

Parkin-catalyzed Ubiquitin-Ester Transfer Is Triggered by PINK1-dependent Phosphorylation*

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Background: Parkin is a ubiquitin ligase activated by a decrease in the mitochondrial membrane potential ($\Delta\Psi_m$). However, details regarding its mechanism remain limited.

Results: PINK1-dependent phosphorylation of Parkin at Ser-65 following dissipation of $\Delta\Psi_m$ triggers ubiquitin-ester transfer by the RING2 domain of Parkin to Cys-431.

Conclusion: Parkin catalyzes trans- (ubiquitin-thioester)ification upon PINK1-dependent phosphorylation.

Significance: The molecular process of Parkin-catalyzed ubiquitylation has been determined.

PINK1 and *PARKIN* are causal genes for autosomal recessive familial Parkinsonism. *PINK1* is a mitochondrial Ser/Thr kinase, whereas Parkin functions as an E3 ubiquitin ligase. Under steady-state conditions, Parkin localizes to the cytoplasm where its E3 activity is repressed. A decrease in mitochondrial membrane potential triggers Parkin E3 activity and recruits it to depolarized mitochondria for ubiquitylation of mitochondrial substrates. The molecular basis for how the E3 activity of Parkin is re-established by mitochondrial damage has yet to be determined. Here we provide *in vitro* biochemical evidence for ubiquitin-thioester formation on Cys-431 of recombinant Parkin. We also report that Parkin forms a ubiquitin-ester following a decrease in mitochondrial membrane potential in cells, and that this event is essential for substrate ubiquitylation. Importantly, the Parkin RING2 domain acts as a transthiolation or acyl-transferring domain rather than an E2-recruiting domain. Furthermore, formation of the ubiquitin-ester depends on PINK1 phosphorylation of Parkin Ser-65. A phosphorylation-deficient mutation completely inhibited formation of the Parkin ubiquitin-ester intermediate, whereas phosphorylation mimics, such as Ser to Glu substitution, enabled partial formation of the intermediate irrespective of Ser-65 phosphorylation. We propose that PINK1-dependent phosphorylation of Parkin leads to the ubiquitin-ester transfer reaction of the RING2 domain, and that this is an essential step in Parkin activation.

Parkinson disease is a neurodegenerative disorder that commonly arises sporadically. In some cases, however, the disease is familial and inherited. *PINK1* and *PARKIN* have been identified as the causal genes responsible for hereditary recessive early-onset Parkinsonism (1, 2). *PINK1* is a mitochondrial Ser/Thr kinase, whereas Parkin is a ubiquitin ligase (E3) that catalyzes ubiquitin transfer from the ubiquitin-activating enzyme (E1) and the ubiquitin-conjugating enzyme (E2) to specific substrates. Although the molecular mechanisms underlying sporadic Parkinson disease and familial Parkinsonism are complex, *PINK1* and Parkin have been shown to cooperate in the identification, labeling, and clearance of damaged mitochondria (3, 4). Dysfunction of either likely causes an accumulation of low-quality depolarized mitochondria, which triggers familial Parkinsonism (3–5).

The molecular basis of how *PINK1* and Parkin maintain mitochondrial integrity has eluded researchers for many years; however, relatively recent data have provided significant insights. After escaping mitochondrial membrane potential ($\Delta\Psi_m$)-dependent degradation (6–9), *PINK1* selectively localizes on low-quality mitochondria and is subsequently activated by an autophosphorylation mechanism (10). *PINK1* then recruits the latent form of Parkin from the cytosol to the same mitochondria. Once $\Delta\Psi_m$ decreases, the E3 activity of Parkin is activated (6) and it ubiquitylates outer mitochondrial membrane substrates such as hexokinase I, MitoNEET/CISD1, mitofusin (Mfn),⁴ miro, and voltage-dependent anion channel 1 (see Refs. 4 and 11–16 and references therein). The ubiquitylated mitochondrial proteins are degraded via the proteasome. As a consequence, damaged mitochondria are quarantined by decreased mitochondrial fusion, separated from the destination by a pause in kinesin-dependent trafficking, and/or

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⁴ The abbreviations used are: Mfn, mitofusin; HHARI, human homologue of ariadne; MEF, mouse embryonic fibroblast; CCCP, carbonyl cyanide *p*-chlorophenylhydrazone; IB, immunoblotting; Ub-VS, ubiquitin-vinyl sulfone; NEM, *N*-ethylmaleimide.

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degraded via autophagy. The final destination of low-quality mitochondria remains controversial (11, 17–23). Furthermore, PINK1 may also possess alternative functions other than Parkin recruitment (24, 25). One of the most poorly understood events of PINK1/Parkin-mediated mitochondrial quality control is how the E3 activity of Parkin is re-established by damaged mitochondria.

Mechanistic insights into the ubiquitin-ligating reaction of Parkin have been developing since 2000 (26–32). Parkin possesses multiple RING finger motifs. *In vitro* reconstitution assays revealed that the most carboxyl-terminal RING finger motif (RING2) encompasses the E3 catalytic core (31, 33). The RING1 and RING2 finger motifs are spanned by an in-between RING (IBR) domain, thus this type of E3 is categorized as a RING-IBR-RING (RBR) E3 class. In addition to Parkin, a number of E3s, such as HOIP (HOIL-1L interacting protein) and human homologue of ariadne (HHARI), belong to this class of ligases (34, 35). Recently, HHARI and HOIL-1L interacting protein were shown to form a thioester adduct with ubiquitin on a consensus cysteine in the RING2 domain, similar to the ubiquitin-cascading reaction of HECT (homologous to E6-AP carboxyl terminus)-type E3s (36–38). These results suggest that Parkin may also form a ubiquitin-thioester intermediate, even though it was not observed in the aforementioned paper (36). In 2013, Lazarou *et al.* (39) showed that ubiquitin-oxyester formation of a Parkin C431S mutant depended on a decrease in the mitochondrial membrane potential, thereby partially solving the aforementioned contradiction. The impact of that article, however, was diminished by the lack of biochemical evidence demonstrating ubiquitin-thioester formation with recombinant Parkin and the absence of a mechanism for how PINK1 regulates a ubiquitin-thioester adduct on the catalytic cysteine of Parkin.

In this study, we found that Parkin forms the ubiquitin-thioester intermediate on Cys-431 both *in vitro* and in cells, and revealed that the function of the RING2 domain during ubiquitylation is not E2 recruitment as suggested but ubiquitin-thioester transfer. We further determined that PINK1-dependent phosphorylation of Ser-65 in Parkin leads to formation of the ubiquitin-ester intermediate. These results provide crucial insights into the mechanisms of Parkin activation.

EXPERIMENTAL PROCEDURES

Cells, Plasmids, and Reagents—*PINK1*^{-/-} MEFs complemented by wild type (WT) or various mutants PINK1 were established by infecting *PINK1*^{-/-} MEFs with recombinant retroviruses as follows. *PINK1* mutants were subcloned into a pMXs-puro vector, transfected into PLAT-E retrovirus packaging cells (40), and cultured at 37 °C for 24 h. After changing the medium, PLAT-E cells were further incubated at 37 °C for 24 h and the viral supernatant was collected and used for infection. *PINK1*^{-/-} MEFs (41) were plated on 35-mm dishes 24 h before infection, and the medium was replaced with the undiluted viral supernatant described above with 8 μg/ml of Polybrene (Sigma). Two days later, transformants were selected in medium containing 5 μg/ml of puromycin.

HeLa cells and MEFs were cultured at 37 °C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Sigma) contain-

ing 10% fetal bovine serum, penicillin/streptomycin, 1× non-essential amino acids (Invitrogen), and 1× sodium pyruvate (Invitrogen). To depolarize the mitochondria, cells were treated with 10–30 μM CCCP (Sigma) for 60–90 min unless otherwise specified.

Plasmids for expressing WT or various PINK1 and Parkin mutants have been described previously (6, 10, 11, 42) or were newly constructed by conventional methods. Plasmid transfections were performed using the transfection reagent FuGENE6 (Roche Applied Science) for HeLa cells. For *PINK1*-complemented *PINK1*^{-/-} MEFs, the transfection reagent polyethylenimine (Polyscience) and the electroporation device Neon (Life technologies) were used.

