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PINK1- Phosphorylated Mitofusin 2 is a Parkin Receptor for Culling Damaged Mitochondria

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Abstract

Senescent and damaged mitochondria undergo selective mitophagic elimination through mechanisms requiring two Parkinson's disease factors, the mitochondrial kinase PINK1 and the cytosolic ubiquitin ligase Parkin. The nature of the PINK-Parkin interaction and identity of key factors directing Parkin to damaged mitochondria are unknown. We show that the mitochondrial outer membrane GTPase mitofusin (Mfn) 2 mediates Parkin recruitment to damaged mitochondria. Parkin bound to Mfn2 in a PINK1-dependent manner; PINK1 phosphorylated Mfn2 and promoted its Parkin-mediated ubiqitination. Ablation of Mfn2 in mouse cardiac myocytes prevented depolarization-induced translocation of Parkin to the mitochondria and suppressed mitophagy. Accumulation of morphologically and functionally abnormal mitochondria induced respiratory dysfunction in Mfn2-deficient mouse embryonic fibroblasts and cardiomyocytes, and in Parkin-deficient *Drosophila* heart tubes, causing dilated cardiomyopathy. Thus, Mfn2 functions as a mitochondrial receptor for Parkin, and is required for quality control of cardiac mitochondria.

Mitochondria are endosymbiotic organelles derived from primitive aerobic bacteria. Healthy mitochondria are essential energy generators for most metazoan cell functions, whereas senescent or damaged mitochondria are sources of toxic reactive oxygen species. Thus, mitochondrial biogenesis and mitophagic elimination are carefully orchestrated, and their mutational disruption causes chronic degenerative diseases (1). Genetic studies have linked Parkinson's disease to mutations of two mitophagy genes, the E3 ubiquitin ligase Parkin and the serine-threonine protein kinase PINK1 (PTEN-induced putative kinase protein 1) (2). Loss of the mitochondrial inner membrane electrochemical gradient stabilizes PINK1 on damaged organelles, tagging them for Parkin binding, ubiquitination, and mitophagic elimination (3). The specific nature of the molecular interaction between PINK1 and Parkin is unclear, and Parkin receptor proteins on damaged mitochondria have not been identified.

Mitofusins (Mfn) 1 and 2 are mitochondrial outer membrane fusion proteins and Parkin ubiquitination substrates (4, 5). Combined genetic ablation of Mfn1 and Mfn2 in mouse hearts induces mitochondrial dysfunction and fragmentation that should stimulate mitophagic removal, but instead results in proliferation of abnormal organelles (6). Because mitophagy is stimulated by Parkin-mediated ubiquitination of mitochondrial proteins (7, 8), we tested whether Mfn1 or Mfn2 might mediate signaling activity of the PINK1-Parkin.

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Parkin co-immunoprecipitated with Mfn2, but not Mfn1, from extracts of human embryonic kidney (HEK) cells transfected with tagged mitofusins and Parkin. The association of Mfn2 and Parkin was greatly enhanced in cells also transfected to overexpress PINK1 (Figs. 1A, 1B, fig. S1).

The oxidative phosphorylation inhibitor carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) depolarizes mitochondria and stimulates PINK1-mediated translocation of Parkin to mitochondria, thus targeting damaged organelles for mitophagy (3) (fig. S2). Consistent with a role for Mfn2 in this process, FCCP treatment stimulated Parkin binding to endogenous fibroblast Mfn2 (Fig. S3). Overexpression of PINK1 enhanced, whereas PINK1 suppression with RNAi inhibited, this effect of FCCP (fig. S3). We developed an ex vivo model of this pathway in cardiomyocytes because they express endogenous Mfn2 and Parkin in large amounts and have abundant mitochondria. In genetically normal cardiomyocytes, mitochondrial uncoupling with FCCP (fig. S4) directed redistribution of Parkin to intracellular punctae especially profuse in the mitochondrial-rich perinuclear region (Fig. 1C, left panels). Parkin translocation was stimulated by FCCP in Mfn1-null, but not Mfn2-null, cardiomyocytes (Fig. 1C, middle and right panels). In Mfn2-null cells Parkin remained diffusely cytosolic, demonstrating a requirement for endogenous Mfn2 to promote Parkin localization to depolarized mitochondria. Because Mfn1 and Mfn2 are each expressed in normal amounts in hearts lacking their counterpart (fig. S5), and recombinant Mfn1 does not bind Parkin (Fig. 1A), Parkin recruitment appears to be a unique property of Mfn2.

