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# Parkin-induced ubiquitination of Mff promotes its association with p62/SQSTM1 during mitochondrial depolarization

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## Abstract

The ubiquitin ligase Parkin and autophagic adapter protein p62 are known to function in a common pathway controlling mitochondrial autophagy (mitophagy). However, the evidence supporting that p62 is directly recruited by ubiquitinated proteins remains undetermined. Here, we demonstrate that mitochondrial fission factor (Mff) associates with Parkin and carbonyl cyanide *m*-chlorophenyl hydrazone treatment significantly increases the affinity of Parkin with Mff. After recruitment to depolarized mitochondria, Parkin mediates poly-ubiquitination of Mff at lysine 251. Replacement of lysine 251 by arginine (K251R) totally abrogates Parkin-stimulated ubiquitination of Mff. Subsequently, the ubiquitinated Mff promotes its association with p62. Mff knockout interferes with p62 translocation to damaged mitochondria. Only re-transfection of Mff WT, but not K251R mutant, rescues this phenotype. Furthermore, loss of Mff results in failure of Parkin translocation and final clearance of damaged mitochondria. Thus, our data reveal functional links among Mff, p62, and the selective autophagy of mitochondria, which are implicated in the pathogenesis of neurodegeneration diseases.

**Key words:** Parkin, Mff, p62/SQSTM1, mitophagy, ubiquitination

## Introduction

In addition to the generation of adenosine triphosphate, mitochondria are also involved in other cellular processes, such as cell growth and division, as well as cell death [1]. Abnormal mitochondrial function has been implicated in many neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease [2,3]. For these important roles of mitochondria, cells develop precise mechanisms to maintain mitochondrial homeostasis. Unveiling these mechanisms by which mitochondria separate themselves from damaged ones will help us to understand the pathogenesis of diseases.

Mitophagy plays the most important role in mitochondrial quality control. Parkinson's disease-associated protein PINK1 and Parkin have been identified as the new players involved in this process. Upon carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) treatment, Parkin is recruited to mitochondrial outer membrane (MOM) via PINK1 [4–7]. Parkin's translocation to mitochondria dramatically changes the ubiquitination states of the mitochondrial proteome [8–10], and then those ubiquitinated mitochondria are recognized by autophagic adapter protein p62/SQSTM1 and finally degraded through autophagy [11,12]. However, none of these studies have identified which ubiquitinated protein on MOM is essential for the recruitment of cytoplasmic p62 to mitochondria.

Mitochondrial fission factor (Mff) is classically known as a mitochondrial morphology control factor [13]. The molecular structure of Mff contains three distinct domains: an N terminal Drp1 binding

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domain, a central coiled-coil domain, and a transmembrane domain at C terminus. Mff is localized on MOM, where it serves as receptor of Drp1 [14]. It has long been considered that mitochondrial fission and fusion are closely related to mitophagy. Mitochondrial fragmentation and increased autophagy are observed in neurodegenerative diseases including Alzheimer's and Parkinson's diseases [15]. Mammalian cells have three structurally distinct classes of recruitment factors on their MOM: Fis1, Mff, and two related proteins MiD49 and MiD51 [16]. Although mutations in Fis1 lead to an accumulation of large LC3 aggregates in Caenorhabditis elegans, it is still unclear whether Mff is involved in mitophagy [17].

In this study, we report that Parkin associates with Mff and ubiquitinates it at K251 upon CCCP treatment. Loss of Mff expression disrupts recruitment of Parkin and p62-mediated final clearance of damaged mitochondria.

## Materials and Methods

### Plasmid construction

The Mff full-length cDNA was obtained from Addgene (Cambridge, USA). The full-length and truncated Mff cDNAs were amplified by polymerase chain reaction (PCR) and cloned into the *Bam*HI and *Xho*I sites of the pcDNA3.1-zeo-NFlag Vector (BioAtom, Chengdu, China). Invariant-coding mutagenesis K251R, K264R, and K268R of pcDNA3.1-zeo-Flag-Mff were performed by using the site-directed mutagenesis kit (NEB, Ipswich, USA). For the expression of ubiquitin and Mff fusion protein, ubiquitin cDNA and wild-type Mff cDNA were subject to PCR amplification with indicated primers and Gibson Assembly (NEB) to generate pcDNA3.1-zeo-Flag-UbG76V-Mff. pcDNA3.1-HA-Parkin and pcDNA3.1-HA-p62 expression vectors were all obtained from BioAtom. All constructs were confirmed by DNA sequencing. The sequences of the PCR primers used are listed in **Supplementary Table S1**.

### Cell lines and reagents

HEK293 cells were obtained from the ATCC (Manassas, USA). Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Cat #0452) was obtained from Tocris Bioscience (Missouri, UK). Valinomycin (V0627) and DTBP (D2388) were obtained from Sigma (St Louis, USA). Transient transfections of HEK293 cells were performed using the Mega Tran 1.0 (OriGene, Rockville, USA) according to the manufacturer's instructions.

### Generation of stable expression cell lines

The coding sequence of EGFP-Parkin from pcDNA3.1-EGFP-Parkin was transferred into plenti6-LVP lentiviral vector (Life Technologies, Boston, USA). Viruses were generated and used to transduce HEK293 cells. One day after the transduction, puromycin was added to the cell culture medium at the final concentration of 2 μg/ml to generate stable polyclonal HEK293 (EGFP-Parkin) cell lines.