In Vitro Ubiquitylation Assay—To obtain maltose-binding protein (MBP)-fused Parkin and MBP-IBR-RING2, *PARKIN*, or *IBR-RING2*, the respective domains were subcloned into a pMAL vector (New England Biolabs) and transfected into a BL21(DE3) RIL codon plus *Escherichia coli* strain (Stratagene). Recombinant proteins were purified by conventional methods in elution buffer containing 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM dithiothreitol (DTT), 100 μM ZnSO₄, and 10 mM maltose. *In vitro* ubiquitylation assays were performed essentially as described previously with a reaction volume of 40 μl in each experimental condition (31). Briefly, the purified MBP-Parkin protein (10 μg/ml) was incubated in reaction buffer (50 mM Tris-HCl (pH 8.5 unless otherwise specified), 5 mM MgCl₂, 2.5 mM ATP, 2 mM DTT) with 300 μg/ml of ubiquitin (Sigma), 100 nM recombinant mouse E1, and 1/100 diluted E2 UbcH7 (BioMol) at 32 °C for 3 h, and then subjected to immunoblotting (IB) with an anti-Parkin antibody. For identification of ubiquitin in Parkin C431S mutants, recombinant MBP-Parkin C431S or MBP-IBR-RING2 C431S proteins were subjected to the *in vitro* ubiquitylation assay described above with 210 μg/ml of HA-ubiquitin (R&D Systems) instead of intact ubiquitin.

For labeling with a ubiquitin-vinyl sulfone probe (Ub-VS), recombinant MBP-Parkin or MBP-IBR-RING2 proteins (about 20 μg/ml) were incubated with saturating amounts (about 25 μg/ml) of Ub-VS (Boston Biochem) in 30 μl of reaction buffer (50 mM Tris-HCl (pH 8.5), 50 mM NaCl) at room temperature for 3 h. Preincubation with *N*-ethylmaleimide (NEM, Wako chemicals) was performed for 10 min at room temperature at a final concentration of 10 mM.

IB, immunoprecipitation, and Immunofluorescence—To detect ubiquitylation via IB, lysates of HeLa cells or MEFs were collected in TNE-N⁺ buffer (150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1% Nonidet P-40) in the presence of 10 mM NEM to protect ubiquitylated proteins from deubiquitylase activity. For IB-based phosphorylation analyses, lysates from MEFs, HeLa, or HEK293T cells described above were collected in the presence of PhosSTOP (Roche Applied Science) to protect phosphorylated proteins from phosphatase activity. For immunoprecipitation experiments, lysates of HeLa cells transiently expressing HA-Parkin with or without Myc₆-ubiquitin were extracted by TNE-N⁺ buffer and reacted with HA-agarose (Sigma) for 1 h at 4 °C. After washing completely, SDS-PAGE sample buffer was added to the precipitates and eluates were subjected to IB. The anti-Parkin antibody PRK8 (Sigma,

1:1,500 dilution), anti-Mfn2 antibody ab56889 (Abcam, 1:500 dilution), anti-LDH antibody ab2101 (Abcam, 1:1,000 dilution), anti-HA antibody TANA2 (MBL, 1:1,000 dilution), anti-Myc antibody 9E-10 (Santa Cruz, 1:500 dilution), and anti-PINK1 antibody BC100-494 (Novus, 1:1,000 dilution) were used for the IB experiments. For oxyester detection, HeLa cells expressing a Parkin C431S mutant treated with or without CCCP were incubated at 37 °C for 20 min with 0.1 N NaOH before being subjected to SDS-PAGE.

For immunofluorescence experiments, HeLa cells were fixed with 4% paraformaldehyde, permeabilized with 50 μ g/ml of digitonin, and stained with a primary antibody (1:500 dilution of anti-HA antibody F7 or 1:3,000 dilution of anti-Tom20 antibody FL-145, Santa Cruz Biotechnology) and a 1:2,000 dilution of the secondary antibody (Alexa Fluor 488- or 568-conjugated anti-mouse or rabbit IgG antibody, Invitrogen). Cells were imaged using a laser-scanning microscope (LSM510; Carl Zeiss, Inc.) and image contrast and brightness were adjusted in Photoshop (Adobe).

Cell Fractionation—For fractionation experiments, HEK293 cells were treated with 10 μ M CCCP for 90 min and subsequently treated with 1 mM dithiobis(succinimidyl propionate) (Pierce) in PBS \pm CCCP for 90 min on ice, inactivated by 10 mM glycine in PBS three times, and suspended in chappell-perry buffer (0.15 M KCl, 20 mM HEPES-NaOH, pH 8.1, 5 mM MgCl₂, protease and phosphatase inhibitor (Roche)). Cells were disrupted by passaging 30 times through a 25-gauge needle (1-ml syringe), debris was removed by centrifugation at 1,000 \times g for 7 min, and the supernatant was subjected to 10,000 \times g for 10 min to separate the mitochondria-rich fraction from the cytosol-rich fraction.

Phos-tag Assay and Protein Phosphatase Treatment—To detect phosphorylated proteins via SDS-PAGE, 7.5–8% polyacrylamide gels containing 50 μ M Phos-tag acrylamide (Wako chemicals) and 100 μ M MnCl₂ were used. After electrophoresis, Phos-tag acrylamide gels were washed with gentle shaking in transfer buffer containing 0.01% SDS and 1 mM EDTA for 10 min and then incubated in transfer buffer containing 0.01% SDS without EDTA for 10 min according to the manufacturer's protocol. Proteins were transferred to PVDF membranes and analyzed by conventional IB as described above.

For protein phosphatase treatment, cell lysates of intact HEK293T cells \pm CCCP treatment were incubated with λ -protein phosphatase (New England Biolabs) for 1 h at the indicated temperature in reaction buffer prepared according to the manufacturer's instructions, and then subjected to Phos-tag PAGE described above.

LC-MS/MS Analysis of GST-Parkin—GST-Parkin from CCCP-treated and untreated cells was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. GST-Parkin protein bands were excised, reduced, alkylated, and digested with trypsin (Promega) in 50 mM ammonium bicarbonate for 16 h at 37 °C. The resultant peptides were analyzed on a Q Exactive mass spectrometer (Thermo Scientific) with the raw data processed using Xcalibur (Thermo Scientific). The Mascot generic format files were searched against the NCBI non-redundant protein database restricted to *Homo sapiens* using the MS/MS ion search tool of the Mascot program (Matrix Science).

Cell-free Ubiquitylation Assays—HeLa cells expressing GFP-Parkin, HA-Parkin, or HA-Parkin with various mutations were homogenized in cell-free assay buffer (20 mM HEPES-KOH (pH 7.5), 220 mM sorbitol, 10 mM KAc, 70 mM sucrose) supplemented with protease inhibitor mixture minus EDTA (Roche). Cells were disrupted by passaging 30 times through a 25-gauge needle and cell homogenates were centrifuged at 800 \times g for 10 min at 4 °C to obtain a postnuclear supernatant and then cytosolic fractions were collected by further centrifugation at 20,400 \times g for 10 min at 4 °C. The final yields were 100 μ l from 1 ml of confluent cell culture. For mitochondrial isolation, HeLa cells or MEFs expressing only endogenous or exogenous PINK1-FLAG were treated with 10 μ M CCCP for 3 h followed by homogenization in the aforementioned cell-free assay buffer. Postnuclear supernatants were obtained by centrifugation as above and mitochondria were pelleted by further centrifugation at 10,000 \times g for 20 min at 4 °C.

To initiate the cell-free ubiquitylation assay, HeLa cytosols with exogenous Parkin were supplemented with 2 mM DTT, 5 mM MgCl₂, 5 mM ATP, and 1% glycerol. Mitochondria isolated from CCCP-treated confluent cells in 10 ml of medium were re-suspended in 100 μ l of Mg/ATP-supplemented cytosol and incubated at 30 °C for 90 min.

RESULTS

A Parkin C431S Mutation Inhibits Substrate Ubiquitylation via Ubiquitin-Oxyester Adduct Formation—Pioneering work by Klevit's group (36) showed that the active cysteine (Cys-357) in the RING2 domain of RBR-type E3 HHARI forms a ubiquitin-thioester intermediate during the ubiquitin-ligating reaction. In the case of Parkin, Cys-431 is equivalent to HHARI Cys-357. Perplexingly, however, ester-linked ubiquitin of Parkin was not observed in that report even under thioester-stabilizing conditions (36). We previously demonstrated that the enzymatic function of Parkin in cells is activated upon dissipation of $\Delta\Psi_m$ (6), and thus examined whether the ubiquitin-thioester formation on Cys-431 is specifically observed when cells were treated with the mitochondrial uncoupler CCCP. In this experiment, Parkin Cys-431 was mutated to Ser thereby converting an unstable ubiquitin-thioester bond to a stable ubiquitin-oxyester bond. When hemagglutinin (HA)-tagged Parkin (HA-Parkin) with the C431S mutation was expressed in HeLa cells, a higher molecular mass population compared with wild type Parkin (WT-Parkin) was observed following CCCP treatment (Fig. 1A, lane 6). The modification resulted in a 6–7 kDa increase in the molecular weight of Parkin, suggesting ubiquitin-oxyester formation at Ser-431. This modification disappeared with a C431A ester-deficient mutation (lane 4). Co-expression of Myc₁-tagged ubiquitin retarded the mobility of this band (Fig. 1B). When Myc₆-tagged ubiquitin was co-expressed with the HA-Parkin C431S mutant and Parkin was subjected to immunoprecipitation with an anti-HA antibody, the retarded band was specifically detected by the anti-Myc antibody. These results confirmed that the modification was derived from ubiquitin conjugation (Fig. 1C).

