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ORIGINAL ARTICLE

Parkin mitochondrial translocation is achieved through a novel catalytic activity coupled mechanism

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Pink1, a mitochondrial kinase, and Parkin, an E3 ubiquitin ligase, function in mitochondrial maintenance. Pink1 accumulates on depolarized mitochondria, where it recruits Parkin to mainly induce K63-linked chain ubiquitination of outer membrane proteins and eventually mitophagy. Parkin belongs to the RBR E3 ligase family. Recently, it has been proposed that the RBR domain transfers ubiquitin to targets via a cysteine~ubiquitin enzyme intermediate, in a manner similar to HECT domain E3 ligases. However, direct evidence for a ubiquitin transfer mechanism and its importance for Parkin's *in vivo* function is still missing. Here, we report that Parkin E3 activity relies on cysteine-mediated ubiquitin transfer during mitophagy. Mutating the putative catalytic cysteine to serine (Parkin C431S) traps ubiquitin, and surprisingly, also abrogates Parkin mitochondrial translocation, indicating that E3 activity is essential for Parkin translocation. We found that Parkin can bind to K63-linked ubiquitin chains, and that targeting K63-mimicking ubiquitin chains to mitochondria restores Parkin C431S localization. We propose that Parkin translocation is achieved through a novel catalytic activity coupled mechanism.

***Keywords*:** Pink1; Parkin; mitophagy; E3 ubiquitin ligase; Parkinson's disease; mitochondria

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Introduction

Mitochondria, as the cellular energy and biosynthesis center, are monitored by a variety of surveillance mechanisms to ensure their proper function [1]. Pink1 and Parkin, a duo of mitochondrial kinase and cytosolic E3 ubiquitin ligase, identified from analysis of early-onset Parkinson's disease mutations, have been shown to function in mitochondrial maintenance [2]. Loss of Pink1 or Parkin in *Drosophila* causes significant mitochondrial degeneration in indirect flight muscle, providing genetic evidence that both are involved in mitochondrial maintenance, and, interestingly, Parkin overexpression can partially suppress the phenotype of Pink1 mutant flies, indicating that Parkin may function downstream of Pink1 [3-6]. Subsequently, Parkin was found to translocate from the cytosol to the surface of depolarized mitochondria, and then trigger mitochondrial aggregation and autophagy (mitophagy), defining a new quality control that clears damaged mitochondria [7]. There is evidence that Pink1 and Parkin may also regulate mitochondrial fusion, fission and motility [8-11]. The exact consequence of Parkin mitochondrial function may depend on the extent of mitochondrial damage. Besides this newly-defined mitochondrial function, another well-studied disease-related function of Parkin is to regulate protein degradation [12, 13] or promote protein aggresome formation through K63-linked ubiquitination [14].

How Pink1 senses mitochondrial status and regulates Parkin's function has been intensively studied recently. Pink1 was found to be essential for Parkin translocation to mitochondria [10, 15-19]. Pink1 import into mitochondria was shown to be sensitive to mitochondrial membrane potential. When mitochondrial potential is normal, Pink1 is imported into mitochondria, and then cleaved by proteases and degraded [20]; when mitochondrial membrane potential is impaired, Pink1 import is blocked and its kinase domain remains outside facing the cytosol and

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triggers Parkin mitochondrial recruitment. Presumably, as a kinase, Pink1 can phosphorylate some mitochondrial surface proteins or itself serve as an anchor for Parkin. The possibility that there are unique mitochondrial anchors has been ruled out by a recent study: when Pink1 is ectopically targeted to peroxisomes, Parkin is redirected to peroxisomes and, interestingly, it induces their autophagy [21]. In line with this observation, Pink1 has been shown to interact with Parkin [22]. Furthermore, recent studies showed that Pink1 can autophosphorylate itself and directly or indirectly trigger Parkin phosphorylation, which may activate its E3 ligase activity [23-25].

Parkin belongs to the RBR (RING-in-Between-RING) E3 ligase family, which has two tandem RING domains. Traditional RING E3 ligases activate direct transfer of ubiquitin from E2~Ub to substrate; in contrast, HECT domain E3 ligases first transfer ubiquitin to a catalytic cysteine before passing it to substrate [26]. It was recently found, however, that HHARI and HOIP, both members of the RBR E3 ligase family, appear to function like HECT domain E3 ligases [27-29]. An intermediate cysteine~ubiquitin transfer step can be detected with both the HHARI and HOIP RBR domains in an *in vitro* assay. But for unknown reasons, the same thioester adduct cannot be demonstrated on the Parkin RBR domain, even though the putative catalytic cysteine is conserved. The Parkin RING2 domain, containing the putative catalytic cysteine, may coordinate two Zn^{2+}; while the HHARI RING2 domain, which transfers ubiquitin, has only one Zn^{2+} [30, 31]. Moreover, the idea that the RBR domain has an intermediate ubiquitin transfer step is based on the use of UbcH7, an E2 unable to pass ubiquitin directly to lysine. However, Parkin has been shown to function with other E2s that are competent to transfer ubiquitin directly to lysine [27, 32, 33]. Therefore, whether Parkin function relies on ubiquitin transfer via a catalytic cysteine needs to be investigated.

In this study, we found that Parkin indeed functions by ubiquitin transfer via a cysteine~ubiquitin intermediate during mitophagy. Furthermore, we proved that Parkin E3 ligase activity is essential for Parkin mitochondrial translocation, and Parkin activated by Pink1 can bind to K63-linked ubiquitin chains. These unexpected findings suggest that Parkin mitochondrial translocation is achieved through a novel catalytic activity coupled mechanism.

Results and Discussion

The putative catalytic cysteine in Parkin RBR domain is essential for mitophagy and mitochondrial translocation

Mutation of the putative catalytic cysteine, Cys431, to phenylalanine has been found in Parkinson disease patients, indicating the importance of this cysteine for Parkin function [34]. However, whether the mutation affects ubiquitination of other Parkin substrates or its newly-defined mitophagy function is not known. Using *in vitro* E3 ligase activity assays, Parkin has been shown to function with several E2s in addition to UbcH7. In contrast to UbcH7, those E2s can pass ubiquitin directly to lysine. Among them, Ube2N/Ube2V1 (UbcH13/Uev1a) has intrinsic K63-linked ubiquitin chain synthesis activity [35]. K63-linked ubiquitin chains are abundant on mitochondria during Parkin-mediated mitophagy, and are known to signal the recruitment of autophagy machinery [15, 36, 37], making Ube2N/Ube2V1 an attractive candidate E2 for Parkin-mediated mitophagy. If Ube2N/Ube2V1 was Parkin's partner during mitophagy, the newly-proposed ubiquitin transfer function of RBR domain might not be necessary.

