing heterozygous mutant flies with w cl(l) FRT19A /Dp(1;Y); ey-FLP flies. The eye images were then acquired on a Leica M205FA Stereo Zoom microscope.

**Mitochondrial fractionation**
Thirty 3rd instar larvae were collected and washed with chilled mitochondrial isolation buffer (210mM mannitol, 70mM sucrose, 1mM EGTA, 5mM HEPES, and 0.5% BSA). The larvae were then transferred into a 1.5ml centrifuge tube containing 250µl of chilled mitochondrial isolation buffer. Micro-pestles were used to homogenize the samples, keeping the sample on ice. 300 µl of mitochondrial isolation buffer was added to the lysate. Lysate was centrifuged at 200G for 5 mins at 4°C to remove large debris. The supernatant was then centrifuged at 1500G for 5mins at 4°C and the pellet was discarded. The supernatant was centrifuged at 8000G for 15mins at 4°C. The supernatant containing the cytoplasmic fraction was processed for western blot by adding Laemmli buffer (0.004% bromophenol blue, 20% glycerol, 4% SDS and 0.125M Tris-HCl pH 6.8) having 5% beta-mercaptoethanol and heated at 98°C for 5mins. The pellet was resuspended in 550µl fresh mitochondrial isolation buffer and again centrifuged at 8000G for 15 mins at 4°C. The pellet containing the mitochondrial fraction was resuspended in 100µl of Laemmli buffer having 5% beta-mercaptoethanol and heated at 98°C for 5mins.

**Western blot**

3rd instar larvae were crushed in RIPA lysis buffer [50mM Tris,150mM NaCl, 0.2% Triton X 100 and 1X protease and phosphatase inhibitor cocktail (Thermo Fisher - A32965,A32957 respectively)], followed by centrifugation at 16,000g for 10 mins at 4°C. Clear fat free supernatant was used for total protein estimation. Lysate was mixed with equal volume of 1X Laemmli buffer (0.004% bromophenol blue, 20% glycerol, 4% SDS and 0.125M Tris-HCl pH 6.8) having 5% beta-mercaptoethanol and heated at 98°C for fine minutes, centrifuged, and 25µg of protein was loaded in each well and resolved on 4-15% gradient Tris-Glycine gel (Bio-Rad - 4561086). Semi-dry transfer was done onto 0.2µm Nitrocellulose membrane as per Trans-Blot Turbo Kit (Bio-Rad - 1704270) for seven minutes. Blocking in either 5% Blotto
(Santa Cruz sc - 2325) or 5% BSA made in 1X TBS with 0.1% Tween-20 (1X TBSTw20) for 1 hour at room temperature followed by primary antibody incubation overnight at 4°C. After washing thrice in 1X TBSTw20, membranes were incubated in HRP conjugated secondary antibodies (Table2) for 2 hours at room temperature. After washing, they were developed using Clarity Western ECL Substrate (Bio-Rad - 1705061) and visualized using Vilber-Lourmat chemidoc. Band intensities were quantified using Fiji and normalized with βActin.

**Real-time PCR**

3rd instar larvae were used for RNA isolation using TRIzol (Ambion life tech - 15596018) method. cDNA conversion for 1µg of RNA was carried out using a cDNA conversion kit (Thermo Fisher - 4368814). qPCR was carried out in 96 well plates in three technical replicates for each of the three biological replicates. Marf qPCR was done using the iTaq SYBR Green supermix (Bio-Rad -1725121) using LightCycler 96 (Fig. 5F).

Following primers were used :

Marf-Fwd-5’-CGAGTGCCAGGAATCGGTTA-3’,
Marf-Rev5’-ATCTGAAAGCCCTCGGCAAT-3’,
RP49-Fwd-5’-TCCTACCAGCTTCAGATGAC-3’,
RP49-Rev-5’-CACGTTGTGAACCAGGAACT-3’.

**TMRE Staining**

3rd instar larvae were dissected in Schneider's Insect media (Himedia - IML003-500ml) . The larvae were transferred to media containing 100nM TMRE (Thermo Fisher - T669) in Schneider's Insect media and incubated for 20 mins. The wing discs were dissected and mounted in Schneider's media using a coverslip. The tissues were live imaged using Leica Stellaris 5 confocal microscope at 63X oil objective.
Mitochondrial morphology analysis

Wing discs immunostained for Complex-V were imaged using Leica Stellaris 5 confocal microscope at 63X oil objective. The mitochondria were segmented on Fiji using the Trainable Weka segmentation plugin (94). The segmented images were then used to find out mitochondrial area using Particle Analyze Tool on Fiji.