We next checked whether Cys-431 is essential for substrate ubiquitylation. Amino-terminal fused lysine-rich proteins can function as Parkin pseudosubstrates (31). Consequently, lysine-

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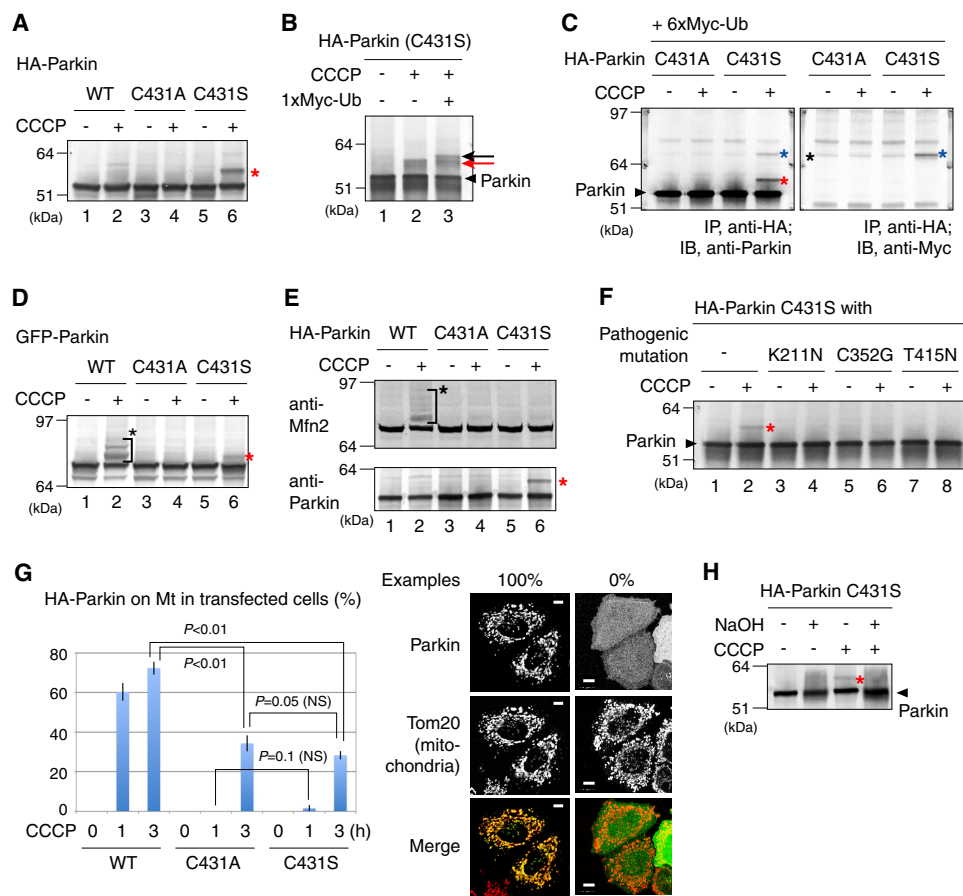


FIGURE 1. *A*, a higher molecular mass population (indicated by the *red asterisk*) was specifically observed in the Parkin C431S mutant following CCCP treatment in HeLa cell lysates. *B*, immunoblotting of the Parkin C431S mutant was repeated in the absence or presence of Myc₁-ubiquitin (*Ub*) co-expression. The slower migrating band resolved as a doublet because of the endogenous-ubiquitin adduct (indicated by a *red arrow*) and the Myc₁-ubiquitin adduct (*black arrow*). *C*, HeLa cell lysates co-expressing HA-Parkin and Myc₆-ubiquitin were immunoprecipitated with an anti-HA antibody, followed by immunoblotting with the indicated antibodies. The anti-Myc antibody specifically detected the modified Parkin(C431S) mutant. *Red asterisk* shows Parkin with the endogenous-ubiquitin adduct; *blue asterisk* shows Parkin with the exogenous Myc₆-ubiquitin adduct; *black asterisk* indicates the cross-reacting band. *D* and *E*, Cys-431 of Parkin is important for substrate ubiquitylation. Both ubiquitylation of a pseudo-substrate (*D*) and a genuine substrate Mfn2 (*E*) were inhibited in the transfected cells by C431A and C431S mutations of Parkin. The *red asterisks* indicate the oxyester-linked ubiquitin and *black asterisks* indicate ordinary substrate ubiquitylation. *F*, HeLa cells expressing HA-Parkin mutants harboring the C431S mutation and one of the disease-relevant mutations (K211N, C352G, and T415N) were subjected to immunoblotting following CCCP treatment. The *red asterisk* indicates the ubiquitin-oxyester band. *G*, Parkin co-localization with mitochondria was analyzed in >100 cells per Cys-431 mutation. Example figures indicative of robust colocalization (counted as 1) and the absence of colocalization (counted as 0) are shown on the right (bars, 10 μ m). Error bars represent the mean \pm S.D. values of three experiments. Statistical significance was calculated using Welch's *t* test; NS, not significant. *H*, the ubiquitylated form of C431S Parkin (marked by a *red asterisk*) is sensitive to NaOH treatment, confirming the presence of the oxyester adduct.

rich GFP can be ubiquitylated in cells when fused in-frame with Parkin (6). The HA tag, in contrast, does not contain lysine residues and thus cannot function as a ubiquitylation pseudo-substrate. This is the reason why ubiquitylation of WT HA-Parkin was not observed in Fig. 1A. GFP-Parkin was ubiquitylated following CCCP treatment (Fig. 1D, lane 2), whereas the C431A mutation completely blocked ubiquitylation (lane 4). The GFP-Parkin C431S mutant was observed as a doublet with only a single additional band, which was putatively derived from ubiquitin-oxyester-Parkin (Fig. 1D, lane 6). Mitofusin 1/2 (Mfn1/2) is a genuine substrate of Parkin and is ubiquitylated upon dissipation of $\Delta\Psi_m$ (17, 43–45). WT HA-Parkin ubiquitylated Mfn2 following CCCP treatment (Fig. 1E, lane 2), whereas no Mfn2 ubiquitylation was observed with either the C431A or the C431S mutation (lanes 4 and 6), confirming that Cys-431 is essential for substrate ubiquitylation. During preparation of this article, Lazarou *et al.* (39) independently published results showing that the Parkin C431S mutant forms a

ubiquitin-oxyester upon CCCP treatment and is unable to ubiquitylate Mfn1. To examine whether pathogenic mutations of Parkin affect the thioester formation, we selected three mutants (K211N, C352G, and T415N) as representative defects for mitochondrial translocation, mitochondrial ubiquitylation, and E3 activity, respectively (6, 42). When HA-Parkin mutants harboring C431S and one of the disease-relevant mutations above (K211N, C352G, or T415N) were subjected to immunoblotting following CCCP treatment, no ubiquitin-oxyester adduct was observed (Fig. 1F), indicating that the three Parkin pathogenic mutations examined compromised formation of the ubiquitin-ester. The monoubiquitylated form of C431S Parkin was sensitive to NaOH treatment (Fig. 1H), confirming oxyester-linked ubiquitylation.