To test whether the presumptive catalytic cysteine is important for Parkin-mediated mitophagy, we mutated the conserved Cys431 to serine or alanine to create Parkin C431S or C431A. We expressed N-terminally Flag-tagged wild-type (WT) Parkin, Parkin C431S and C431A in HeLa cells, which have very low endogenous Parkin levels and have been used in other studies [15-17]. Their ability to clear mitochondria damaged by CCCP (carbonyl cyanide m-chlorophenylhydrazone, a mitochondrial uncoupler) was measured by immunostaining (Figure 1A). In the absence of CCCP, WT Parkin, Parkin C431S and C431A (Figure 1A and Supplementary information, Figure S1A) were cytosolic. After 1 h of CCCP treatment, WT Parkin was clearly detected on mitochondria, and after 3 h, mitochondrial aggregation became apparent in cells expressing WT Parkin as reported [7, 15]. In stark contrast, neither Parkin C431S nor Parkin C431A was able to trigger mitochondrial aggregation and mitophagy, nor were they recruited to mitochondria, remaining in the cytosol throughout 24 h of treatment (Figure 1A). The impact of this cysteine mutation on Parkin mitochondrial translocation was unexpected, as the cysteine to serine mutation was reported not to affect the overall structure of the RBR domain [27]. If this cysteine plays a catalytic role, according to the current model, Parkin C431S and C431A should still be able to translocate onto damaged mitochondria. One plausible explanation is that the mutation may affect the interaction between Pink1 and Parkin. To test this possibility, we co-expressed the C-terminally HA-tagged Pink1ΔMTS (mitochondrial translocation signal) with N-terminally Flag-tagged Parkin WT and Parkin C431S in HeLa cells. We found that Parkin WT and Parkin C431S immunoprecipitated using Flag-conjugated beads could pull down a similar amount

of Pink1ΔMTS (Figure 1B), indicating that the C431S mutation does not affect interaction between Parkin and Pink1. The following results indicate that the defect of Parkin CS431S mitochondrial translocation is due to other mechanisms.

Mutation of the putative catalytic cysteine to serine can trap ubiquitin

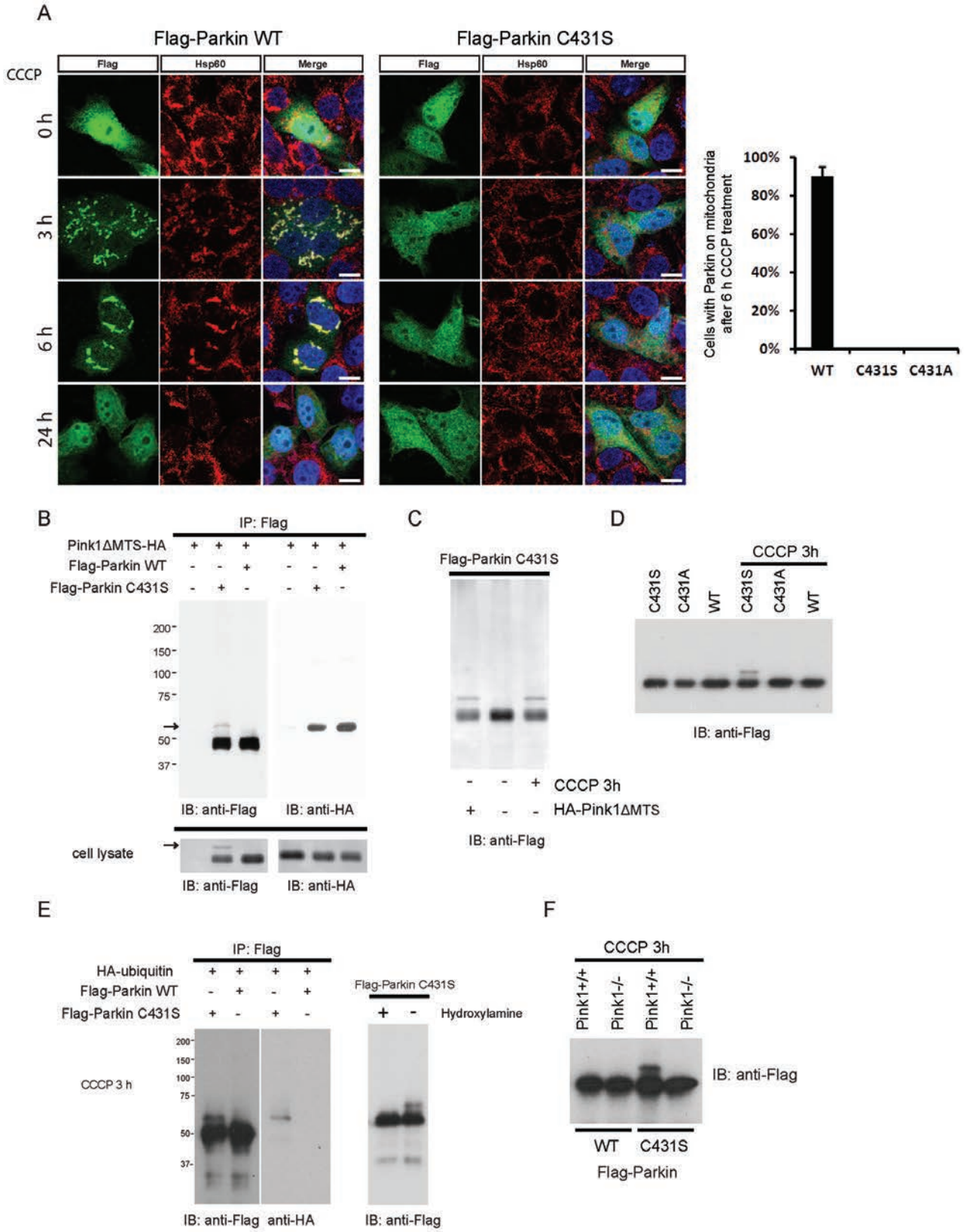
Interestingly, when Pink1ΔMTS was co-expressed with Parkin, an extra higher molecular weight species, ~8 kDa above the main band, was detected for Parkin C431S but not Parkin WT (Figure 1B). Parkin C431S also exhibited the same extra band when cells were treated with CCCP, but this extra band was absent in untreated cells expressing Parkin C431S (Figure 1C). The size of the extra band is equal to that of a single ubiquitin-modified Parkin. Notably, Parkin C431A did not exhibit the extra band in cells treated with CCCP (Figure 1D). As serine, but not alanine, can trap ubiquitin through the formation of an oxyester bond, the extra band might be due to the formation of a ubiquitin adduct via S431. This CCCP-induced extra band in Parkin C431S is not dependent on the presence of the Flag tag or the use of HeLa cells, as it could be detected with 6× His-tagged Parkin C431S and in 293 cells (data not shown).