Blind test: For qualitative assessment of mitochondrial morphology in larval muscle, we renamed a set of images containing mitochondria from larval muscles with random numbers. The images from different genotypes (control, ben\(^4\), ppr\(^4\), and ppr\(^4\) ben\(^4\)) were pooled and were assessed for the presence of different mitochondrial morphologies, including presence or absence of mitochondria network, large globular mitochondria, ring shaped mitochondria and mitochondrial aggregates. Multiple images were used for the assessment, 40 images from 11 larvae for control, 24 images from 7 larvae for ben\(^4\), 27 images from 7 larvae for ppr\(^4\), and 32 images from 9 larvae for ppr\(^4\)ben\(^4\).

Statistics analysis

At least three independent experiments were used for all quantifications, the n values for each experiment is indicated in their respective figure legends. Two-tailed paired t-test was used to analyze data obtained from clonal analysis, One sample t-test was used to analyze the data in Supplementary Fig. 5. Two-tailed unpaired t-test was used to analyze all other data sets. Significance of the data was represented as * for p<0.05, ** for p<0.01, and *** for p<0.0001. Details of the test used and the significance is mentioned in respective figure legends.
| Genotype                                                                 | Source                  |
|-------------------------------------------------------------------------|-------------------------|
| **Figure 1**                                                            |                         |
| \( y' \text{ ppr}^A \text{ w* P\{neoFRT\}19A} \)                     | FBst0067166 (29)        |
| \( \text{Marf::HA} \) (Genomic tag on Chromosome III)                   | (32)                    |
| \( \text{Ubiquitin}>\text{GFP hsFLP [122], P\{neoFRT\}19A} \)        | Hugo Bellen             |
| \( \text{Ubiquitin}>\text{GFP, hsFLP [122], P\{neoFRT\}19A ;; Marf::HA(III) \) | This study              |
| \( \text{Tom20-mCherry (Genomic construct Chromosome III) } \) (95)     |                         |
| \( \text{Ubiquitin}>\text{GFP, hsFLP [122], P\{neoFRT\}19A;; Tom20-mCherry} \) | This study              |
| \( \text{w;; pacman Opa1::HA::Flag VK31 (III) (Opa1::HA) } \)         | This study              |
| \( \text{Ubiquitin}>\text{GFP, hsFLP [122], P\{neoFRT\}19A ;; Opa1::HA::Flag VK31} \) | This study              |
| \( y' \text{ w* ; P\{w[+mC]\}=FLAG-FlAsH-HA-Drp1\}3, Ki[1] } \)     | FBst0042208 (96)        |
| Expression | Source |
|------------|--------|
| Ubiquitin>GFP, hsFLP [122], P{neoFRT}19A ;; P{w+[mC]=FLAG-FlAsH-HA-Drp1}3, Ki[1] | This study |
| y¹ w* P{neoFRT}19A | (30) |

**Figure 2**

| Expression | Source |
|------------|--------|
| y¹ ppr¹ w* P{neoFRT}19A | FBst0067166 (29) |
| w ;; Actin>Gal4 | Hugo Bellen |
| w ;; Actin>Gal4, Marf::HA (III) | This study |
| y¹ ppr¹ w* P{neoFRT}19A;; Actin>Gal4, Marf::HA (III) | This study |
| w*; P{UAS-Probeta6[1].B}2B (II) | FBst0006786 (42) |

**Figure 3**

| Expression | Source |
|------------|--------|
| y¹ ppr¹ w* P{neoFRT}19A | FBst0067166 (29) |
| w* Pink1<sup>5</sup> | FBst0051649 (48) |
| y¹ ppr¹ Pink1<sup>5</sup> P{neoFRT}19A | This study |
| w*;; park<sup>121</sup> (III) | FBst0051652 |
| y\textsuperscript{I} ppr\textsuperscript{A} w* P\{neoFRT\}19A;; park\textsuperscript{A21} Marf::HA | This study |
| Ubiquitin\textgreater;GFP, hsFLP [122], P\{neoFRT\}19A ;; park\textsuperscript{A21} | This study |
| Ubiquitin\textgreater;GFP, hsFLP [122], P\{neoFRT\}19A; P\{Marf::HA\}2 | This study |