Direct Biochemical Evidence for a Ubiquitin-ester Adduct on Parkin Cys-431—Results shown in Fig. 1, D and E, imply that ubiquitin-ester formation on Cys-431 is essential for Parkin-catalyzed ubiquitylation. However, because the Parkin C431F

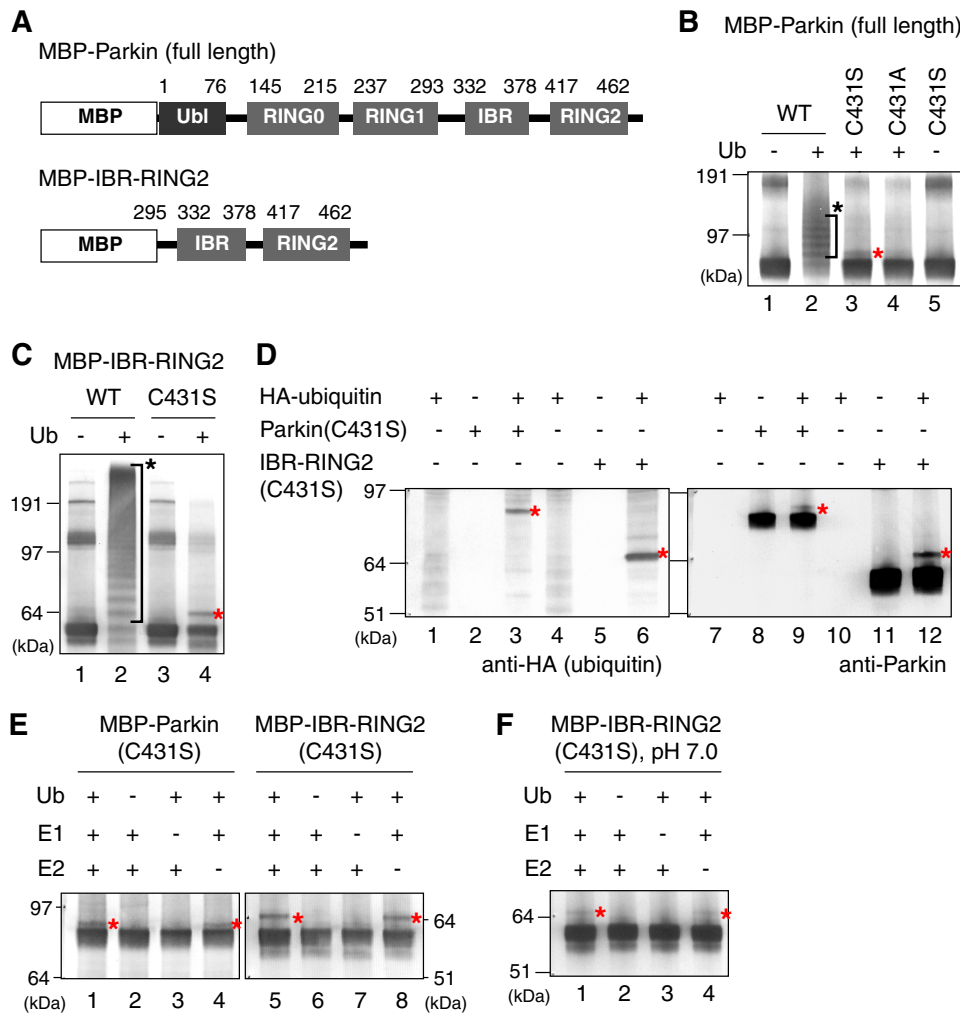


FIGURE 2. Reconstitution of ubiquitin-oxyster formation using recombinant MBP-Parkin protein *in vitro*. *A*, schematic depiction of the domain structure of Parkin and the deletion mutant (IBR-RING2). *B*, *in vitro* E3 activity of Parkin C431 mutants. MBP-Parkin with the C431A or C431S mutation was purified from *E. coli* and reconstituted with ATP, ubiquitin, E1, and E2. Ub indicates ubiquitin, the red asterisk indicates the oxyster-linked ubiquitin and the black asterisk indicates conventional isopeptide-linked ubiquitylation, unless otherwise specified. *C*, E3 activity of the Parkin IBR-RING2 domain \pm C431S mutation. *D*, observed variances in the MBP-Parkin C431S and MBP-IBR-RING2 C431S mutants are the result of ubiquitylation. *In vitro* ubiquitylation was performed with recombinant HA-ubiquitin and followed by immunoblotting with the indicated antibodies. The anti-HA antibody specifically detected the modified Parkin(C431S) mutants. *E*, ubiquitin-oxyster formation of MBP-Parkin and MBP-IBR-RING2 with the C431S mutation in the absence of ubiquitin, E1 or E2, or in the presence of all three components. *F*, ubiquitin-oxyster formation of MBP-IBR-RING2 (C431S) was repeated as *E*, except at neutral pH conditions (pH 7.0).

and C431S mutants do not translocate to the mitochondria following CCCP treatment (39, 46), we are hesitant to over-interpret the previous results. We consequently further confirmed that both mutations strongly (albeit not completely) inhibited translocation of Parkin to depolarized mitochondria (Fig. 1G). Thus the defect in Mfn2 ubiquitylation shown in Fig. 1E could be attributable to mislocalization of the Parkin C431A/S mutants rather than enzymatic dysfunction. To separate the effect of subcellular mislocalization from biochemical function, we next measured the E3 activity of Parkin mutants.

Previously, we reconstituted the E3 activity of Parkin *in vitro* using recombinant Parkin or the IBR-RING2 domain (Fig. 2A) purified from *E. coli*, and found that the C431F pathogenic mutation completely inhibited E3 activity (31), indicating that Cys-431 is essential for E3 activity. We consequently performed an *in vitro* reconstitution assay using recombinant MBP-fused Parkin with the C431S mutation. Similar to the GFP-Parkin ubiquitylation observed in cells (Fig. 1D), MBP-Parkin *in vitro*

exhibited multiple ubiquitylation bands, whereas the MBP-Parkin C431S resolved as only a doublet, which is equivalent to the singly ubiquitylated form (Fig. 2B, lanes 2 and 3). This band was not observed in the ester-deficient C431A mutant (lane 4). Furthermore, exclusion of ubiquitin from the reaction completely quenched modification of the Parkin C431S mutant (lane 5). Clearer results were obtained when the Parkin deletion mutant (MBP-IBR-RING2; Fig. 2A) was used (Fig. 2C). WT IBR-RING2 exhibited multiple ubiquitylation bands (lane 2), whereas the IBR-RING2 C431S mutant was observed as a singly ubiquitylated form (lane 4). When *in vitro* ubiquitylation was performed with recombinant HA-ubiquitin and followed by immunoblotting with anti-HA and anti-Parkin antibodies, the anti-HA antibody specifically detected the modified C431S mutants (Fig. 2D), confirming that *in vitro* modification was, as shown in cells, derived from ubiquitin conjugation. Next, we excluded each reaction component (ubiquitin, E1, and E2) from the assay because we previously showed that Parkin can catalyze E2-in-

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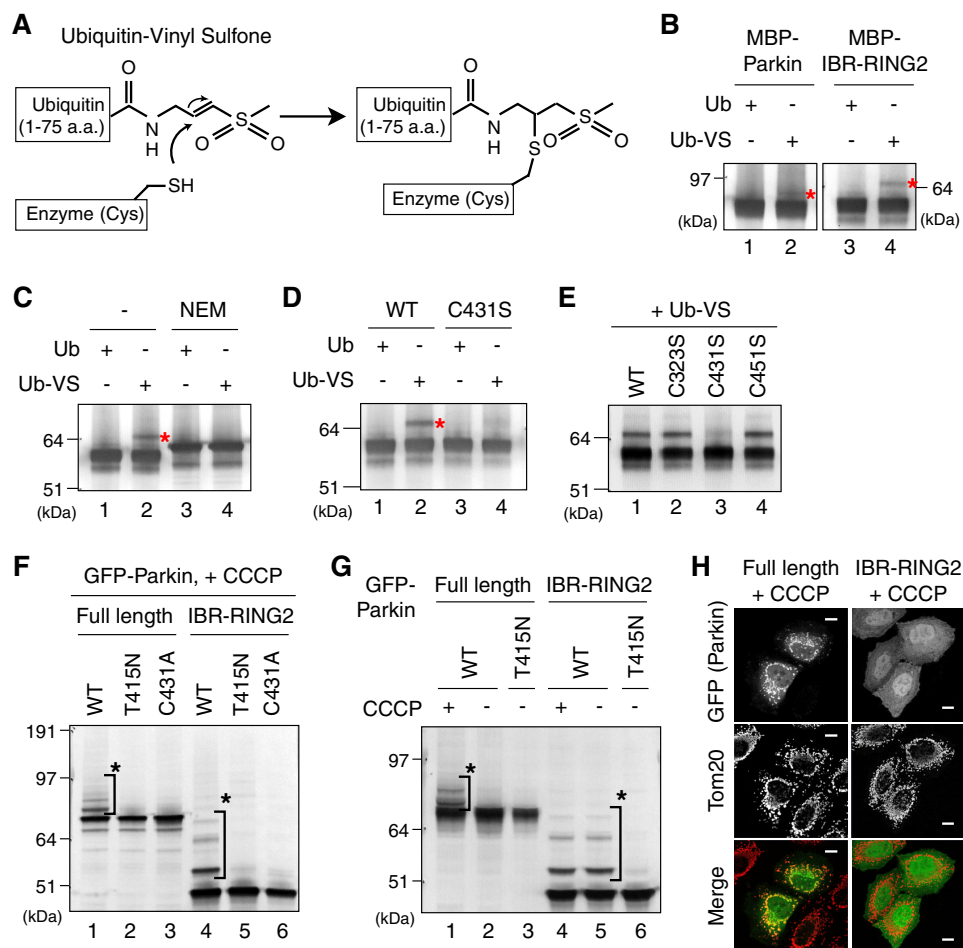