To determine whether this extra band was ubiquitin-modified Parkin, we transfected HeLa cells with Flag-tagged Parkin C431S or Parkin WT together with HA-tagged ubiquitin. After 3 h of CCCP treatment, cell lysates were prepared with 1% SDS-containing hot buffer, and then diluted and subjected to immunoprecipitation with anti-Flag (mouse) antibody conjugated beads. The immunoprecipitates were analyzed by immunoblotting using anti-Flag (rabbit) and anti-HA antibodies (rat). The anti-HA antibodies recognized a band at exactly the same position as the higher molecular weight Parkin band detected by the anti-Flag antibodies (Figure 1E, left panel). As the oxyester bond of serine-trapped ubiquitin is labile to alkaline treatment, the immunoprecipitate was treated with hydroxylamine as described [38]. The higher molecular weight band disappeared after alkaline treatment (Figure 1E, right panel). To examine whether the formation of the ubiquitin~Parkin C431S adduct after CCCP treatment depends on Pink1, we expressed N-terminally Flag-tagged Parkin WT and Parkin C431S in Pink1-null and WT fibroblasts, respectively. The ubiquitin-trapped band of Parkin C431S could only be detected in CCCP-treated WT but not in Pink1-null cells (Figure 1F), indicating that Pink1 is required for ubiquitin trapping by Parkin. These data suggest that Parkin-mediated mitophagy indeed relies on an intermediate ubiquitin transfer step, consistent with the recent *in vitro* biochemical findings with the HHARI and HOIP RBR E3 ligases [27-29]. The fact that Parkin C431S ubiquitin trapping only happens when mitochondria are depolarized and Pink1 is present, implies that Parkin E3 ligase activity is switched from an inactive to an active state under these conditions, in a manner regulated by Pink1.

A recent study suggests that the E3 ligase activity of Parkin is inhibited by its N-terminal ubiquitin like (UBL) domain [32]. Deletion of the UBL domain increased Parkin E3 ligase activity and led to self-ubiquitination in an *in vitro* ubiquitination assay. On this basis, one might predict that removal of the UBL domain would trigger formation of the ubiquitin-trapped form of Parkin C431S. However, we were unable to detect the ubiquitin-Parkin adduct in UBL-deleted Parkin C431S with or without CCCP treatment (Supplementary information, Figure S1B). UBL-deleted Parkin could still localize to mitochondria and trigger mitochondrial aggregation (data not shown), although this response was delayed compared with Parkin WT, as reported [15, 17]. These data imply that the UBL domain has additional roles besides auto-inhibition.

Co-expression of WT Parkin restores Parkin C431S mitochondrial translocation

As Parkin C431S can trap ubiquitin and still bind to Pink1ΔMTS, we inferred that the overall structure of Parkin is intact in this mutant. One intriguing interpretation of the loss of mitochondrial localization is that Parkin E3 ligase activity may be required for Parkin mitochondrial accumulation. A direct consequence of loss of E3 activity is a lack of ubiquitin chain formation on mitochondria. We hypothesized that ubiquitin chains may play some role in Parkin localization. A simple idea was that restoration of ubiquitin chain formation on mitochondria might be able to rescue the localization of Parkin C431S. As no tools are available to selectively catalyze ubiquitin chain formation on mitochondria, we tested whether co-expression of WT Parkin could rescue Parkin C431S localization by co-expressing Flag-tagged WT Parkin and GFP-tagged Parkin C431S at a 1:1 ratio of vector DNA in HeLa cells. Interestingly, expression of WT Parkin was able to completely rescue the mitochondrial localization of Parkin C431S upon CCCP treatment (Figure 2A). The protein ratio of GFP-Parkin C431S versus Flag-Parkin WT was about 3:1, as detected by anti-Parkin antibody immunoblotting (Figure 2A, right). These data support our hypothesis that ubiquitin chains are required for recruitment of Parkin to damaged mitochondria, but an alternative explanation is that Parkin may form dimers or multimers when mitochondria are depolarized, which could drive recruitment of additional


A
Flag-Parkin WT
Flag-Parkin C431S
Flag
Hsp60
Merge
CCCP
0 h
3 h
6 h
24 h
Cells with Parkin on mitochondria after 6 h CCCP treatment
100%
80%
60%
40%
20%
0%
WT
C431S
C431A
B
IP: Flag
Pink1ΔMTS-HA
Flag-Parkin WT
Flag-Parkin C431S
200
150
100
75
50
37
IB: anti-Flag
IB: anti-HA
cell lysate
C
Flag-Parkin C431S
CCCP 3h
HA-Pink1ΔMTS
IB: anti-Flag
D
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anti-HA
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F
CCCP 3h
Pink1+/+
Pink1-/-
IB: anti-Flag
WT
C431S
Flag-Parkin

Parkin molecules. To test whether Parkin formed multimers, we co-expressed GFP- and Flag-tagged Parkin in HeLa cells. When Flag-Parkin was immunoprecipitated with anti-Flag conjugated beads, we could not detect GFP-Parkin in the immunoprecipate from either CCCP-treated or -untreated cell lysate (Supplementary information, Figure S1C). Nevertheless, these negative results cannot entirely exclude the possibility that Parkin multimers formed through weak protein-protein interactions might play a role in Parkin recruitment to damaged mitochondria.

To test whether targeting the RBR domain alone to the outer surface of mitochondria is sufficient to rescue mitochondrial localization of Parkin C431S, we made a new chimeric protein by replacing the Pink1 kinase domain (Pink1 157-581 aa) with the Parkin RBR domain (216-465 aa), adding a Flag tag sequence between them. When the PINK1Δkinase-Flag-RBR protein was expressed in HeLa cells, it triggered mitochondrial aggregation and mitophagy after CCCP treatment similar to WT Parkin (Supplementary information, Figure S1D). Co-expression of PINK1Δkinase-Flag-RBR protein also rescued the Parkin C431S mitochondria localization, whereas when the catalytic cysteine in RBR domain was mutated to serine, it failed to do so (Figure 2B). Both the PINK1Δkinase-Flag-RBR WT and PINK1Δkinase-Flag-RBR C431S proteins accumulated on the mitochondria after CCCP treatment, as judged by immunostaining. These results suggest that Parkin catalytic activity is important for recruitment and Parkin multimer formation is unlikely to be solely responsible for the ability of WT Parkin to restore Parkin C431S mitochondrial localization.