Mfn2 can be a Parkin ubiquitination substrate (8). This might require PINK1-stimulated association of Parkin and Mfn2 with mitochondria, so we tested the functional significance of PINK1-Mfn2-Parkin interactions on ubiquitination. We observed complete concordance of ubiquitination with PINK1-stimulated Mfn2 binding to Parkin in HEK cells (Fig. 2A), and with FCCP-stimulated binding of endogenous cardiac Mfn2 to Parkin in isolated perfused mouse hearts (Fig. 2B). Conversely, Mfn2 (but not Mfn1) gene ablation in cardiac myocytes decreased mitochondrial ubiquitination stimulated by mitochondrial depolarization, following the same pattern seen for Parkin translocation (Fig. 2C, fig. S6). Moreover, the mitophagic response measured as punctal accumulation of the mitophagy adaptor protein p62 (also called sequestosome 1[SQSTM1]) was impaired in FCCP-treated Mfn2-deficient cardiac myocytes (Fig. 2D, fig. S6). Together, these data support a model in which Mfn2 functions as a receptor to which cytosolic Parkin binds on depolarized mitochondria, provoking ubiquitination of mitochondrial proteins that target the organelle for autophagic elimination.

Enhancement of Mfn2-Parkin association by PINK1 indicated that Mfn2 might be phosphorylated. We noted a slight electrophoretic mobility shift in a fraction of Mfn2 when PINK1 was co-transfected, suggesting a possible post-translational modification (Fig. 3A, top). This mobility shift was augmented on Phos-Tag gels, indicating that Mfn2 might be phosphorylated at one or mores sites (Fig. 3A, bottom; fig. S7). Indeed, phosphoserine immunoreactivity of immunoprecipitated Mfn2 was also increased in cells overexpressing PINK1 (Fig. 3B). If PINK1-mediated phosphorylation of Mfn2 is required for Mfn2-Parkin interactions, then catalytically inactive PINK1 should be ineffective. Consistent with this notion, the catalytically inactive PINK1 K219A/D362A/D384A mutant (9) failed to promote Mfn2-Parkin binding or to induce the characteristic change in Mfn2 mobility on Phos-Tag gels (Fig. 3C).

To establish that Mfn2 is a substrate of PINK1 and the mitochondrial binding partner for Parkin, we mapped the Mfn2 PINK1 phosphorylation sites and examined their functional consequences. Bioinformatics analysis identified three highly conserved potential Mfn2

phosphorylation sites: T111, S442, and Y448 (figs. S8 and S9). As PINK1 is a serine-threonine kinase, we mutated Mfn2 T111 and S442 to alanine, preventing their phosphorylation. Both Mfn2 mutations decreased PINK1-stimulated Mfn2-Parkin binding without eliminating it (Fig. 3D), whereas simultaneous mutation of both residues (T111A/S442A) completely abrogated PINK1-stimulated interactions between Mfn2 and Parkin (Fig. 3E). In agreement with these loss-of-function data, Mfn2 mutations that mimic PINK1 phosphorylation of Mfn2 (T111E/S442E) conferred PINK1-independent binding activity to Parkin (Fig. 3E). Collectively, these studies reveal a potential mechanism by which Mfn2 orchestrates the PINK1-Parkin mitochondrial quality control apparatus.

We tested whether decreased Parkin translocation caused by the absence of its mitochondrial binding partner would adversely impact long-term mitochondrial homeostasis. Because it has no Parkin binding activity, deletion of Mfn1 had no effect on mitochondrial morphometry or respiratory function of cardiomyocytes (fig. S10 A and B). However, deletion of Mfn2 provoked mitochondrial enlargement, which we measured by ultrastructural examination (Fig. 4A, fig. S10A) and flow cytometric forward light scatter (fig. S10C). Mfn2-null cardiac myocytes also exhibited decreased substrate-dependent O_2 consumption, pointing to mitochondrial respiratory impairment (Fig 4B).