FIGURE 3. *A*, reaction scheme for *in vitro* labeling experiments performed in *B* to *E* using the active site-directed probe Ub-VS. *B*, Ub-VS conjugates to Parkin and IBR-RING2 proteins. *C* and *D*, the Ub-VS adduct was inhibited by preincubation of IBR-RING2 with NEM (*C*) or the C431S mutation (*D*). *E*, C431S is the lone free cysteine mutation to specifically inhibit Ub-VS conjugation. *F*, E3 activity of Parkin lacking the Ubl and RING1 domains in cells. HeLa cells expressing GFP-Parkin or GFP-IBR-RING2 with the C431A or T415N mutation were treated with CCCP and subjected to immunoblotting. *G*, GFP-IBR-RING2 catalyzes autoubiquitylation in cells irrespective of a decrease in $\Delta\Psi_m$. *H*, cytosolic localization of GFP-IBR-RING2 following CCCP treatment. The mitochondrial localization of GFP-Parkin following CCCP treatment is shown as a control.

dependent ubiquitylation at high pH *in vitro* (30). Ubiquitin-oxyster formation of the Parkin C431S mutant was completely inhibited by the exclusion of ubiquitin or E1 from the reaction (Fig. 2*E*, lanes 2, 3, 6, and 7). In contrast, even in the absence of E2, the ubiquitin-oxyster adduct was observed (lanes 4 and 8), suggesting that the IBR-RING2 domain catalyzes ubiquitin-oxyster formation not by E2 recruitment but by discharge and transfer of the ubiquitin-thioester moiety to itself (see "Discussion"). We checked the pH dependence of this reaction, and found that ubiquitin-oxyster formation was weak at pH 7.0 (Fig. 2*F*) but became evident when the reaction pH was increased to 8.5 (Fig. 2*E*). This is consistent with our previous results demonstrating that the E3 *in vitro* activity of MBP-Parkin is the highest under weak alkaline conditions (31). We speculate that the reactivity of the nucleophiles involved in the ubiquitin transfer reaction is affected by the relatively high pH.

We further examined whether ubiquitin is attached to the WT Parkin catalytic cysteine. Ubiquitin- or ubiquitin-like protein-derived probes with electrophilic C-terminal ends, such as Ub-VS and ubiquitin-vinylmethyl ester (Ub-VME) react specifically with the cognate-conjugating and -deconjugating enzymes (47, 48). For example, Ub-VME attached to E1, E2,

and HECT-type E3s (47). Importantly, these adducts are formed through a Michael addition reaction of the active-site cysteine thiol of the conjugating or deconjugating enzyme with the C-terminal electrophilic (*e.g.* vinyl sulfone) moiety (48) (Fig. 3*A*).

When Parkin was incubated with Ub-VS, it led to the appearance of a faint modification with a molecular weight consistent with a Parkin-Ub-VS adduct (Fig. 3*B*, lane 2). A clearer signal was obtained when MBP-IBR-RING2 was used (lane 4). Incubation of Parkin with WT ubiquitin lacking the C-terminal vinyl sulfone moiety did not lead to this modification (lanes 1 and 3), suggesting that covalent modification of Parkin depends on the C-terminal electrophilic substituent of ubiquitin. This modification by Ub-VS was blocked by pretreatment with the cysteine-directed sulphydryl alkylating agent NEM, confirming the cysteine-dependence of adduct formation (Fig. 3*C*, lanes 2 and 4). Moreover, this reaction was not observed in the Parkin C431S mutant (Fig. 3*D*, lane 4). The IBR-RING2 domain has 17 cysteine residues, 14 of which coordinate with zinc ions (49, 50). Consequently only 3 cysteine residues are free in IBR-RING2 domain: Cys-323, Cys-431, and Cys-451. We serially substituted these 3 Cys residues with Ser, and found that Ub-VS still

conjugated to C323S and C451S mutants equivalent to WT (Fig. 3E), suggesting that Cys-431 is the only ligatable cysteine within the IBR-RING2 domain. Taken together, we conclude that Cys-431 is an active-site cysteine in Parkin crucial for ubiquitin ligation and is consequently labeled by Ub-VS.

Collectively, the results shown in Figs. 2 and 3 reveal that Parkin forms a ubiquitin-thioester on Cys-431, and suggest that impaired substrate ubiquitylation by the Parkin C431S mutant (Fig. 1, D and E) is attributable to both aberrant subcellular localization and the trapping of ubiquitin in this dead-end pseudo-intermediate on Ser-431. We observed no difference in the mitochondrial localization between the C431A (ubiquitin-ester deficient) and C431S (ubiquitin-oxyster stabilized) mutants (Fig. 1G), suggesting that the ubiquitin-ester itself does not promote the translocation of Parkin to depolarized mitochondria.

We also examined the ubiquitylation activity of the IBR-RING2 domain of Parkin toward a pseudosubstrate (GFP) in cells, and found that GFP-IBR-RING2 catalyzed ubiquitylation, which was blocked by a T415N or C431A mutation (Fig. 3F). The RING1 domain of HHARI functions as the “ubiquitin-conjugated E2” recruiting domain and is essential for ubiquitin ligation (36), thus a lone IBR-RING2 domain without the Parkin RING1 domain catalyzing pseudosubstrate ubiquitylation both *in vitro* (Fig. 2C) and in cells (Fig. 3F) is unexpected. We consequently examined the CCCP dependence and subcellular localization of GFP-IBR-RING2, and found that GFP-IBR-RING2 undergoes autoubiquitylation irrespective of CCCP treatment and mitochondrial localization (Fig. 3, G and H). These results suggest that IBR-RING2 becomes a constitutively active form because the autoinhibitory effect is prevented. The Parkin structure (50) is consistent with this result as RING0 occludes Cys-431 of RING2 via RING0-RING2 interactions. Although IBR-RING2 can catalyze ubiquitylation, the results shown in Figs. 2 and 3 do not indicate that RING1 and the interaction with E2 are physiologically dispensable because there are many pathogenic missense mutations in the Parkin RING1 domain (3). Rather Figs. 2 and 3 imply that the underlying mechanism during the ubiquitin ligating reaction is different between the RING1 and RING2 domains, even though both domains contribute cooperatively to ubiquitin ligation (see “Discussion”).

PINK1 Is Essential for Formation of the Ester-linked Parkin-Ubiquitin Intermediate—We next checked the effect of PINK1 on the ubiquitin-ester formation of Parkin. In MEFs prepared from *PINK1* knock-out (*PINK1*^{-/-}) mice (41), the formation of the ubiquitin-oxyster in the Parkin C431S mutant was completely impeded even following CCCP treatment (Fig. 4A, lane 1). Subsequent transfection of WT PINK1 complemented the defect (lane 2), revealing that PINK1 is essential for Parkin ubiquitin-oxyster formation. To investigate the role of mitochondrial localization, kinase activity, and the effect of various pathogenic mutations of PINK1 on Parkin ubiquitin-oxyster formation, we co-expressed the various PINK1 mutants with the Parkin C431S mutant in *PINK1*^{-/-} MEFs. A PINK1 N-terminal deletion mutant lacking the terminal 155 amino acids, which are critical for mitochondrial localization of PINK1 (51), and kinase-dead (KD) mutations (K219A, D362A, and D384A) that abolish PINK1 kinase activity (52), completely blocked