### Generation of Mff knockout cell lines

Mff knockout cell lines were generated from HEK293 cells with the CRISP/Cas9 system designed in our laboratory. HEK293 cells were first transfected with expression vectors of Cas9 and two gRNAs, which targeted the AGTCGAATTCAGTACGAAA and GAAAAGTTAAAGTAGCAC sequences in exon 1 of Mff, respectively. After 24 h, the cells were diluted and seeded in 96-well plates at 1 cell/well to isolate monoclonal cell lines without Mff expression as determined by western blot analysis.

### Generation of p62-knockout cell lines

To generate *p62−/−* cells, HEK293 cells were first transfected with expression vectors of a pair of TALEN, p62-T2L and p62-T2R (designed by Viewsolid Biotechnology, Suzhou, China), which targeted the GCGCTGGCTGCTCCCT and GGCCGAACTGGGGACCC sequences in intron 1 of p62, respectively. After 24 h, the cells were diluted and seeded in 96-well plates at 1 cell/well to isolate monoclonal cell lines without p62 expression as determined by western blot analysis.

### Immunocytochemistry

Cells grown on cover slips were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. They were then permeabilized with 0.2% Triton X-100 for 15 min, blocked with 5% goat serum for 1 h and incubated with primary antibodies at 4°C overnight. After being washed with PBS, cells were incubated with Alexa Flour 488 or 568-conjugated secondary antibodies (Life Technologies) for 2 h, stained with DAPI solution for 5 min and mounted for fluorescence microscopy examination. Fluorescent microscopy was performed with an Olympus FV1000 confocal microscope.

### Immunoprecipitation

HEK293 cells were transfected with indicated plasmids. After 24 h, the cells were lysed in immunoprecipitation (IP) buffer consisting of 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM EDTA, and Halt Protease Inhibitor Cocktail (Thermo Scientific, Boston, USA) for 20 min on ice. Cell lysates were spun at 14,000 *g* at 4°C for 15 min to remove cellular debris. The supernatant from each sample then underwent 2 h incubation with 2 μg of indicated antibodies followed by 1 h incubation with 10 μl Protein G agarose beads (EMD Millipore, Darmstadt, Germany). The beads were then spun down, washed three times with washing buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 1% NP-40) and re-suspended in 1× SDS protein sample buffer.

### Isolation of mitochondria from cultured cells

Cells were harvested by digestion with 0.05% Trypsin-EDTA solution and centrifugation. After two times wash with ice-cold PBS, cells were re-suspended in 1 ml ice-cold Mitochondria Purification Buffer (10 mM HEPES-KOH, 1 mM EDTA, 1 mM EGTA, 0.21 M sucrose, and 70 mM mannitol) and disrupted with Qsonic Sonicator XL-2000 (Misonix, Farmingdale, USA) at the lowest power setting until the cell suspension became transparent. Undisrupted cells and cell debris were removed by low-speed centrifugation at 1000 *g* for 10 min. The supernatant was transferred into a new tube and mitochondria were pelleted by high-speed centrifugation at 20,000 *g* for 20 min. The mitochondria were then solubilized in 0.2 ml of 1× SDS protein sample buffer.

### Western blot analysis

Protein samples in SDS sample buffer were separated by SDS–PAGE and transferred onto Immobilon-FL polyvinylidene difluoride membrane (EMD Millipore). The membrane was then blocked with 5% non-fat dry milk in PBST (PBS, pH 7.4, 0.2% Tween-20) and incubated at 4°C overnight with primary antibodies. It was then washed three times with PBST and incubated with Dylight 680 and/or Dylight 800-conjugated secondary antibodies (KPL, Gaithersburg, USA) in the dark for 2 h. After three washes with PBST, the image was acquired

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by a Li-Cor Odyssey Clx Infrared Imaging System (LI-COR Biosciences, Lincoln, USA). The following primary antibodies were used for western blot analysis in this study: anti-Mff antibody (Proteintech, Chicago, USA), anti-p62/SQSTM1 antibody (Proteintech), anti-Tomm20 antibody (Proteintech), anti-VDAC1 antibody (Mitosciences, Eugene, USA), anti-Flag tag antibody (Prospec, East Brunswick, USA), anti-HA tag antibody (Proteintech), anti-GFP antibody (Immunology Consultants Laboratory, Portland, USA), anti-GAPDH antibody (Zen Bioscience, Chengdu, China), anti-β-Tubulin antibody (Zen Bioscience), anti-β-Actin antibody (Proteintech), anti-HtrA2 antibody (Proteintech), and anti-Parkin antibody (Covance, Princeton, USA).

### Statistical analysis

Data were expressed as the mean ± SEM of three independent experiments. The two-tailed Student's *t*-test was used for statistical analysis. $P < 0.05$ was considered of significant difference.

## Results

### Mff interacts with Parkin

Previous studies have shown that mitochondrial depolarization induced PINK1 accumulation on MOM not only recruits Parkin to mitochondria, but also phosphorylates Parkin at S65, which actives its ubiquitin ligase activity [18,19]. Given the important role of Parkin in mitophagy, we tried to identify the downstream effectors of Parkin-induced mitophagy. A small-scale screen was performed by co-IP Flag-Parkin with EGFP-tagged mitochondrial proteins. Flag-Parkin co-precipitated with PINK1–EGFP and EGFP–Mff but not with EGFP, Pen2–EGFP, Aph–EGFP, Diablo–EGFP, VDAC1–EGFP, or EGFP–SENP5 (**Fig. 1A**). The interaction between Parkin and PINK1 has been extensively characterized [20], but study on Parkin and Mff interaction has not been reported. Mff localizes on MOM, where Parkin is recruited during mitochondrial depolarization. To explore the functional relationship between Mff and Parkin, we further determined the affinity between Parkin and Mff upon CCCP treatment by co-IP. As shown in **Fig. 1B,C**, CCCP treatment significantly increased the affinity of HA-Parkin with Flag-Mff. Similar results were also obtained after IP with anti-Parkin antibody, showing that Parkin interacts with endogenous Mff, with or without CCCP treatment (**Fig. 1D**). Interestingly, Parkin was dramatically decreased in CCCP-treated cell lysates (**Fig. 1B**). The reason for this was that Parkin was translocated to large protein aggregates after 2 h exposure to CCCP (**Fig. 1E**), and these aggregates could not be solubilized by cell lysis buffer containing 1% mild detergent, such as NP-40.