Mitochondrial function deteriorates over time in senescent hearts, contributing to increased prevalence of heart failure with age (10). Because mitochondrial abnormalities caused by Mfn2 ablation resemble those of age-related cardiomyopathy, we assessed in vivo cardiac chamber dilation and contractile function over time in hearts from cardiac-specific Mfn1 and Mfn2 knockout mice. Hearts lacking Mfn1 appeared normal (fig. S11A) and had normal heart function assessed through non-invasive echocardiography (fig. S11B) or invasive hemodynamic measurements of contractility (+dP/dt) (fig. S11C). However, hearts lacking Mfn2 dilated with increasing age (fig. S11D), developed impaired contractile performance assessed by echocardiography (Fig. 4C, fig. S11E), and were insensitive to β-adrenergic stimulation (fig. S11F). If cardiomyopathy in hearts lacking Mfn2 is caused by the defect in Parkin signaling, then primary Parkin deficiency should recapitulate the organelle and organ phenotypes. Indeed, in *Drosophila* lacking Parkin (11) cardiomyocyte mitochondria were enlarged (Fig. 4D), heart tubes exhibited a defect in respiration (Fig. 4E), chamber dilatation and contractile impairment (Fig. 4F, fig. S12), and Parkin-deficient cardiomyocyte mitochondria were not effectively ubiquitinated after FCCP stimulation (Fig. 4G). The common properties of Mfn2 deficient mouse hearts and Parkin deficient *Drosophila* heart tubes are characteristic of maladaptive remodeling and dilated cardiomyopathy. The inability of Parkin to promote ubiquitin-dependent mitophagy in both models may contribute to accumulation of abnormal mitochondria that ultimately impairs cellular respiration and compromises cardiac function.

Our results implicate the mitochondrial fusion protein Mfn2 as a Parkin receptor on damaged mitochondria, linking mitochondrial regeneration with selective organelle culling. Studies have suggested a role for Mfn2 in mitophagy downstream of Parkin (4, 5), but it also appears also to function upstream to help translate the PINK1 signal for Parkin translocation. We envision that PINK1 stabilized in depolarized mitochondria phosphorylates Mfn2 that attracts and binds Parkin to promote mitophagy. In the absence of Mfn2, the PINK1-Parkin pathway of mitophagic quality control is interrupted, abnormal mitochondria accumulate, and cardiac toxicity ensues. A broad role for Mfn2 as Parkin receptor is indicated by mitochondrial abnormalities also observed with liver- and neuron-specific Mfn2 ablation (12, 13). Parkin translocation to mitochondria in cultured embryonic fibroblasts lacking Mfn2 (8) indicates activity for one or more compensatory Parkin binding mechanisms, although respiratory compromise suggests that these secondary mechanisms are incompletely effective (fig. S13). Like tethering to endoplasmic reticulum (14), the

unique Parkin receptor function of Mfn2 distinguishes it from Mfn1. Defective Parkin binding may have importance in hereditary neuropathies genetically linked to mutations of Mfn2 and characterized by accretion of abnormal mitochondria (15). The adverse consequences of interrupting Parkin-mediated mitophagy in the heart might also provide insight into the previously unexplained epidemiological link between Parkinson's disease and cardiomyopathy in the elderly (16, 17).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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17. Yun Chen performed experiments and analyzed data. Gerald W. Dorn, II designed the studies, analyzed data and wrote the manuscript. We thank Poonam Bhandaria and Sohail Anwar for assistance with Drosophila studies; Yingqiu Liu, Qingling Huang, and Yan Zhang for technical assistance. Supported by National Institutes of Health R01 HL059888 and R21 HL107276.