Parkin binds K63-linked ubiquitin chains

In a recent study, a Parkin peptide array was used to map the binding region for its own UBL domain [32]. Interestingly, two motifs, one in RING0 and the other in the RBR region (the exact positions are not provided in the report) were found to have ubiquitin-binding capacity. This observation prompted us to test whether Parkin can bind to polyubiquitin chains directly. For this purpose, we expressed His-Sumo-GST and His-Sumo-Parkin in *E. coli* (the His-Sumo tag generated higher levels of soluble Parkin compared with GST or His tag). GST-p62 UBA (384-440 aa) was used as a positive binding control for K63-linked ubiquitin chains. Using an *in vitro* binding assay for K63- and K48-linked ubiquitin chains, we found that His-Sumo-Parkin specifically pulled down K63- but not K48-linked polyubiquitin chains (Figure 3A). Unfortunately, we were unable to express sufficient amounts of soluble Parkin RING0 and RBR domains to further dissect the region required for K63-linked ubiquitin chain binding. Parkin does not contain a conventional ubiquitin-binding domain, but there are other examples in which ubiquitin binding has been observed in the absence of a conventional ubiquitin-binding motif. For example, the Rsp5 HECT domain E3 ligase contains a ubiquitin-binding site within its catalytic domain required for ubiquitin chain formation [39, 40].

Figure 1 Mutation of the putative catalytic cysteine to serine in the Parkin RBR domain blocks mitochondrial translocation and leads to ubiquitin trapping. **(A)** Mutation of the putative catalytic cysteine 431 to serine in the Parkin RBR domain abrogated its mitochondrial translocation. HeLa cells transfected with Flag-tagged Parkin WT or Parkin C431S were treated with CCCP. The cells were stained with anti-Flag (green), anti-Hsp60 (red) and Hoechst 33342 (blue). Scale bars, 10 μm. The graph corresponds to means ± SD of percent of cells exhibiting mitochondrial recruitment in three independent experiments, with 100 anti-Flag staining-positive cells being counted per sample. **(B)** Interaction between Pink1 and Parkin was not affected by C431S mutation. HeLa cells were transfected with C-terminally HA-tagged Pink1ΔMTS (mitochondrial translocation signal) and Flag-Parkin WT or Flag-Parkin C431S. Parkins were immunoprecipitated using anti-Flag M2 antibody conjugated beads; the immunoprecipitated Parkin was detected with anti-Flag antibody (rabbit) and the co-precipitated Pink1ΔMTS-HA was detected with anti-HA antibody (rat) by immunoblotting. An extra higher molecular weight species, ~8 kDa above the main band, was detected for Parkin C431S but not Parkin WT as indicated by arrows. **(C)** Co-expression of Pink1ΔMTS-HA or CCCP treatment led to an extra higher molecular weight band of Parkin C431S. HeLa cells were transfected with Pink1ΔMTS-HA and Flag-Parkin C431S or Flag-Parkin C431S alone. HeLa cells transfected with Flag-Parkin C431S were treated with CCCP for 3 h or untreated. Flag-Parkin C431S was detected with anti-Flag antibody (mouse) by immunoblotting. **(D)** The extra band in Parkin C431S was serine dependent. HeLa cells were transfected with Flag-tagged Parkin WT, Parkin C431S or Parkin C431A, and then treated with CCCP for 3 h. Flag-Parkins were detected by immunoblotting using anti-Flag antibody. **(E)** The extra band of Parkin C431S is caused by serine-trapped ubiquitin. HeLa cells were transfected with HA-ubiquitin and Flag-Parkin WT or Flag-Parkin C431S. Flag-Parkins were immunoprecipitated by anti-Flag M2 antibody conjugated beads. The co-precipitated HA-ubiquitin conjugates were detected by immunoblotting with anti-HA rat antibody (left panel). The immunoprecipitated Flag-Parkin C431S containing the serine-trapped ubiquitin was treated with sodium hydroxylamine as described in Materials and Methods, and detected by anti-Flag rabbit antibody (right panel). **(F)** Pink1 was essential for ubiquitin trap of Parkin C431S. Pink1-null and WT mouse fibroblasts were transfected with Flag-tagged Parkin WT or Parkin C431S, and treated with CCCP for 3 h. Parkin was detected by immunoblotting with anti-Flag antibody.