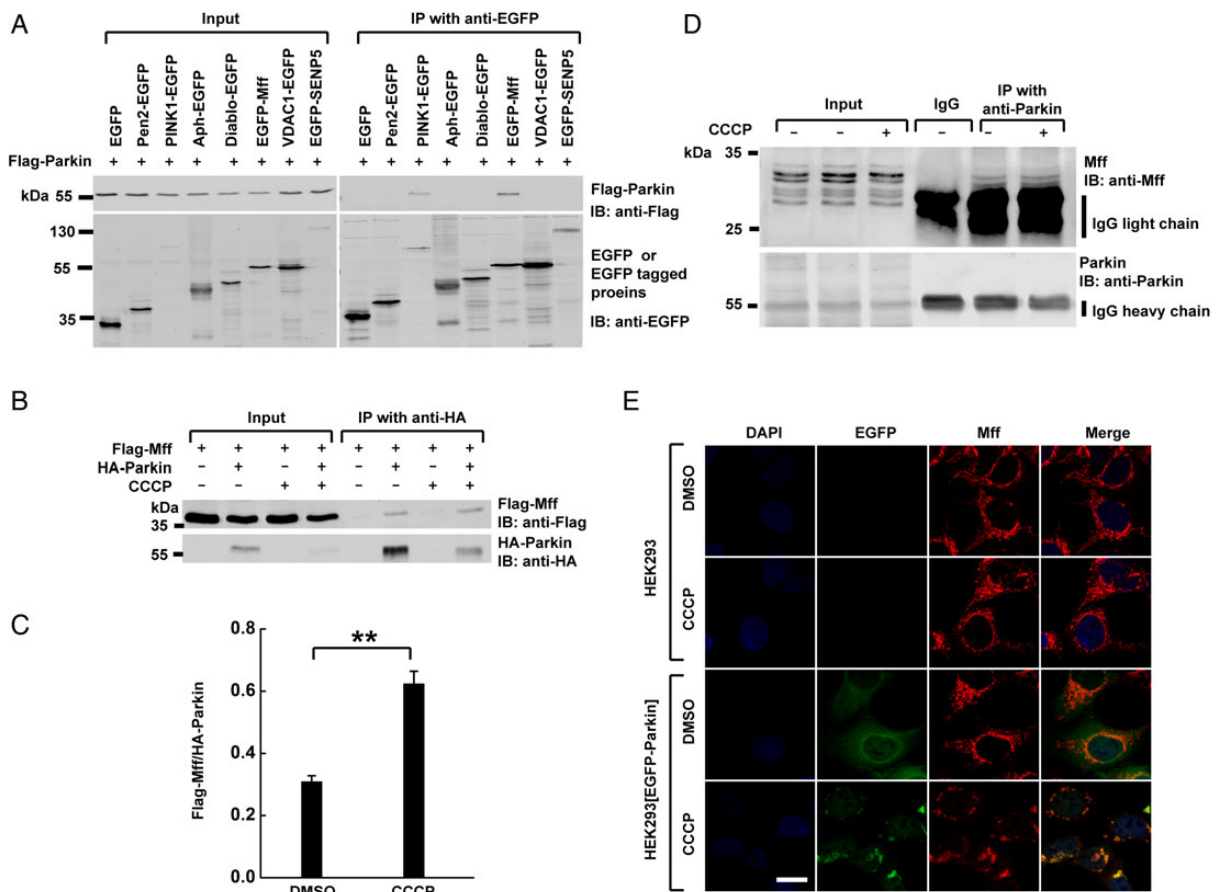


**Figure 1. Mff interacts with Parkin** (A) HEK293 cells were transfected with plasmids indicated and immunoprecipitiated with anti-EGFP tag antibody and detected with anti-Flag tag and anti-EGFP tag antibodies. (B) HEK293 cells were transfected with indicated plasmids for 24 h, then treated with CCCP (10 μM, 2 h). Cell lysates were immunoprecipitiated with anti-HA tag antibody and detected with anti-Flag tag and anti-HA tag antibodies. (C) Quantitative analysis of (B). Data are shown as the mean ± SEM of three independent experiments. $^{**}P < 0.01$. (D) HEK293 cells were exposed to 10 μM CCCP or DMSO for 2 h, and then were treated with 5 mM chemical crosslinker DTBP. Cell lysates were subject to IP with anti-Parkin antibody or IgG. Then, the immune complexes were analyzed by western blotting using anti-Mff antibody. (E) HEK293 or HEK293[EGFP-Parkin] cells were treated with DMSO or CCCP (10 μM) for 2 h and immunostained with anti-Mff antibody. Scale bar, 20 μm.

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Because the molecular weight of endogenous Parkin is the same as IgG heavy chain, we could not detect Parkin in immunoprecipitated complex. Taken together, these observations demonstrate that Mff associates with Parkin in cells.