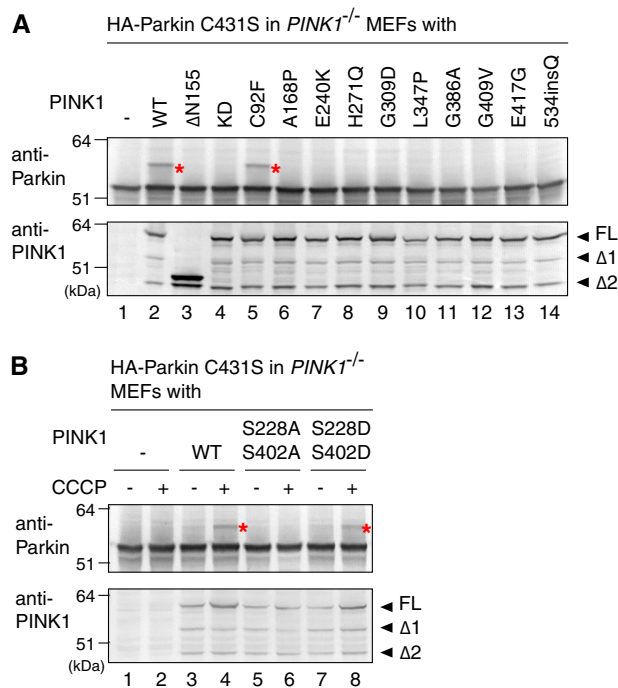


FIGURE 4. A and B, *PINK1*^{-/-} MEFs co-expressing C431S Parkin mutant and various pathogenic (A) or autophosphorylation-relevant (B) PINK1 mutants were subjected to immunoblotting using an anti-Parkin antibody for detection of ubiquitin-oxyster formation (upper panel) or an anti-PINK1 antibody to confirm expression of the PINK1 mutants (lower panel). The red asterisks indicate the ubiquitin-oxyster band. FL, full-length PINK1; Δ1 and Δ2, the amino-terminal processed forms as reported in Ref. 6.

complementation of ubiquitin-oxyster formation in the Parkin C431S mutant (Fig. 4A, lanes 3 and 4). Among the various pathogenic PINK1 mutations that cause early-onset familial Parkinson disease, the C92F mutant supported Parkin ubiquitin-oxyster formation equivalent to WT PINK1 (Fig. 4A, lane 5). In contrast, the other PINK1 point mutations (*i.e.* A168P, E240K, H271Q, G309D, L347P, G386A, G409V, E417G, and 534insQ) severely hindered ubiquitin-oxyster formation (lanes 6–14). The C92F mutation was identified from a sporadic case carrying the compound heterozygote missense mutations (C92F and R464H) but not identified in the lineage (53), suggesting that C92F may represent a natural rare variant and is not a true disease-causing mutation.

We next examined the effect of PINK1 autophosphorylation on formation of the Parkin-ubiquitin intermediate. We recently showed that dissipation of ΔΨ_m triggers PINK1 autophosphorylation of Ser-228 and Ser-402. This autophosphorylation is critical for Parkin recruitment to the same mitochondria (10). When WT PINK1, PINK1 with a S228A/S402A double mutation (autophosphorylation-deficient form), or PINK1 with a S228D/S402D double mutation (autophosphorylation mimic form) were expressed in *PINK1*^{-/-} MEFs at the appropriate expression level, we found that the S228D/S402D mutant promoted ubiquitin-oxyster formation of the Parkin C431S mutant similar to WT PINK1 (Fig. 4B, lane 8). The S228A/S402A mutant, in contrast, failed to support formation of the ubiquitin adduct on Parkin (lane 6). Taken together, the results shown in Fig. 4 suggest that mitochondrial localization, kinase activity, and autophosphorylation of PINK1 are essential for

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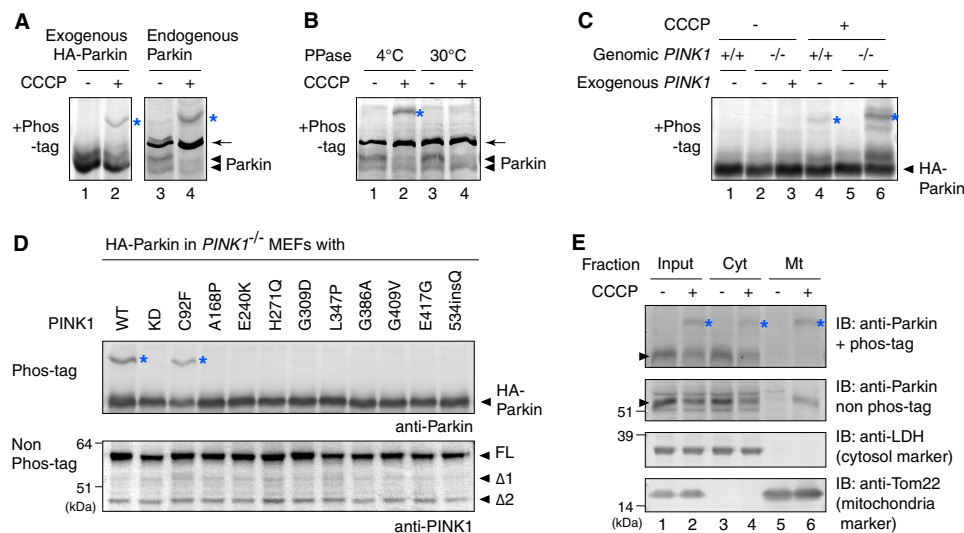


FIGURE 5. *A*, exogenous or endogenous Parkin underwent phosphorylation following CCCP treatment. The cell lysate of exogenous Parkin-expressing HeLa cells or intact HEK293T cells \pm CCCP treatment were subjected to Phos-tag PAGE (58) and thus molecular weight markers are not shown. The blue asterisks indicate phosphorylated Parkin, whereas the arrows indicate the cross-reacting band unless otherwise specified. *B*, protein phosphatase treatment in cell lysates caused the high-molecular shift of endogenous Parkin to disappear. *C*, phosphorylation of Parkin following CCCP treatment in cells depends on PINK1. Parkin was not phosphorylated in *PINK1*^{-/-} MEFs following CCCP treatment, whereas exogenous PINK1 complemented the aforementioned defect. *D*, phosphorylation of Parkin was severely compromised by various pathogenic mutations of PINK1. Parkin C431S mutant-expressing *PINK1*^{-/-} MEFs complemented by disease-relevant PINK1 were treated with CCCP and subjected to immunoblotting. FL, full-length PINK1; $\Delta 1$ and $\Delta 2$, the amino-terminal processed form as described in the legend to Fig. 4A. *E*, intact HEK293T cells \pm CCCP treatment were subjected to fractionation experiments. Cyt and Mt indicate the cytosol-rich supernatant and the mitochondria-rich membrane pellet, respectively. Arrowheads indicate endogenous Parkin.

formation of the ubiquitin-thioester intermediate on Parkin Cys-431.

Parkin Phosphorylation Is Dependent on Both $\Delta\Psi_m$ Dissipation and PINK1—Because PINK1 is a Ser/Thr kinase, the simplest model is that PINK1 phosphorylation of Parkin accelerates formation of the ubiquitin-thioester intermediate. However, PINK1 phosphorylation of Parkin has, until recently, been controversial (7, 54–56). To detect a potentially phosphorylated form of Parkin with high sensitivity, we performed electrophoresis using polyacrylamide gels conjugated with a 1,3-bis[bis(pyridin-2-yl-methyl)amino]propan-2-olato diMn(II) complex (referred to hereafter as Phos-tag). Because Phos-tag can capture phosphomonoester dianions (ROPO_3^{2-}), the phosphorylated Parkin can be easily distinguished from the non-phosphorylated form as a slower migrating band (57). Because mobility in Phos-tag PAGE does not reflect the molecular weight (58), the necessity of molecular weight markers is not critical.