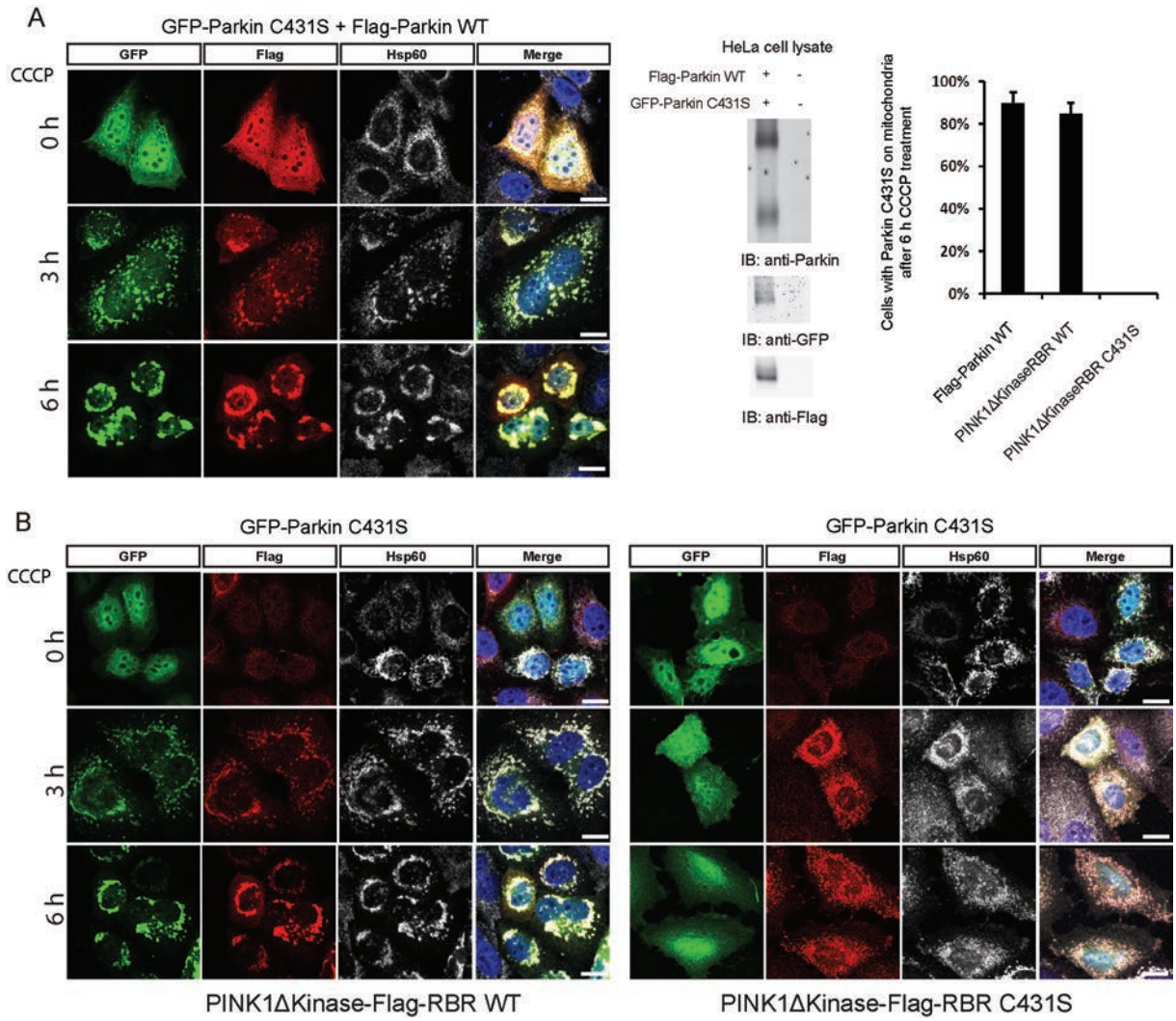


Figure 2 Co-expression of WT Parkin restores Parkin C431S mitochondrial translocation. **(A)** Parkin C431S localizes to damaged mitochondria when co-expressed with WT Parkin. HeLa cells transfected with N-terminal GFP-Parkin C431S and Flag-Parkin WT, and then treated with CCCP. The cells were stained with anti-Flag antibody (red), anti-Hsp60 antibody (white) and Hoechst 33342 (blue). The relative levels of GFP- and Flag-tagged Parkin were determined by immunoblotting using anti-Parkin antibody; untransfected HeLa cells were used as control. The blot was also probed by anti-GFP and anti-Flag antibody, respectively. **(B)** A chimeric fusion of Pink1ΔKinase and Parkin RBR domain was sufficient to restore Parkin C431S mitochondrial localization. HeLa cells were transfected with GFP-Parkin C431S and Pink1ΔKinase-Flag-RBR WT or Pink1ΔKinase-Flag-RBR C431S, and then treated with CCCP and stained with anti-Flag antibody (red), anti-Hsp60 antibody (white) and Hoechst 33342 (blue). Scale bars, 10 μm. The graph shows means ± SD of percent of cells showing mitchondrial recruitment in three independent experiments, with 100 anti-Flag staining- and GFP-positive cells being counted per sample.

The tertiary structure of linear ubiquitin chains is similar to that of K63-linked ubiquitin chains, because K63 is spatially close to the N-terminal methionine of ubiquitin and both chains have highly extended conformations [41]. Therefore, linear ubiquitin chains can in principle be used to mimic K63-linked ubiquitin chains. Knowing that Parkin can bind to K63-linked ubiquitin chains *in vitro*, we next tested whether the mitochondrial localization of Parkin C431S could be restored by co-expression of a chimeric Pink1 derivative in which its kinase domain is replaced by four tandem copies of ubiquitin G76V, which mimic a linear ubiquitin chain but cannot be cleaved in the cell by the ubiquitin processing machinery. When Pink1ΔKinase-Flag-1×ubiquitinG76V or Pink1ΔKinase-Flag-4×ubiquitinG76V and GFP-Parkin C431S were co-expressed in HeLa cells, we found that after CCCP