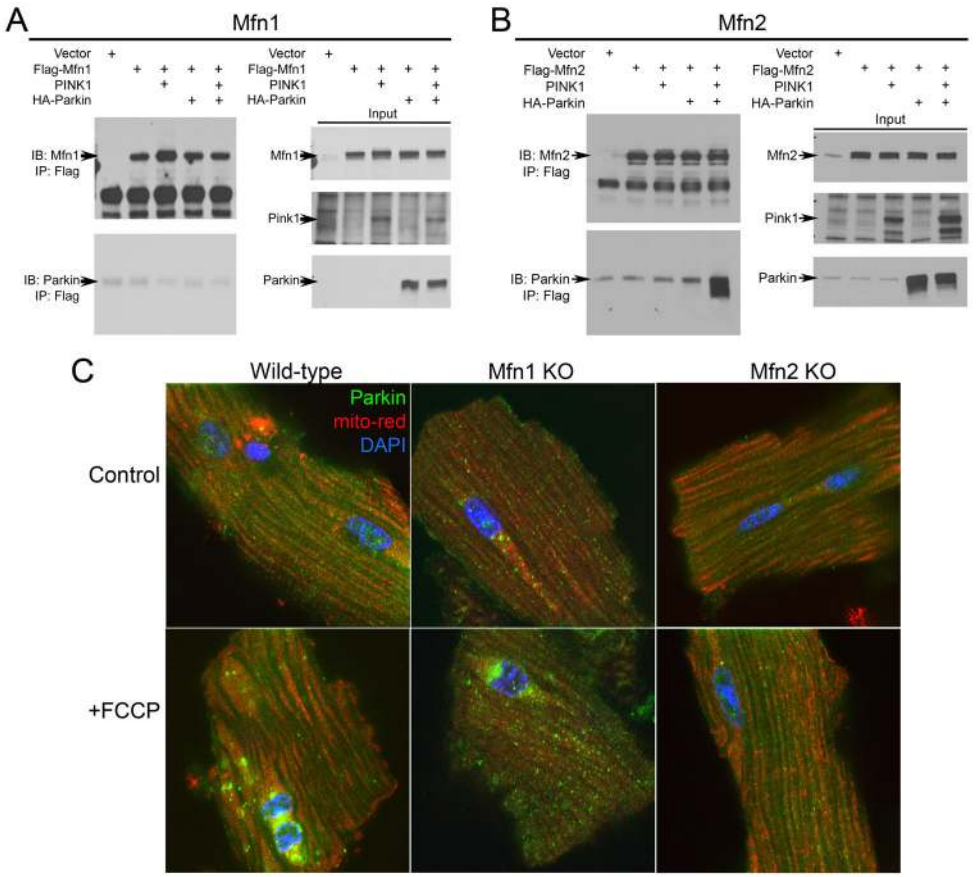

Fig. 1. Interaction of Mfn2 with Parkin in a PINK1-dependent manner and its requirement for Parkin translocation to depolarized mitochondria

(A and **B)** Fibroblasts were transfected with Flag-Mfn1 (A) or Mfn2 (B), PINK1, and/or HA-Parkin, immunoprecipitated with anti-Flag, and immunoblotted (IB). Right panels show IB of input homogenates. **(C)** Subcellular Parkin redistribution (green) induced by mitochondrial depolarization with FCCP in wild-type, Mfn1-deficient, and Mfn2-deficient mouse cardiomyocytes.