## Mff is ubiquitinated during mitochondrial depolarization

CCCP-induced Parkin translocation to mitochondria mediates ubiquitination of MOM proteins [8]. The interaction and co-localization between Mff and Parkin implicates that Mff might be another substrate of Parkin on MOM when mitochondria lose their membrane potential. To test this hypothesis, we generated EGFP-Parkin and EGFP-Parkin(C431F) stable-expressing HEK293 cell lines. As shown in **Fig. 2A**, CCCP or Valinomycin ($K^+$ ionophore) exposure-induced molecular weight shift of Mff was only observed in HEK293[EGFP-Parkin] cells, but not in HEK293 or HEK293[EGFP-Parkin(C431F)] cells, suggesting that the modification of Mff was dependent on Parkin's E3 ligase activity. Meanwhile, the appearance of modified endogenous Mff coincided with the increase in Parkin autoubiquitination (**Fig. 2A**). There are at least six splicing forms of endogenous Mff [13], so it is hard to identify which one is modified by Parkin. So, we transiently expressed Flag-Mff (protein sequence was shown in **Supplementary Fig. S1**) and HA-Parkin in HEK293 cells. After CCCP treatment, two upper bands were seen in Parkin and Mff co-overexpressed cells (**Fig. 2B**).

To demonstrate that the observed modifications were due to ubiquitination, Myc-Mff was re-transfected into *mff*−/−[EGFP-Parkin] cells. After IP with anti-Mff antibody, western blot analysis of Mff immunoprecipitating with anti-Ub antibody revealed that highly ubiquitinated species were pulled down only from cell lysates treated with CCCP (**Fig. 2C**).

Different linkages of poly-ubiquitin chain determine the substrates to be degraded by different pathways, such as proteasome pathway and autophagy pathway. To explore whether the ubiquitinated Mff was degrade by proteasome, Parkin and Mff co-expressing cells were treated with CCCP and proteasome inhibitor MG132. As shown in **Supplementary Fig. S1B**, both the modified Mff and unmodified Mff were not increased by inhibition of proteasome activity, indicating that ubiquitinated Mff was not degraded by proteasome. Next, we separated mitochondria from total lysates and identified that ubiquitinated Mff was only present in mitochondrial-rich fraction (**Fig. 2D**). Additionally, the appearance of modified endogenous Mff coincided with an increase in p62 in mitochondrial-rich fraction (**Fig. 2E**). These results suggest that Mff is ubiquitinated by Parkin in response to mitochondrial depolarization.

## Parkin ubiquitinates Mff at lysine 251

To determine which lysine residue of Mff is the Ub acceptor site, we performed a series of deletion and mutation analysis. Deletion analysis