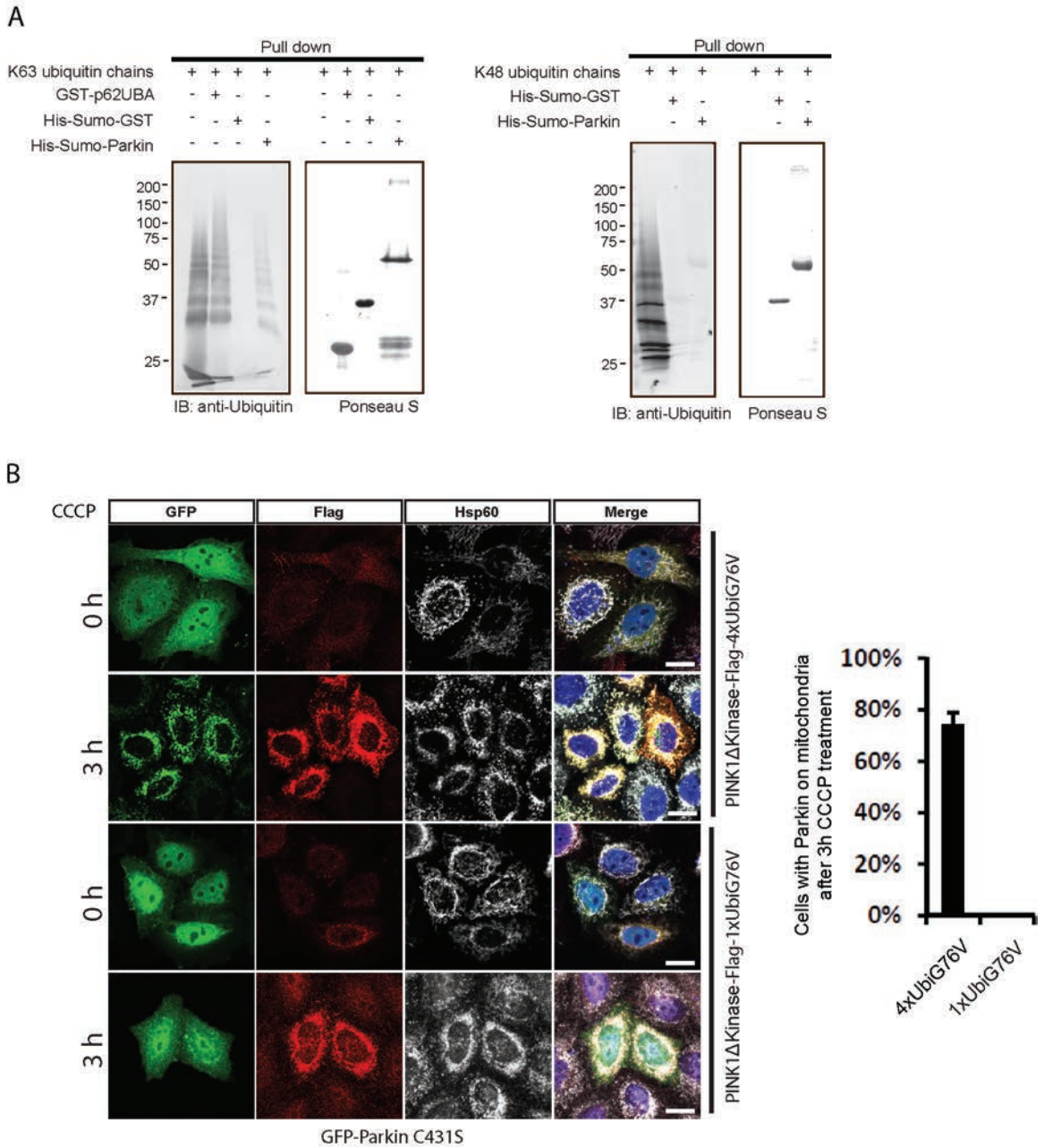
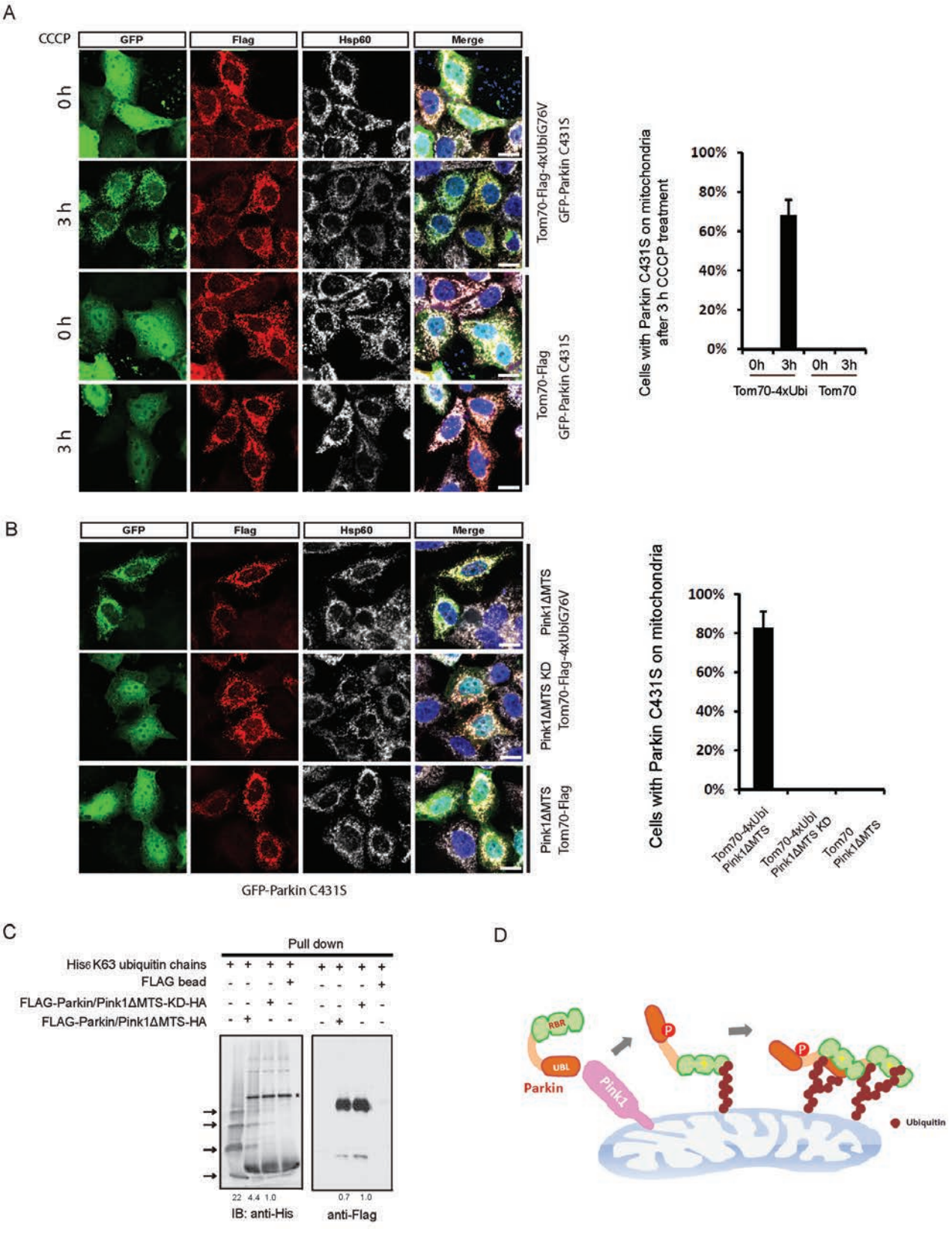


Figure 3 Parkin binds K63-linked ubiquitin chains. **(A)** Recombinant Parkin can pull down K63-linked polyubiquitin chains. GST-p62UBA was used as a positive control for K63-linked chain binding. The bound K63-linked ubiquitin chains were detected by anti-ubiquitin antibody (left panel); K48-linked ubiquitin chains were detected by anti-ubiquitin chain antibody (right panel). The amount of recombinant proteins was determined by Ponceau S staining. **(B)** Expression of a chimeric Pink1 whose kinase domain was replaced by a linear ubiquitin chain comprising four copies of G76V can restore Parkin C431S mitochondria localization. HeLa cells were transfected with GFP-Parkin C431S and Pink1ΔKinase-Flag-1×ubiquitinG76V or Pink1ΔKinase-Flag-4×ubiquitinG76V, and then treated with CCCP. The cells were stained with anti-Flag antibody (red), anti-Hsp60 antibody (white) and Hoechst 33342 (blue). Scale bars, 10 μm. The graph shows means ± SD of percent of cells exhibiting mitochondrial recruitment in three independent experiments, with 100 anti-Flag staining- and GFP-positive cells being counted per sample.

treatment, Pink1ΔKinase-Flag-4×ubiquitinG76V but not Pink1ΔKinase-Flag-1×ubiquitinG76V was able to restore GFP-Parkin C431S translocation to mitochondria (Figure 3B).