**Figure 4**

| y\textsuperscript{I} w* ben\textsuperscript{A} P\{neoFRT\}19A | FBst0057057 (30) |
| Ubiquitin\textgreater;GFP, hsFLP [122], P\{neoFRT\}19A ;; Marf::HA (III) | This study |
| Ubiquitin\textgreater;GFP, hsFLP [122], P\{neoFRT\}19A ;; Tom20::mCherry (III) | This study |
| w; P\{Marf::HA\}2 P\{w[+mW.hs]=en2.4-GAL4\}e16E, P\{w[+mC]=UAS-RFP.W\}2 | This study |
| w\textsuperscript{*}; P\{UAS.ben::V5.w+mC\} | This study |
| y\textsuperscript{I} ppr\textsuperscript{A} w* P\{neoFRT\}19A | FBst0067166 (30) |
| y\textsuperscript{I} ppr\textsuperscript{A} w* ben\textsuperscript{A} P\{neoFRT\}19A | This study |

**Figure 5**
| Genotype                                                                 | Ref. |
|-------------------------------------------------------------------------|------|
| y¹ w⁺; P{Marf::HA}2 P{w[+mW.hs]=en2.4-GAL4}e16E, P{w[+mC]=UAS-RFP.W}2 | This study |
| w⁺; P{w[+mC]=UAS-Pink1.C}A                                               | FBst0051648 (48) |
| y¹ w⁺ ben⁴ P{neoFRT}19A ; P{Marf::HA}2                                   | This study |
| P{w[+mW.hs]=en2.4-GAL4}e16E, P{w[+mC]=UAS-RFP.W}2                      | |
| Ubiquitin>GFP, hsFLP [122], P{neoFRT}19A;;P{w[+mC]=UAS-Pink1.C}A        | This study |
| P{Pink1-9Myc} (Pink1::Myc) (II)                                         | FBtp0022940 (48) |
| y¹ w⁺ ben⁴ P{neoFRT}19A; P{Pink1-9Myc}                                   | This study |
| y¹ w⁺ P{neoFRT}19A; P{Pink1-9Myc}                                        | This study |
| **Figure 6**                                                            |      |
| y¹ w⁺ P{neoFRT}19A                                                      | (30) |
| y¹ w⁺ ben⁴ P{neoFRT}19A                                                 | FBst0057057 (30) |
| y¹ ppr⁴ w⁺ P{neoFRT}19A                                                 | FBst0067166 (29) |
| y¹ ppr⁴ ben⁴ P{neoFRT}19A                                               | This study |
\[ cl^1 \ w^* \ FRT19A/ Dp(1;Y)y+ v+; \ ey-FLP \] (30)

**Supplementary Figure 1**

| y' \ ppr^4 \ w^* \ P\{neoFRT\}19A | FBst0067166 |
|------------------------------------|-------------|
|                                     | (29)        |

| y' \ ppr^2 \ w^* \ P\{neoFRT\}19A | FBst0067167 |
|------------------------------------|-------------|
|                                     | (29)        |

\[ P\{Ubi-mRFP.nls\}, \ w^* \ P\{hsFLP\}, \ P\{neoFRT\}19A \] FBst0031418

\[ w^*:P\{w[+mC]=Marf-gHA\}2 \ (Genomic \ construct) \]

\[ P\{Ubi-mRFP.nls\}, \ w^* \ P\{hsFLP\}, \ P\{neoFRT\}19A; P\{Marf-gHA\}2 \]

**Marf::mCherry** (Genomic rescue construct, Chromosome III)

Ubiquitin>\(\text{GFP, hsFLP [122]}, \ P\{neoFRT\}19A \); \(\text{Marf::mCherry (III)}\)

\[ y' \ w^* \ P\{neoFRT\}19A \] (30)

Ubiquitin>\(\text{GFP hsFLP [122], P\{neoFRT\}19A}\)