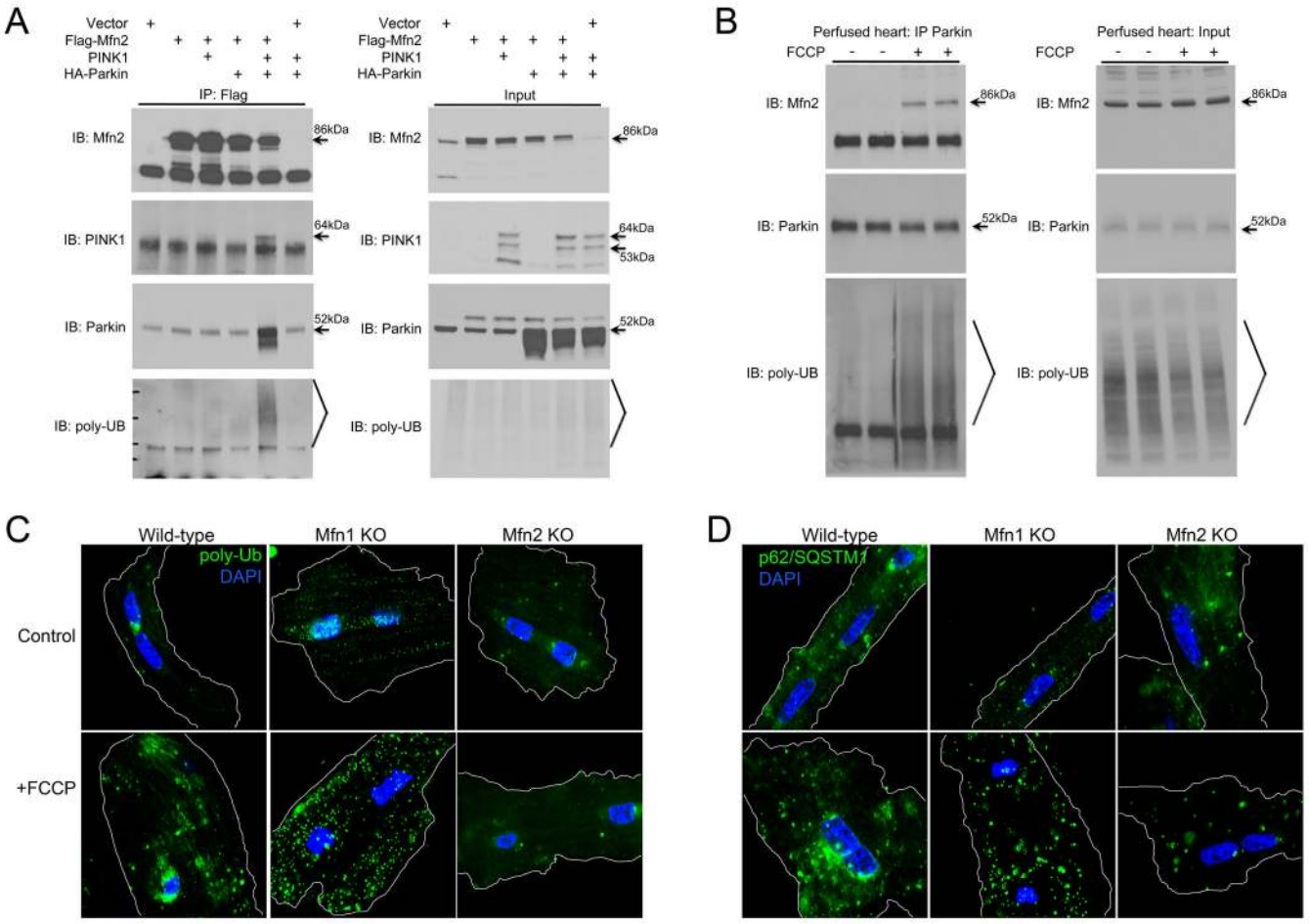

Fig. 2. Defective ubiquitination and mitophagy in Mfn2-deficient mouse hearts

(A) Mfn2 ubiquitination in PINK1/Parkin-containing HEK cell immune complexes (left). Input proteins are on right. **(B)** Protein ubiquitination stimulated by FCCP in Mfn2/Parkin immune complexes from mouse heart. **(C)** Mitochondrial ubiquitination (green) induced by FCCP in wild-type, Mfn1-, and Mfn2-deficient mouse cardiomyocytes. Cell borders are outlined in white. **(D)** Parallel studies of mitophagy-adaptor protein p62/SQSTM1 (green).