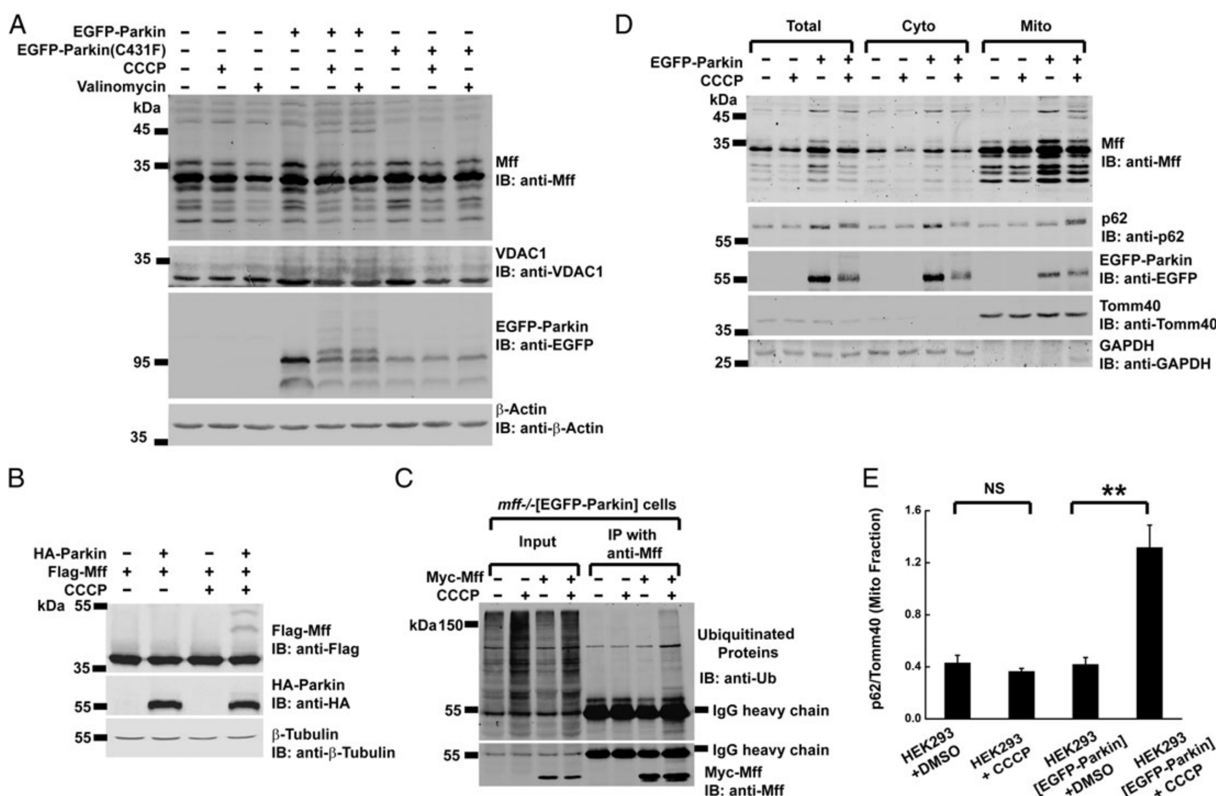


**Figure 2. Mff is ubiquitinated during mitochondrial depolarization** (A) Western blot analysis of Mff ubiquitination in HEK293, HEK293[EGFP-Parkin], and HEK293 [EGFP-Parkin(C431F)] cell lines. Different cells were treated with CCCP (10 μM, 2 h) or Valinomycin (10 μM, 2 h) before being harvested by SDS sample buffer. (B) Western blot analysis of Mff ubiquitination in HEK293 cells transiently co-expressing HA-Parkin with Flag-Mff upon CCCP treatment (10 μM, 2 h). (C) *mff*−/−[EGFP-Parkin] cells were transfected with Myc-Mff for 24 h. After being treated with DMSO or CCCP (10 μM, 4 h), cell lysates were immunoprecipitated with anti-Mff antibody and the purified complexes were analyzed by western blotting using anti-Ub antibody. (D) HEK293 or HEK293[EGFP-Parkin] cells were treated with DMSO or CCCP (10 μM, 2 h), then crude mitochondria were isolated from the lysates. Tomm40 was detected and used as a mitochondrial marker. (E) Quantitative analysis of p62 in mitochondrial-rich fraction. Data are shown as the mean ± SEM of three independent experiments. $^{**}P < 0.01$. NS, non-significant.

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showed that full-length Mff, truncated protein 29–291, and 89–291 were all ubiquitinated by Parkin (**Fig. 3A,B**), indicating that the Ub acceptor site(s) should be in truncated protein 89–291. Then, the potential Ub acceptor sites (K251, K264, and K268) were mutated within the coiled-coil domain to arginine. As shown in **Fig. 3C**, replacement of lysine 251 by arginine (K251R) totally abrogated CCCP-stimulated ubiquitination of Mff. We further did sequence alignment with Mff in different species to explore the significance of ubiquitination on lysine 251. Amazingly, it was found that lysine 251 of Mff showed 100% identity from fly to mammals (**Fig. 3D**).

## K251R mutant of Mff losses its p62 binding activity

Gandre-Babbe and van der Bliek [13] have reported that Mff forms oligomer through its coiled-coil domain. To examine the effect of ubiqutination of Mff on its self-interaction, the ability of self-interaction of Mff wild-type (WT) and K251R was assessed by IP. CCCP-induced ubiquitination of Mff did not affect its self-interaction, and similar to Mff WT, MffK251R mutant had no effect on its self-interaction (**Supplementary Fig. S2A**). We also examined whether Parkin-induced ubiquitination of Mff affects its Drp1 binding activity. As shown in **Supplementary Fig. S2**, the interactions between Flag-Mff or Flag-MffK251R and HA-Drp1 were not altered in DMSO or CCCP-treated cells (**Supplementary Fig. S2B**). Although the shift of the mitochondrial dynamics toward enhanced fission is essential for the clearance of damaged mitochondria, our data suggest that CCCP-induced mitochondrial fission is not dependent on Mff, because mitochondria in Mff knockout cells also showed fragmented morphology after CCCP treatment (**Supplementary Fig. S2C**).

The most important step of ubiquitination-induced peroxisome autophagy is the recruitment of p62 to damaged organelles [21]. A recent study suggested that p62 may be involved in the Parkin-mediated clearance of the depolarized mitochondria [11], but Narendra *et al.* [22] reported that p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy. To analyze the functional significance of p62 in the Parkin-dependent mitophagy, p62 was knockout in HEK293 cells. After 24 h of CCCP treatment, there was a significant loss of mitochondrial clearance in p62-knockout cells when compared with control cells (**Supplementary Fig. S3**). These data show the importance of p62 as an adaptor protein for Parkin-directed mitophagy [23]. p62 contains classic ubiquitin binding domain, which promotes us to investigate the relationship between p62 recruitment and Mff ubiquitination. For this, we co-expressed Flag-Mff WT or K251R mutant with HA-p62, and assessed the association of Mff with p62 by IP. Compared with Flag-Mff WT, Flag-MffK251R lost its affinity with p62 during CCCP treatment, supporting the hypothesis that ubiquitinated Mff might function as one of p62 receptors on mitochondrial surface (**Fig. 4A,B**). It is unlikely that this result is an artifact caused by overexpression, as we observed that endogenous Mff interacted with p62 only after CCCP treatment (**Fig. 4C**). In order to determine the interaction between ubiquitinated Mff and p62 in more detail, we attached Ub (containing the G76V mutation to prevent cleavage by ubiquitin-specific protease) to the N-terminal of Mff. Co-IP assay showed that p62 strongly associated with Ub-Mff without CCCP treatment, but not with Mff WT and Drp1 binding defective mutants (**Fig. 4D**). The affinity of Mff WT and Ub-Mff with Drp1 was also assayed by co-IP. As shown in **Supplementary Fig. S3D**, Ub-Mff did not affect its Drp1 binding activity.

If ubiquitinated Mff serves as mitochondrial receptor of p62, loss of Mff should affect mitochondrial translocation of cytosolic p62. To test this hypothesis, we analyzed mitochondrial p62 in *mff*+/+, *mff*−/−, and *mff*−/− rescued cells. Compared with *mff*+/+ cells, p62 in mitochondrial-rich fraction was significantly decreased in *mff*−/− cells (**Fig. 4E,F**). Only re-transfection of wild-type Mff, but not K251R mutant, could rescue this phenotype (**Fig. 4E,F**), confirming that Parkin-mediated ubiquitination of Mff promotes p62 recruitment to mitochondria during CCCP treatment.