Hugo Bellen

**Supplementary Figure 2**

\[ y' \ w^* \ HUWE1^B \ P\{neoFRT\}19A \] FBst0052343
| Strain Description                                                                 | Source     |
|-----------------------------------------------------------------------------------|------------|
| `ppr^4 HUWE19 P{neoFRT}19A`                                                        | This study |
| `Ubiquitin>GFP, hsFLP [122], P{neoFRT}19A ;; Marf::HA (III)`                     | This study |
| `MUL1^{46} (III)`                                                                | FBal0301081|
| `y^{1} ppr^4 w* P{neoFRT}19A ;; MUL1^{46}`                                        | This study |
| `Ubiquitin >GFP, hsFLP [122] ,P{neoFRT}19A ;; MUL1^{46}`                          | This study |
| `y^{1} w* P{neoFRT}19A`                                                           | (30)       |
| `w^{1118};P{w[+mW.hs]=en2.4-GAL4}e16E, P{w[+mC]=UAS-RFP.W}2`                    | FBst0030577|
| `w; P{Marf::HA}2 P{w[+mW.hs]=en2.4-GAL4}e16E, P{w[+mC]=UAS-RFP.W}2`             | This study |
| `y^{1} ppr^4 w* P{neoFRT}19A;P{Marf::HA}2`                                        | This study |
| `P{w[+mW.hs]=en2.4-GAL4}e16E, P{w[+mC]=UAS-RFP.W}2`                              |            |
| `y^{1} v^{1}; P{y[+t7.7] v[+t1.8]=TRiP.JF02007}attP2 (RNAi against crc)`        | FBst0025985|
| Ubiquitin >GFP, hsFLP [122], P{neoFRT}19A ;; | This study |
|---------------------------------------------|------------|
| P{y[+t7.7] v[+t1.8]=TRiP.JF02007}attP2 (RNAi against crc) | |
| w^{1118}, P{GD1425}v3781 (RNAi against dve) | FBti0084290 |
| Ubiquitin >GFP, hsFLP [122], P{neoFRT}19A;; | This study |
| P{GD1425}v3781 (RNAi against dve) | |
| y¹ v¹; P{y[+t7.7] v[+t1.8]=TRiP.JF02734}attP2 (RNAi against foxo) | FBst0027656 |
| Ubiquitin >GFP, hsFLP [122], P{neoFRT}19A;; | This study |
| P{y[+t7.7] v[+t1.8]=TRiP.JF02734}attP2 (RNAi against foxo) | |
| P{UAS-rOTC.P} (UAS-OTC) | FBal0291051 |
| (UAS-OTC) | (56) |
| P{UAS-rOTC.d} (UAS-ΔOTC) | FBal0291052 |
| (56) | |
| Supplementary Figure 3 | |
| P{FRT(w¹¹)}2A | FBst0001997 |
| park^{2+I} P{FRT(w¹¹)}2A | This study |
| P{hsFLP}1, y¹ w* P{UAS-mCD8::GFP.L}Ptp4ELL4: P{tubP-GAL80}LL9 P{FRT(whs)}2A | FBst0044404 |
| Pink1^{5} P{neoFRT}19A | This study |
| Expression |
|-----------------|-----------------|
| Ubiquitin>GFP, hsFLP [122], P{neoFRT}19A ;; Marf::HA (III) | This study |
| y¹ w¹* ben® P{neoFRT}19A | FBst0057058 (30) |
| P{Ubi-mRFP.nls}, w¹* P{hsFLP}, P{neoFRT}19A ; P{Marf::HA}2 | This study |
| y¹ ppr¹ w¹*ben® P{neoFRT}19A | This study |
| w¹*; P{UAS-HA::ben.w+mC} | This study |
| y¹ w¹*; P{w[+mC]=tubP-GAL4}LL7 (III) | FBti0012687 |
| w¹*; P{UAS.ben::V5.w+mC} | This study |
| **Supplementary Fig 4** |
| w¹*; P{w[+mC]=UAS-Pink1.C}A | FBst0051648 (48) |
| w; P{Marf::HA}2 P{w[+mW.hs]=en2.4-GAL4}e16E, P{w[+mC]=UAS-RFP.W}2 | This study |
| y¹ w¹* ben® P{neoFRT}19A ; P{Marf::HA}2 | This study |
| P{w[+mW.hs]=en2.4-GAL4}e16E, P{w[+mC]=UAS-RFP.W}2 | |
| P{Pink1-9Myc} (Pink1::Myc) | FBtp0022940 (48) |
TABLE 2