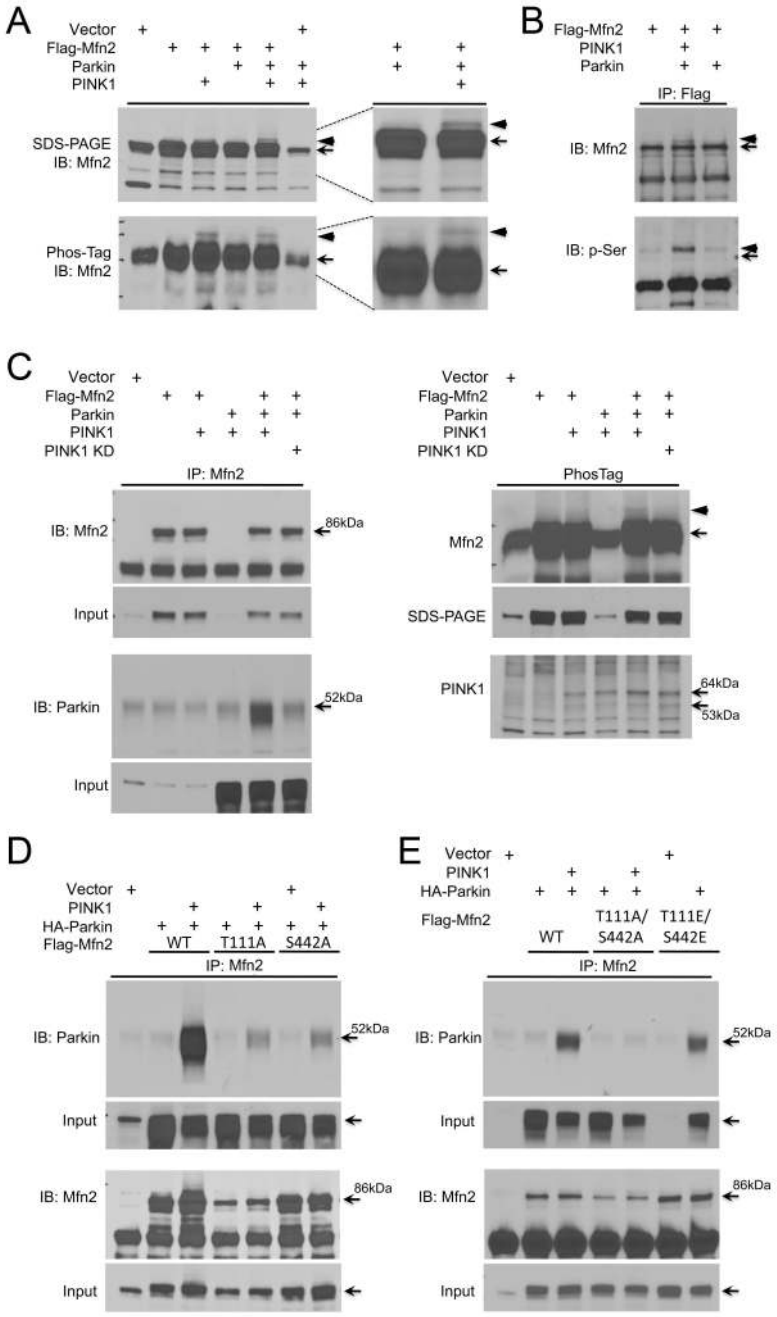

Fig. 3. PINK1 Phosphorylation of Mfn2 T111 and S442 dictates Parkin binding

(**A**) Mfn2 electrophoretic mobility shifts (arrowheads) induced by PINK1 in SDS-PAGE (top) and Phos-Tag (bottom) gels. Exploded view of lanes 4 and 5 is shown to the right. (**B**) PINK1-mediated Mfn2 phosphorylation (arrowheads) by anti-phosphoserine immunoblot (IB). (**C**) (*left*) Mfn2-Parkin co-immunoprecipitation study with functional and kinase-defective (KD) PINK1; (*right*) Mfn2 Phos-Tag phosphorylation study with functional and KD PINK1. (**D**) Effects of Mfn2 T111A and S442A mutations on PINK-stimulated Mfn2-Parkin binding. (**E**) Abrogation of PINK1-stimulated Mfn2-Parkin binding by Mfn2 T111A/S442A mutation and induction of PINK1-independent Mfn2-Parkin binding by Mfn2 T111E/S442E mutation.