A
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Tom70-Flag
GFP-Parkin C431S
Cells with Parkin C431S on mitochondria after 3 h CCCP treatment
100%
80%
60%
40%
20%
0%
0h 3h 0h 3h
Tom70-4xUbi Tom70
B
GFP
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Hsp60
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Pink1ΔMTS
Tom70-Flag-4xUbiG76V
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IB: anti-His
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Ubiquitin

To examine whether the ubiquitin chain itself is sufficient to recruit Parkin to mitochondria independently of Pink1 accumulation induced by CCCP treatment, we expressed Tom 70-Flag-4×ubiquitinG76V and Tom 70-Flag. We found that CCCP treatment was still required to localize GFP-Parkin C431S to mitochondria (Figure 4A), suggesting that Pink1 has a role prior to Parkin ubiquitin chain binding. It should also be noted that expression of Tom 70-Flag-4×ubiquitinG76V *per se* was not sufficient to induce mitophagy in our experimental conditions, although a small percentage of cells did exhibit perinuclear aggregation of mitochondria.

As overexpression of Pink1ΔMTS can activate Parkin, as can be inferred from the presence of ubiquitin-trapped Parkin C431S (Figure 1C), we hypothesized that Pink1 might promote a structural change in Parkin to enhance its ubiquitin-binding ability. As the *in vitro* pull-down assay using His-Sumo-Parkin might simply reflect its basal K63-linked polyubiquitin chain binding activity, we needed to test whether Parkin activation increased K63-linked ubiquitin chain binding. For this purpose, we co-expressed a C-terminally HA-tagged Pink1ΔMTS or Pink1ΔMTS kinase-dead form (KD) together with Tom70-Flag-4×ubiquitinG76V. Tom70-Flag-4×ubiquitinG76V and Pink1ΔMTS co-expression was able to trigger Parkin C431S mitochondrial translocation without CCCP treatment (Figure 4B). Mitochondrially-targeted Tom70-Flag-linked ubiquitin chains were still required, as expression of Pink1ΔMTS with Tom70-Flag was not able to trigger Parkin C431S mitochondrial localization (Figure 4B). When we used Flag-tagged Parkin purified from HeLa cells to pull down 6× His-tagged K63-linked polyubiquitin chain, co-expression of Pink1ΔMTS but not Pink1ΔMTS KD was able to increase Parkin binding to K63-linked ubiquitin chains by ~5-fold (Figure 4C). This suggests that Pink1, either through direct phosphorylation or through an indirect mechanism, increases the ability of Parkin to bind K63-linked ubiquitin chains.

In conclusion, our findings suggest an updated model of Parkin recruitment to damaged mitochondria (Figure 4D). When mitochondria are depolarized, Parkin is first transiently recruited and activated by Pink1, and then starts to catalyze K63-linked ubiquitin chain formation on neighboring mitochondrial proteins; these newly-synthesized K63-linked ubiquitin chains provide secondary docking sites for activated Parkin molecules, which then create additional K63 chains, amplifying the signal. Obviously, one advantage of this mechanism is that it allows a single Pink1 molecule to activate multiple Parkin molecules. Parkin mitochondrial translocation is thus achieved through a catalytic activity coupled mechanism. The finding that Parkin has intrinsic ubiquitin chain-binding capability may also help to explain a pathological observation: Parkin is found in Lewy bodies (ubiquitin-enriched protein aggregate), a pathological hallmark of Parkinson's disease [42].

During the preparation of our manuscript, a similar discovery was reported by Richard Youle's group [43]. Using an elegant cell-free assay system, they found that Pink1 can stimulate the formation of a Parkin-ubiquitin thioester intermediate, and showed that this HECT-like ubiquitin E3 ligase activity is essential for mitochondrial translocation of Parkin in response to CCCP treatment. Interestingly, they found that Pink1 can trigger Parkin self-association independently of Parkin ubiquitin E3 ligase activity, and they further showed that the RBR domain may account for Parkin self-association by targeting a WT RBR domain to mitochondria. An unanswered question is why Parkin E3 ligase activity is required for mitochondrial translocation. In our studies, we found that, unlike the WT RBR domain, a mitochondrially-

Figure 4 Pink1 activates ubiquitin chain binding ability of Parkin. **(A)** Mitochondrially-targeted linear ubiquitin chains alone are not sufficient to recruit Parkin C431S to mitochondria without CCCP treatment. HeLa cells were transfected with GFP-Parkin C431S and Tom70-Flag-4×ubiquitinG76V or Tom70-Flag, and then treated with CCCP. The cells were stained with anti-Flag antibody (red), anti-Hsp60 (white) antibody and Hoechst 33342 (blue). **(B)** Expression of Pink1ΔMTS can stimulate Parkin C431S localization to mitochondria carrying linear ubiquitin chains. HeLa cells were transfected with GFP-Parkin C431S/Tom70-Flag-4×ubiquitinG76V/Pink1ΔMTS-HA; GFP-Parkin C431S/Tom70-Flag-4×ubiquitinG76V/Pink1ΔMTS KD-HA; or GFP-Parkin C431S/Tom70-Flag/Pink1ΔMTS-HA, respectively. 12 h after transfection, cells were stained with anti-Flag antibody (red), anti-Hsp60 antibody (white) and Hoechst 33342 (blue). The graphs show means ± SD of percent of cells exhibiting mitchondrial recruitment in three independent experiments, with 100 anti-Flag staining- and GFP-positive cells being counted per sample. **(C)** Pink1 might enhance Parkin ubiquitin chain binding. HeLa cells were transfected with Flag-Parkin WT together with Pink1ΔMTS-HA or Pink1ΔMTS KD-HA. Parkins were immunoprecipitated and then incubated with 6× His-tagged K63-linked polyubquitin chain for 2 h. The bound ubiquitin chains were detected by anti-His antibody (mouse); Parkin was detected by anti-Flag rabbit antibody. 200 ng of 6× His-tagged K63-linked polyubquitin chains were loaded as a positive control for immunobloting. The total intensities of bound ubiquitin chains and the corresponding immunoprecipitated Parkin were determined by normalizing to the levels of Flag-Parkin without co-expression of Pink1ΔMTS using Odyssey software. **(D)** A model for catalytic activity coupled Parkin mitochondrial recruitment. Details are in the main text.