| Antibody       | Catalog                           | Dilution |
|----------------|-----------------------------------|----------|
| Mouse HA       | Cell Signaling Technology- 2367S  | 1:500 IF |
| Rabbit HA      | Cell Signaling Technology- 3724S  | 1:500 IF |
| Mouse Complex-V| Abcam- 176569                      | 1:2500 WB|
| Rabbit V5      | Cell Signaling Technology- 13202S | 1:500 IF |
| Antibody                      | Vendor                          | Dilution | Method  |
|-------------------------------|--------------------------------|----------|---------|
| Rabbit mCherry                | Cell Signaling Technology- 43590S | 1:500    | IF      |
| Rabbit Actin                  | Cell Signaling Technology- 4967S | 1:5000   | WB      |
| Mouse Actin                   | Invitrogen- MA5- 15739          | 1:5000   | WB      |
| Rabbit Tubulin                | Novus Biologicals- NB100-56459  | 1:1000   | WB      |
| Rabbit Hsp60A (98)            |                                 | 1:200    | IF      |
| Mouse Myc Tag                 | Cell Signaling Technology-2276S | 1:1000   | WB      |
| Rabbit p62                    | Abcam- ab178440                 | 1:500    | IF      |
| Anti-Mouse 488                | Invitrogen- A11029              | 1:500    | IF      |
| Anti Mouse 555                | Invitrogen- A21424              | 1:500    | IF      |
| Anti-Mouse 633                | Invitrogen- A21052              | 1:500    | IF      |
| Anti-Rabbit 488               | Invitrogen- A32731              | 1:500    | IF      |
| Anti-Rabbit 555               | Invitrogen- A32732              | 1:500    | IF      |
| Anti-Rabbit 647               | Invitrogen- A32733              | 1:500    | IF      |
| Anti-Rabbit HRP               | Novus Biologicals- NB7160       | 1:5000   | WB      |
| Anti-Mouse HRP                | Novus Biologicals- NB7539       | 1:5000   | WB      |

IF- Immunofluorescence WB- Western Blot
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Figure 1

**ppr^A** mutant (non GFP)

A

Marf::HA

B

Tom20::mCh

C

Opa1::HA

D

Drp1::HA

E

Control

|               | Marf::HA | Tom20::mCh | Opa1::HA | Drp1::HA |
|---------------|----------|------------|----------|----------|
| **Relative fluorescence intensity** |          |            |          |          |
| 0.6           |          |            |          |          |
| 0.8           |          |            |          |          |
| 1.0           |          |            |          |          |
| 1.2           |          |            |          |          |

***

ppr^A
A Heterozygous mutant cells with one copy of GFP

FLP mediated recombination at FRT sites

+/+

-/-

non GFP

Supplementary Figure. 1

B

ppr^E

Marf::HA

C

ppr^A

Marf::mCh

D

ppr^A

p62

B'

C'

D'

E

E''

E'''

F

Mitochondrial area (µm^2)

Control

ppr^A

*
**Figure 2**

*ppr^A* mutant (non-GFP/RFP) Marf::HA

|                | DMSO                  | Chloroquine          |
|----------------|-----------------------|----------------------|
| **A**          | [Image](#)            | [Image](#)           |
| **A'**         | [Image](#)            | [Image](#)           |
| **B**          | [Image](#)            | [Image](#)           |
| **B'**         | [Image](#)            | [Image](#)           |
| **C**          | [Image](#)            | [Image](#)           |
| **C'**         | [Image](#)            | [Image](#)           |
| **D**          | [Image](#)            | [Image](#)           |
| **D'**         | [Image](#)            | [Image](#)           |
| **E**          | [Image](#)            | [Image](#)           |

**Relative Marf::HA intensity**

- **DMSO**
- **Chloroquine**
- **MG132**
- **Actin>UAS-Proβ6^1**

### Controls

- **Control**
- **ppr^A***
Supplementary Figure. 2

Mutant clones (non GFP) Marf::HA

A

ppr^A HUWE1^B

B

ppr^A ; MUL1^AG

A'

B'

Figure 3

Mutant clones (non GFP) Marf::HA

A

ppr^A

B

ppr^A ; park^Δ21

C

ppr^A Pink1^5

A'

B'