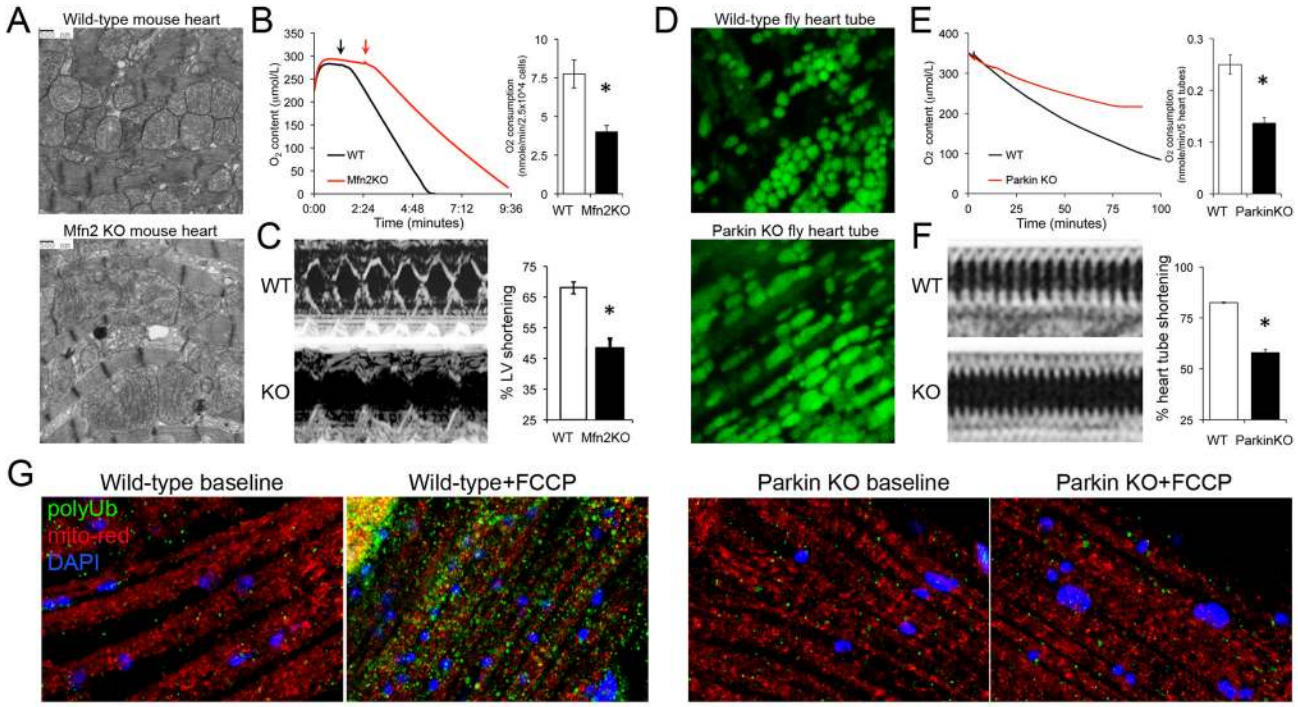

Fig. 4. Progressive cardiomyopathy of Mfn2-deficient mouse hearts and Parkin-deficient Drosophila heart tubes

(A) Transmission electron microscopic examination of mouse cardiomyocyte mitochondria (5,000x). **(B)** Whole cell O_2 consumption studies of cadiomyocytes isolated from wild-type (black) or Mfn2-deficient (red) mouse hearts. **(C)** M-mode echocardiograms of mouse hearts. **(D)** Confocal imaging of *Drosophila* cardiomyocyte mitochondria. **(E)** O_2 consumption studies of heart tubes isolated from wild-type (black) or Parkin-deficient (red) *Drosophila*. **(F)** Optical coherence tomography of *Drosophila* heart tubes. (**G)** Mitochondrial ubiquitination (green) induced by FCCP in wild-type and Parkin-deficient *Drosophila* cardiomyocytes.