When Parkin overexpressed in HeLa cells was subjected to Phos-tag PAGE, a clear mobility shift was observed following CCCP treatment, implying that Parkin underwent phosphorylation in response to mitochondrial damage (Fig. 5A, lanes 1 and 2; note that the ester-ubiquitin derived band was only observed with the C431S mutation and was thus undetectable in this experiment). Unlike HeLa cells, HEK293T cells express the *PARKIN* gene (59, 60). We consequently used this cell line to determine whether endogenous Parkin is also phosphorylated. Although the extended exposure necessary to detect endogenous Parkin resulted in an intense cross-reacting band (Fig. 5A, arrow), we confirmed that endogenous Parkin is also phosphorylated (lanes 3 and 4). λ -Phosphatase treatment abolished the high-molecular weight shift of this endogenous Par-

kin (Fig. 5B), confirming that Parkin is indeed phosphorylated. We used *PINK1*^{-/-} MEFs to further examine the role of PINK1 on Parkin phosphorylation. In *PINK1*^{+/+} MEFs, Parkin underwent phosphorylation upon CCCP treatment (Fig. 5C, lane 4). In contrast, no detectable phosphorylation was observed in *PINK1*^{-/-} MEFs (lane 5). However, the introduction of PINK1 in *PINK1*^{-/-} MEFs complemented the phosphorylation of Parkin (lane 6), confirming that the defect was caused by the lack of endogenous PINK1. Co-expression of pathogenic PINK1 mutations with HA-Parkin in *PINK1*^{-/-} MEFs severely compromised Parkin phosphorylation (Fig. 5D). The lone exception was the C92F mutation. This effect of the PINK1 disease-relevant mutations on Parkin phosphorylation is consistent with the observed effect on Parkin ubiquitin-oxyster formation (compare Fig. 4A with Fig. 5D), implying an underlying link between these two events. In fractionation experiments using HEK293 cells, endogenous Parkin is recovered in the mitochondria-rich fraction following CCCP treatment (6). Phos-tag PAGE confirmed that Parkin recovered in the mitochondrial fraction is the phosphorylated form (Fig. 5E, lane 6), suggesting that Parkin phosphorylation is important for mitochondrial localization.

We next sought to identify the Parkin phosphorylation site. Although numerous papers have reported Parkin phosphorylation, the phosphorylation site(s) remains debatable. To date, Ser-65 (56, 61), Ser-101 (62, 63), Ser-108 (64), Ser-127 (63), Ser-131 (56, 62, 63, 65), Ser-136 (62, 65), Thr-175 (66), Thr-217 (66), Ser-296 (62), and Ser-378 (62, 63) have been reported as phosphorylation sites. We consequently serially substituted these Ser/Thr residues with Asp. When these Parkin mutants were subjected to Phos-tag PAGE following CCCP treatment, almost all of the mutations, excluding S65D (described later),

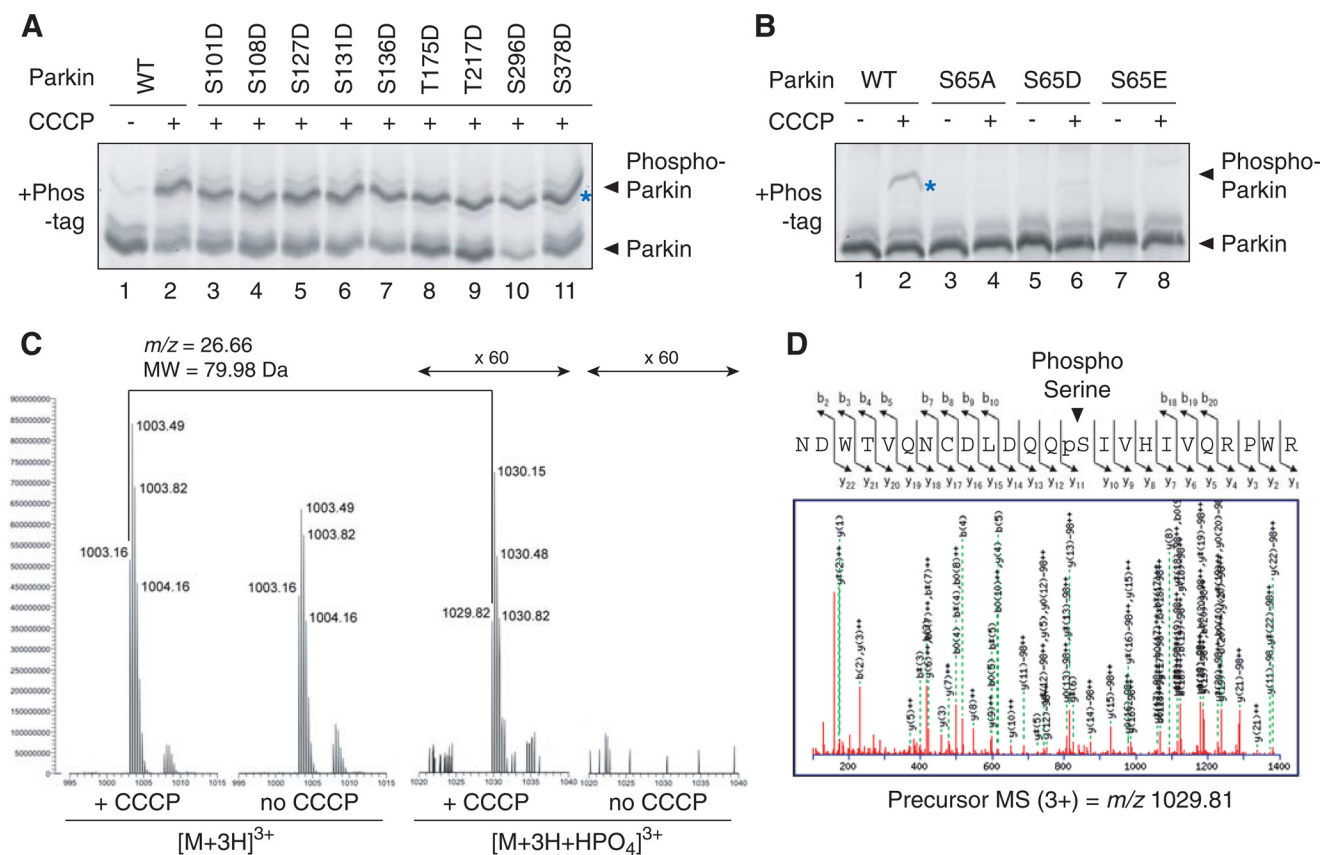


FIGURE 6. Parkin is phosphorylated at Ser-65 following CCCP treatment. *A*, Ser to Asp substitution at various putative phosphorylation sites did not alter the phosphorylation pattern of Parkin in HeLa cells upon dissipation of $\Delta\Psi_m$. Blue asterisks indicate phosphorylated Parkin unless otherwise specified. Note that mobility does not reflect the molecular weight in Phos-tag PAGE (58) and thus molecular weight markers are not shown. *B*, the S65A, S65D, and S65E mutations dramatically decreased the phosphorylation of Parkin in cells. *C*, mass spectrometric analysis of the in cell phosphorylation site of Parkin. GST-Parkin purified from cells \pm CCCP treatment was subjected to LC-MS/MS analysis. A phosphorylated peptide equivalent to amino acids 52–75 was detected only in CCCP-treated cells. *D*, the MS/MS data suggest that phosphorylation occurs at Ser-65.

had a clear phosphorylation-derived signal following CCCP treatment (Fig. 6A). We thus suggest that these amino acids are not the Parkin phosphorylation sites associated with mitochondrial damage. In sharp contrast, mutating Ser-65 in the amino-terminal ubiquitin-like (Ubl) domain of Parkin (Fig. 2A) to Ala, Asp, or Glu prevented phosphorylation following CCCP treatment (Fig. 6B, lanes 4, 6, and 8). This is consistent with the claim by Muqit and Hattori (56, 61) and co-workers that Ser-65 is the genuine Parkin phosphorylation site. To determine the phosphorylation site directly, we performed mass spectrometric analysis of phosphorylated Parkin. Because Parkin autoubiquitylation impedes the detection of phosphorylation, a T415N Parkin mutant was used. Glutathione *S*-transferase (GST)-fused Parkin (T415N) was integrated into the genome of HeLa cells by retrovirus-mediated transformation. GST-Parkin(T415N) was then purified from this stable cell line following \pm CCCP treatment, and subjected to LC-MS/MS analysis after trypsin digestion. A peptide equivalent to amino acids 52–75 (NDWTVQNCDLQQSIVHIVQRPWR) was identified as a putative phosphorylated peptide. Although the unphosphorylated peptide was detected from both CCCP-treated and untreated cells, the phosphorylated peptide was only detected in CCCP-treated cells (Fig. 6C). MS/MS data further suggested that Ser-65 was phosphorylated (Fig. 6D). Taken

together, Fig. 6 indicates that Ser-65 is the Parkin phosphorylation site.