## Mff is required for Parkin-mediated autophagy

If Mff is required for Parkin-mediated mitophagy, knockout of Mff will interfere with Parkin translocation and/or mitochondrial clearance in response to CCCP treatment. In fact, loss of Mff significantly

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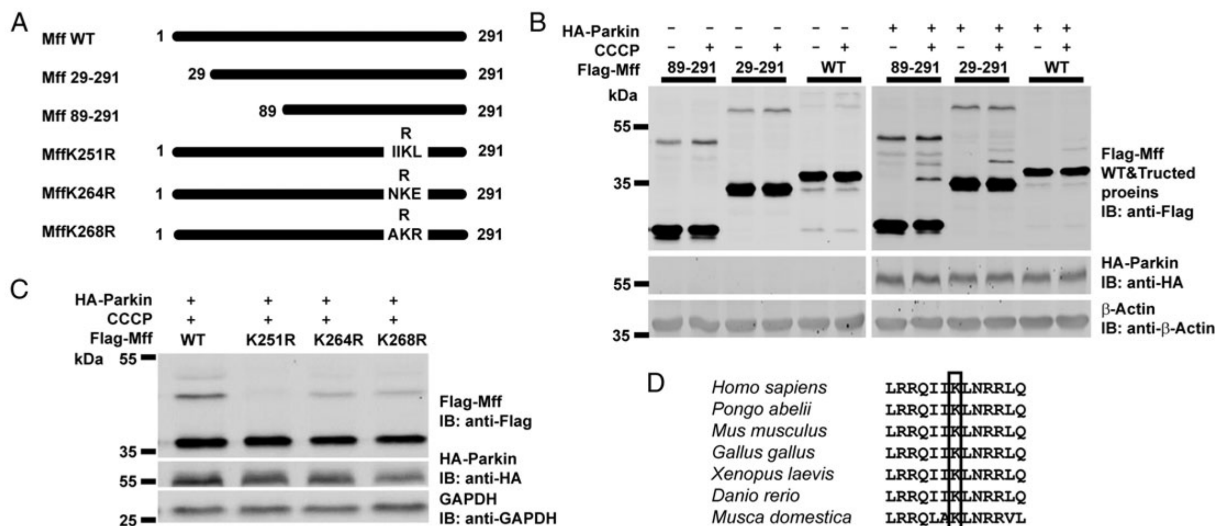


**Figure 3. Parkin ubiquitinates Mff at Lys 251** (A) Structure of Mff WT and mutants used for Parkin-mediated ubiquitination mapping. (B) Transfected HEK293 cells were treated with DMSO or CCCP (10 μM, 2 h), and harvested by SDS sample buffer. Lysates were subject to western blot analysis with indicated antibodies. (C) HEK293 cells were transfected with HA-Parkin and Flag-Mff WT or mutants for 24 h, then treated with DMSO or CCCP (10 μM, 2 h). Western blot analysis was performed using indicated antibodies. (D) Conservation of K251 and its surrounding amino acid sequence in Mff proteins of human, chimpanzee, mouse, chicken, frog, zebrafish, and fly.