C'

D

Relative Marf::HA intensity

Control

ppr^A

park^Δ21

ppr^A Pink1^5

park^Δ21
Figure 4

**En**>*UAS-ben::V5 Marf::HA**

**ppr**<sup>A</sup> **ben**<sup>A</sup> mutant (non-GFP) Marf::HA

**E**

Relative fluorescence intensity

- **Marf::HA**
- **Tom20::mCh**
- **Marf::HA**
- **Marf::HA**

**F**

Fold change

- **Marf mRNA**

**G**

Subcellular localization of Ben

- **Total**
- **Cyto**
- **Mito**

**H**

Tubulin

Complex V

Ben::V5

**Control**

**ben**<sup>A</sup>

**Control**

**ben**<sup>A</sup>

**Control**

**UAS ben::V5**

**Control**

**ppr**<sup>A</sup> **ben**<sup>A</sup>

**Control**

**ben**<sup>A</sup>

**Control**

**ben**<sup>A</sup>

**Control**

**ben**<sup>A</sup>

**Control**

**ben**<sup>A</sup>

**Control**

**ben**<sup>A</sup>
**Supplementary Figure. 4**

| Mutant (GFP) Marf::HA | Mutant (non-GFP) Marf::HA |
|------------------------|---------------------------|
| ![A](parkΔ21)           | ![A'](Pink1^5)            |
| ![B](parkΔ21)           | ![B'](Pink1^5)            |
| ![C](ben^B)            | ![C'](ppr^A ben^B)        |
| ![D](ben^B)            | ![D'](ppr^A ben^B)        |

**G**

**En > UAS-GFP Marf::mCh**

**F**

| Allele | Mutation | Lethality |
|--------|----------|-----------|
| ben^A  | W129Stop | Pupal     |
| ben^B  | P120L    | Pupal     |
| ben^A; Tubulin>UAS-ben::V5 | - | Viable |

**G**

| Ben homologs | Identity | Similarity |
|--------------|----------|------------|
| Human UBE2N  | 121/152 (80%) | 137/152 (90%) |
| Mouse Ube2n  | 121/152 (80%) | 137/152 (90%) |
| Yeast UBC13  | 105/149 (70%) | 125/149 (84%) |

**H**

- ben^A(W129Stop)
- UBQ-Conjugate _E2_ domain
- ben^B (P120L)
Figure 5

En>UAS-Pink1 UAS-GFP Tom20::mCh

En>UAS-Pink1 UAS-RFP MarfHA

A

B

C

A'

B'

C'

D  Relative fluorescence intensities

Tom20::mCh

Marf::HA

E  Relative PINK1::Myc levels

Control  ben^A

100KDa

75KDa

37KDa

PINK1::Myc

Actin

F  Relative levels of cleaved forms of Pink1::Myc

Control  ben^A

1

2

3

4

5 + 6

100KDa

75KDa

37KDa

Actin
Supplementary Figure. 5

| En>UAS-Pink1 | UAS-RFP | ben$^A$ mutant (non-GFP) |
|--------------|---------|-------------------------|
| ![Image](A)  | ![Image](B) | ![Image](C)  |
| Complex-V    | ben$^A$ | Marf::HA                |
| ![Image](A') | ![Image](B') | ![Image](C')  |
|              |         | Mito-EYFP               |
**Figure 6**

| A | Control |
|---|---------|
| B | *ben*<sup>A</sup> |
| C | *ppr*<sup>A</sup> |
| D | *ppr*<sup>A</sup> *ben*<sup>A</sup> |

| E | Control |
|---|---------|
| F | *ben*<sup>A</sup> |
| G | *ppr*<sup>A</sup> |
| H | *ppr*<sup>A</sup> *ben*<sup>A</sup> |

- Filamentous
- Large Globular
- Ring

**Mutant eyes**
Supplementary Figure 6

Larval muscle stained with Complex-V

A. Control

B. benA

C. pprA

D. pprA benA
Figure 7

A  Presence of Bendless

Marf is degraded by proteasome complex

A  Absence of Bendless

PINK1 undergoes proteolysis cleavage

B  Presence of Bendless

Fusion of healthy mitochondria

B  Absence of Bendless

Impaired mitochondria may not segregate

Impaired mitochondria may not segregate

Ben-Pink1

Marf degradation