targeted RBR C431S domain is unable to rescue Parkin C431S localization, indicating that ubiquitin chain formation may explain the phenomenon observed by the Youle group [43]. Indeed, in our hands, targeting linear ubiquitin chains to mitochondria restores Parkin C431S mitochondrial localization. Nevertheless, ubiquitin chains themselves are not sufficient to recruit Parkin in the absence of Pink1 activity. We suspect that Pink1-triggered Parkin self-association may be a prerequisite for stable Parkin-ubiquitin binding. Overall, these two independent studies provide strong evidence of a role for Parkin HECT-like E3 activity during mitophagy, and give us further molecular insights into the mechanism of Parkin mitochondrial translocation.

By using complementary interaction proteomics in an effort to identify all the potential Parkin substrates and their sites of ubiquitylation during Parkin-mediated mitophagy, Wade Harper's group has recently found that numerous Parkin substrates can physically associate with Parkin; interestingly, those interactions were undetectable when Parkin C431S was used [44]. Another recent study reported a new function for Parkin showing that in response to cellular stress, Parkin is recruited to the linear ubiquitin chain assembly complex, and increases linear ubiquitylation of NEMO, a process that is critical for the activation of the NF-κB pathway [45]. Our finding that Parkin can associate with ubiquitin chains attached to substrates may provide an explanation for these observations.

Materials and Methods

Antibodies, plasmids and cell lines

Antibodies used in immunoblotting analysis were as follows: anti-Flag mouse antibody (1:5 000, Sigma, Clone M2), anti-Flag rabbit antibody (1:2 000, Sigma), anti-HA rat antibody (1:2 000, Roche), anti-Parkin mouse antibody (1:1 000, Santa Cruz, PRK8), anti-ubiquitin antibody (1:1 000, Santa Cruz, P4D1), anti-ubiquitin antibody, Lys48 specific (1:1 000, Millipore, Apu2), anti-polyhistidine monoclonal antibody (1:1 000, Sigma, Clone HIS-1). cDNAs for human Parkin and PINK1 were cloned from the MGC human cDNA library. Parkin mutations were generated by QuikChange site-directed mutagenesis kit (Stratagene), and confirmed by sequencing. GFP-Parkin WT, GFP-Parkin C431S and C431A were expressed in pEGFP Clontech vectors, and Flag-tagged versions were expressed in pCMV-3tag Agilent vectors. Pink1ΔMTS-HA was generated by removing the N-terminal 1-110 aa and expressed by pCMV-3tag vector in which the Flag tag was replaced by the HA sequence. Tetra-linearized ubiquitin G76V was generated by repetitive ligation of single ubiquitin G76V. HeLa cells, 293 cells, $PINK1^{-/-}$ and $PINK1^{+/+}$ MEFs were cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. MEFs were transfected with lipofectamine 2000 (Invitrogen), and HeLa cells were transfected with PolyJet™ DNA In Vitro Transfection Reagent (SignaGen). Mitochondrial depolarization was induced by treatment with 10 μM CCCP for HeLa cells or 30 μM CCCP for MEF cells.

Immunocytochemical analysis

Antibodies used in immunocytochemistry were as follows: anti-Flag mouse antibody (1:1 000, Sigma, Clone M2), anti-Hsp60 (1:1 000, Santa Cruz, N-20), Alexa 488, 546, 647, 680 conjugated donkey anti-mouse, donkey anti-goat, anti-rabbit antibodies were used as secondary antibodies at 1:1 000 (Invitrogen). Cells plated on chamber slides (Millipore) were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100. Nuclei were counterstained with Hochest 33342 (Invitrogen). Cells were imaged using laser-scanning microscope systems (LSM 780, Carl Zeiss).

Expression and purification of recombinant proteins and ubiquitin chain pull-down assay

Human Parkin cDNA and GST sequence (from pGEX-6P-1) was cloned into pET SUMO protein expression vector modified from pET-28a by cloning budding yeast SUMO1 ORF downstream of 6× His tag. The UBA domain of p62 was cloned into pGEX-6P-1 vector. All the constructs were transformed into *E. coli* BL21 (DE3) gold (Stratagene). LB media were supplemented with 2 mM $ZnCl_2$. Expression was induced with 1 mM IPTG for 16 h at 16 °C. Cells were harvested and sonicated in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM DTT, bound to TALON® Metal Affinity Resin (Clontech) or GST beads (Sigma) and incubated at 4 °C for 2-3 h. Beads were washed twice with the same extraction buffer, and the third wash was done with ubiquitin-binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% glycerol, 0.2% Triton X-100, 1 mM DTT). 100 μl beads were used in 500 μl binding reaction containing K48- and K63-linked polyubiquitin chains (UC230, UC320 from Boston Biochem) at 5 μg/ml, and incubated at 4 °C for 2 h. The beads were washed five times with ubiquitin-binding buffer, and boiled in 2× SDS-PAGE sample buffer.

Immunoprecipitation and sodium hydroxylamine treatment

Flag-tagged Parkin WT, C431S and Pink1ΔMTS-HA were cotransfected into HeLa cells. Cell lysates were prepared with standard lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% glycerol, 1% Triton X-100 plus protease and phosphatase inhibitor cocktail (Roche)). Anti-Flag M2 antibody conjugated beads were used to immunoprecipitate Flag-tagged proteins. For ubiquitin chain binding assays, His-tagged K63-linked polyubiquitin chains (UCH 330, Boston Biochem) were used at the concentration of 5 μg/ml, and incubated with Flag-Parkin purified from ~10^8 transfected HeLa cells. The ubiquitin binding buffer described above was used. For detection of serine-trapped ubiquitin, cell lysates were prepared with 1% SDS-containing hot buffer, boiled and then 1:10 diluted and subjected to immunoprecipitation by anti-Flag (mouse) antibody conjugated beads. For hydroxylamine treatment, immunoprecipitates were incubated in 1 M sodium hydroxylamine for 2 h at 37 °C. Mock-treated samples were incubated with PBS as described [38].

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)