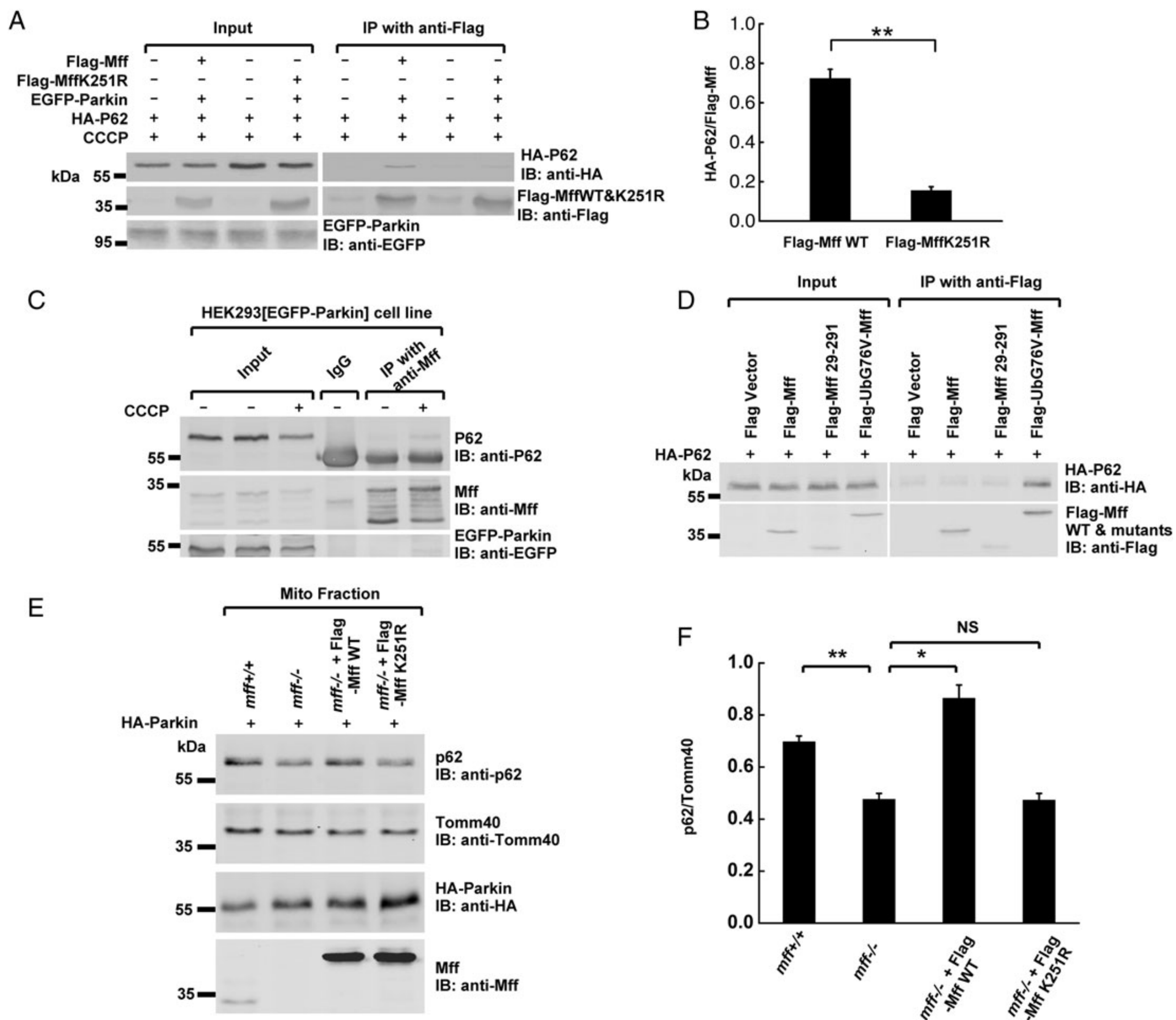


**Figure 4. K251R mutation of Mff decreases its p62 binding activity** (A) Twenty-four hours after transfection, HEK293 cells were treated with DMSO or CCCP (10 μM, 2 h), and then immunoprecipitated with anti-Flag tag antibody, and the associated HA-P62 was detected with anti-HA tag antibody. (B) Quantitative analysis of (A). Data are shown as the mean ± SEM of three independent experiments. $^{**}P < 0.01$. (C) HEK293[EGFP-Parkin] cells were exposed to 10 μM CCCP or DMSO for 4 h, and then treated with 5 mM chemical crosslinker DTBP. Cell lysates were subject to IP with anti-Mff antibody or IgG. Then, the immune complexes were analyzed by western blotting using anti-p62 antibody. (D) HEK293 cells were transfected with indicated plasmids, then immunoprecipitated with anti-Flag tag antibody and the associated HA-p62 was detected with anti-HA tag antibody. (E) *mff−/−* cells were transfected with indicated plasmids. Twenty-four hours after transfection, mitochondria were isolated from different cells after 2 h treatment with CCCP (10 μM). Tomm40 was detected and used as a mitochondrial marker. (F) Quantitative analysis of (E). Data are shown as the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. NS, non-significant.

reduced the translocation of Parkin from the cytosol to damaged mitochondria after 2 h of CCCP treatment, compared with control cells (**Fig. 5A,B**). Both Flag-Mff WT and K251R mutant re-transfection restored Parkin mitochondrial translocation (**Fig. 5A,B**). After 24 h of CCCP treatment, loss of Mff also significantly prevented mitochondrial clearance (**Fig. 5A,B**). Importantly, re-transfection of Flag-Mff WT, but not K251R mutant, significantly restored Parkin-induced mitochondrial clearance.

## Discussion

During past few years, PINK1/Parkin-mediated mitophagy has been extensively studied. Mutations in the PINK1 and ubiquitin E3 ligase Parkin cause the familial form of Parkinson's disease in humans, as well as accumulation of defective mitochondria and cellular degeneration in flies [24,25]. Although several proteins have been identified as the substrates of Parkin [11,26–28], details of Parkin-mediated mitophagy are still undetermined. In this study, we report that Parkin-induced ubiquitination of Mff promotes its association with p62. Loss of Mff results in failure of recruitment of p62 and clearance of damaged mitochondria.

The initiation of mitophagy requires recruitment of cytosolic Parkin to damaged mitochondria via PINK1. Mitochondrial targeting of Parkin catalyzes the formation of different types of poly-ubiquitin chains, which determine the different fates of substrates. When mitofusion and miro proteins are poly-ubiquitinated by Parkin with K48-linked ubiquitin chains, they are targeted to degradation by proteasome [27,28]. However, K27- or K63-linked poly-ubiquitin chains on VDAC1 facilitates recruitment of the autophagic adaptor p62 to mitochondria [11,12,22,24,29]. Parkin-mediated ubiquitination of

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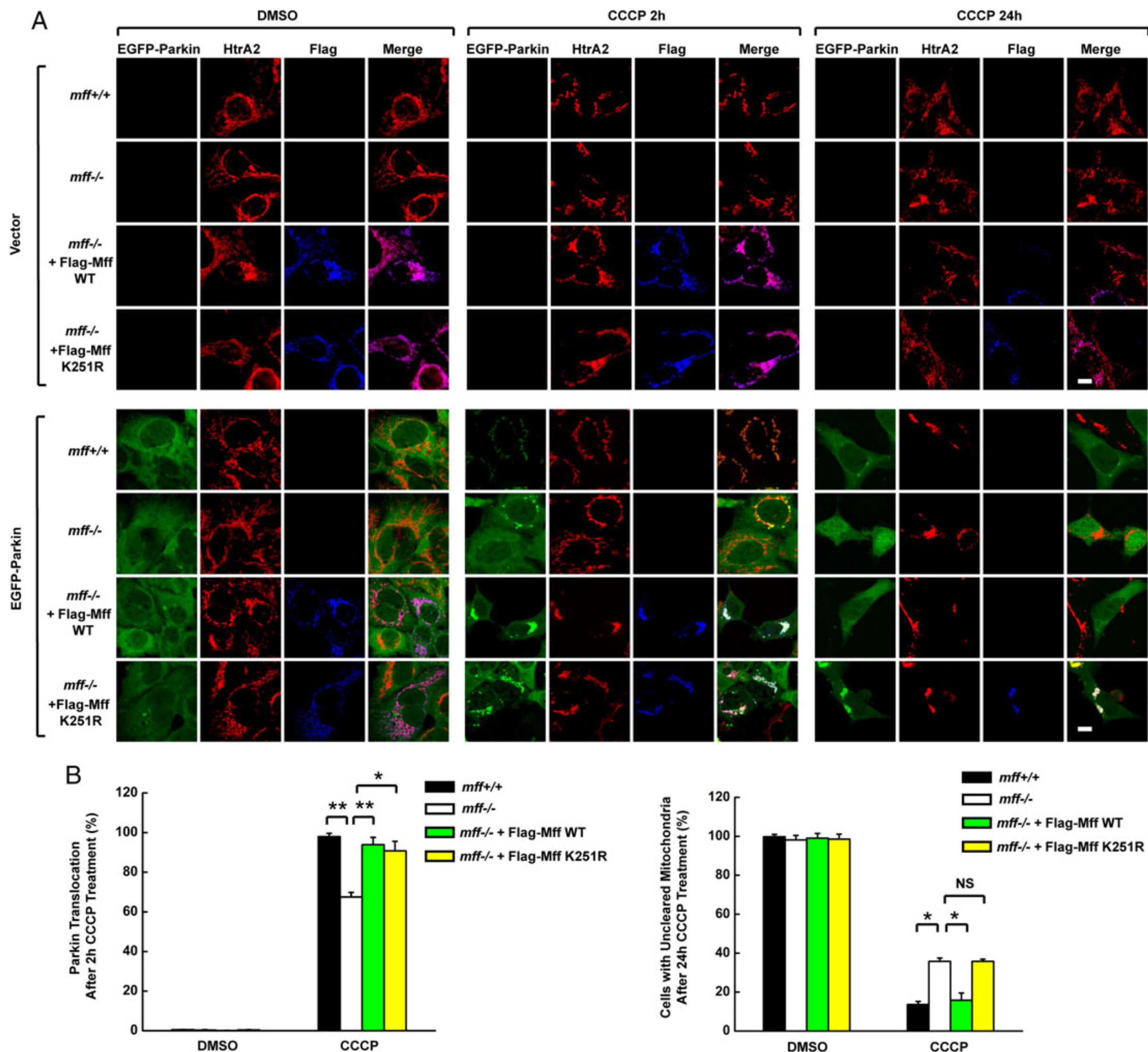


**Figure 5. Mff is required for Parkin-mediated autophagy** (A) *mff*−/− [EGFP-Parkin] and control cells were transfected with the indicated plasmids. Cells were stained with anti-HtrA2/Omi and anti-Flag to visualize Parkin co-localization with impaired mitochondria after 2 h and uncleared mitochondria after 24 h of CCCP (2 μM) treatment. Scale bar, 20 μm. (B) Quantification of (A). Data are shown as the mean ± SEM of three independent experiments. **P* < 0.05, ***P* < 0.01. NS, non-significant.

Mff was not degraded by proteasome, suggesting that modification of Mff should serve as a signal for autophagic adaptor proteins to recognize. The future work should identify which kind of ubiquitin chain is formed by Parkin on Mff K251.

Mitochondrial recruitment of p62 appears to be involved in the control of mitochondrial distribution [12,22], but the exact function of p62 in damaged mitochondrial clearance is still ambiguous [30]. We confirmed that knockout of p62 resulted in failure of Parkin-induced mitochondria clearance in HEK293 cells. In fact, only ubiquitinated Mff associates with p62, indicating that mitochondrial recruitment of p62 happens after Parkin-mediated ubiquitination. Mff WT, but not K251R mutant, rescues Mff knockout-induced failure of p62 recruitment, confirming that Mff ubiquitination is required for p62 translocation from cytosol to mitochondria during CCCP treatment.

Mitochondrial dynamics is recognized as a critical process underlying mitochondrial homeostasis [31]. Inhibiting mitochondrial fission results in the prevention of mitochondrial autophagy and the accumulation of damaged mitochondria [32].

Our observations suggest that mitochondrial fission induced by CCCP is not dependent on Mff, but loss of Mff inhibits Parkin-induced mitochondrial clearance. These data are in contrast to the findings of a recently published report demonstrating that knockdown of Mff in COS7 cells has no effect on Parkin-induced mitochondria clearance [33]. There are two reasons to explain the discrepancy in these findings. First, we monitor Parkin-induced mitophagy in Mff knockout cells, which provides better gene silencing than small interfering RNAs (siRNAs). Secondly, we perform all experiments in HEK293 cells, but not COS7 cells, suggesting that the mechanism of Parkin-mediated mitophagy may be cell type-dependent. Mff knockout fails to inhibit CCCP-induced mitochondria fission, indicating that some other factors may also participate in the regulation of mitochondrial fission during CCCP-induced mitochondrial depolarization.

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Further studies are needed to find these factors by genome-wide siRNA screen.

In summary, our data show that Mff is ubiquitinated by Parkin upon CCCP treatment, and then ubiquitinated Mff promotes its association with p62. Loss of Mff results in failure of mitochondrial recruitment of cytosolic p62 and final clearance of damaged mitochondria as well.

## Supplementary Data

Supplementary data is available at *ABBS* online.

## Funding

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