Phosphorylation of Ser-65 Is Essential for Ubiquitin-Ester Formation of Parkin—We examined the role of this phosphorylation on Parkin ubiquitin-oxyster formation. Phos-tag PAGE analysis of a C431S Parkin mutant harboring the S65A, S65D, or S65E mutations revealed that the phosphorylation-derived band was absent in all cases (Fig. 7A) as well as the individual S65A, S65D, or S65E mutations (Fig. 6B). We subsequently examined formation of the ubiquitin-oxyster in these mutants. The S65A/C431S mutant was unable to form the ubiquitin-oxyster band on HA-Parkin (Fig. 7B, lanes 7–9), whereas the S65D/C431S and S65E/C431S mutants partially complemented ubiquitin-oxyster formation (Fig. 7B, lanes 12 and 15), although neither underwent phosphorylation (Fig. 7A). When these HA-Parkin mutants were co-expressed with Myc₆-tagged ubiquitin and subjected to immunoprecipitation with an anti-HA antibody, the retarded band was specifically detected by the anti-Myc antibody, confirming that the modification was derived from ubiquitin conjugation (Fig. 7C). We also generated a Parkin S65T mutant that can potentially act as a substrate for the Ser/Thr kinase PINK1. The Parkin S65T mutant underwent phosphorylation equivalent to WT Parkin as expected (Fig. 7D), and formed a clear ubiquitin-oxyster band (Fig. 7E).

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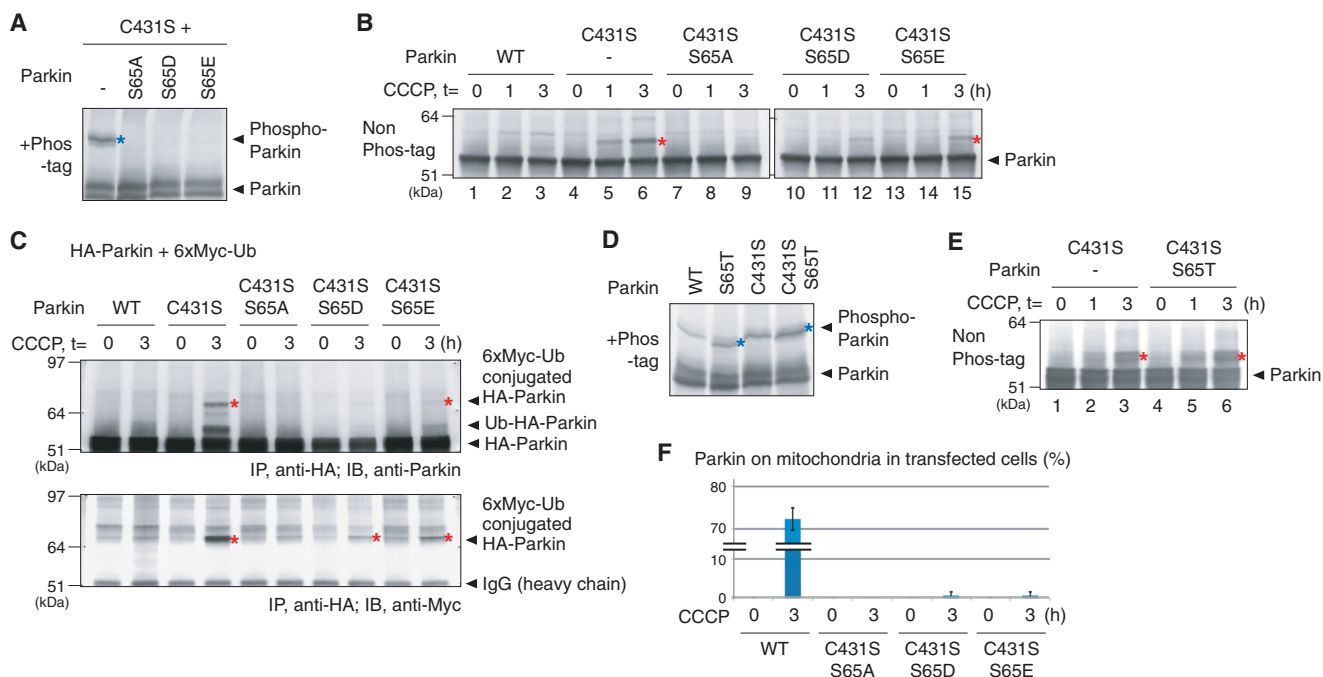


FIGURE 7. *A*, the S65A/S65D/S65E mutations decreased the phosphorylation of ubiquitin ester-stabilized Parkin C431S mutants in cells. *Blue asterisks* indicate phosphorylated Parkin, unless otherwise specified. *B*, straight immunoblotting of HeLa cell lysates expressing Parkin harboring double mutations (*i.e.* C431S with the S65A, S65D, or S65E mutation). The *red asterisks* indicate ubiquitin-oxyster formation unless otherwise specified. The S65A/C431S Parkin mutant disturbed the ubiquitin-ester formation completely, whereas the S65D/C431S and S65E/C431S mutants partially complemented ubiquitin-ester formation. *C*, immunoprecipitation with an anti-HA antibody followed by immunoblotting with the indicated antibodies confirmed that Myc₆-ubiquitin is conjugated to the Parkin C431S, S65D/C431S, and S65E/C431S mutants. *Red asterisks* show Parkin with the Myc₆-ubiquitin adduct. *D* and *E*, the Parkin S65T or S65T/C431S mutants underwent phosphorylation (*D*) and exerted ubiquitin-oxyster formation (*E*) equivalent to WT or C431S mutant in cells. *F*, the number of HeLa cells with Parkin localized to the mitochondria per C431S/S65X mutation following CCCP treatment for 1 h was counted in >100 cells as Fig. 1G. *Error bars* represent the mean \pm S.D. values and statistical significance was calculated using Welch's *t* test.

The Ser-65 mutation affected mitochondrial localization of Parkin(C431S) following CCCP treatment (Fig. 7F). The S65A mutation in particular had a pronounced negative effect on the translocation of Parkin to damaged mitochondria. Thus the S65A mutation could inhibit ubiquitin-oxyster formation via subcellular mislocalization.

To demonstrate the importance of Ser-65 phosphorylation for ubiquitin-ester formation more convincingly, we established the *in vitro* assay referred to by Lazarou *et al.* (39). When cytosol-containing GFP-Parkin derived from mitochondria-intact HeLa cells was incubated *in vitro* with mitochondria isolated from CCCP-treated or non-treated cells, ubiquitylation of GFP-Parkin and Mfn2 were specifically observed in the reaction containing CCCP-pretreated mitochondria (Fig. 8A). Addition of recombinant ubiquitin, E1, and E2 accelerates the ubiquitylation (*lane 6*). Ubiquitylation of GFP-Parkin and Mfn2 was not observed in the reaction containing mitochondria isolated from CCCP-treated *PINK1*^{-/-} MEFs, whereas exogenous *PINK1* complement the ubiquitylation (Fig. 8B), revealing that *PINK1* is essential. A Parkin C431S mutant specifically forms an ubiquitin-oxyster in this assay in the presence of CCCP-pretreated mitochondria (Fig. 8C). We then examined the effect of Ser-65 mutations on ubiquitin-oxyster formation at Ser-431. Even under *in vitro* experimental conditions, the phosphorylation-deficient S65A mutation completely inhibited ubiquitin-oxyster conjugation of HA-Parkin (Fig. 8D, *lane 6*), whereas the S65E/C431S mutation weakly complemented ubiquitin-oxyster formation (Fig. 8D, *lane 8*). These results

suggest that phosphorylation of Parkin Ser-65 is important for ubiquitin-oxyster formation.

DISCUSSION

Biochemical Evidence for Ubiquitin-Ester Conjugation of Parkin Cys-431—Ubiquitin ligases (E3) can be classified into three groups, namely HECT, U-box, and RING-type E3s. In HECT-type E3s, ubiquitin is transferred from the ubiquitin-conjugation enzyme (E2) to a HECT domain as a ubiquitin-thioester relay, and is finally passed to the substrate. On the other hand, the most basic role of RING-type E3 is that it unites both E2 and the substrate facilitating ubiquitin transfer from E2 to the substrate by placing them in close physical proximity. During this process, the RING finger motif functions as an E2-binding domain (67). Since the reports of E3 activity in 2000 (26–28), Parkin has been considered a RING-type E3. This activity was subsequently shown to reside in the RING2 domain (31, 33).

In 2011, based on analysis of the HHARI E3, Wenzel *et al.* (36) proposed that RING-IBR-RING type E3s such as Parkin function as a “RING-HECT hybrid” because HHARI forms an ubiquitin-thioester on a conserved cysteine residue in the rear RING finger motif similar to the HECT domain. Although thought provoking, this hypothesis was inconsistent with results showing that the Parkin C431S mutant was unable to stabilize ubiquitin-oxyster linkage. Using intact cell lysate and mitochondria collected from CCCP-treated cells, Lazarou *et al.* (39) reported formation of ubiquitin-oxyster on a Parkin C431S mutant dependent on *PINK1* and a decrease in $\Delta\Psi_m$,

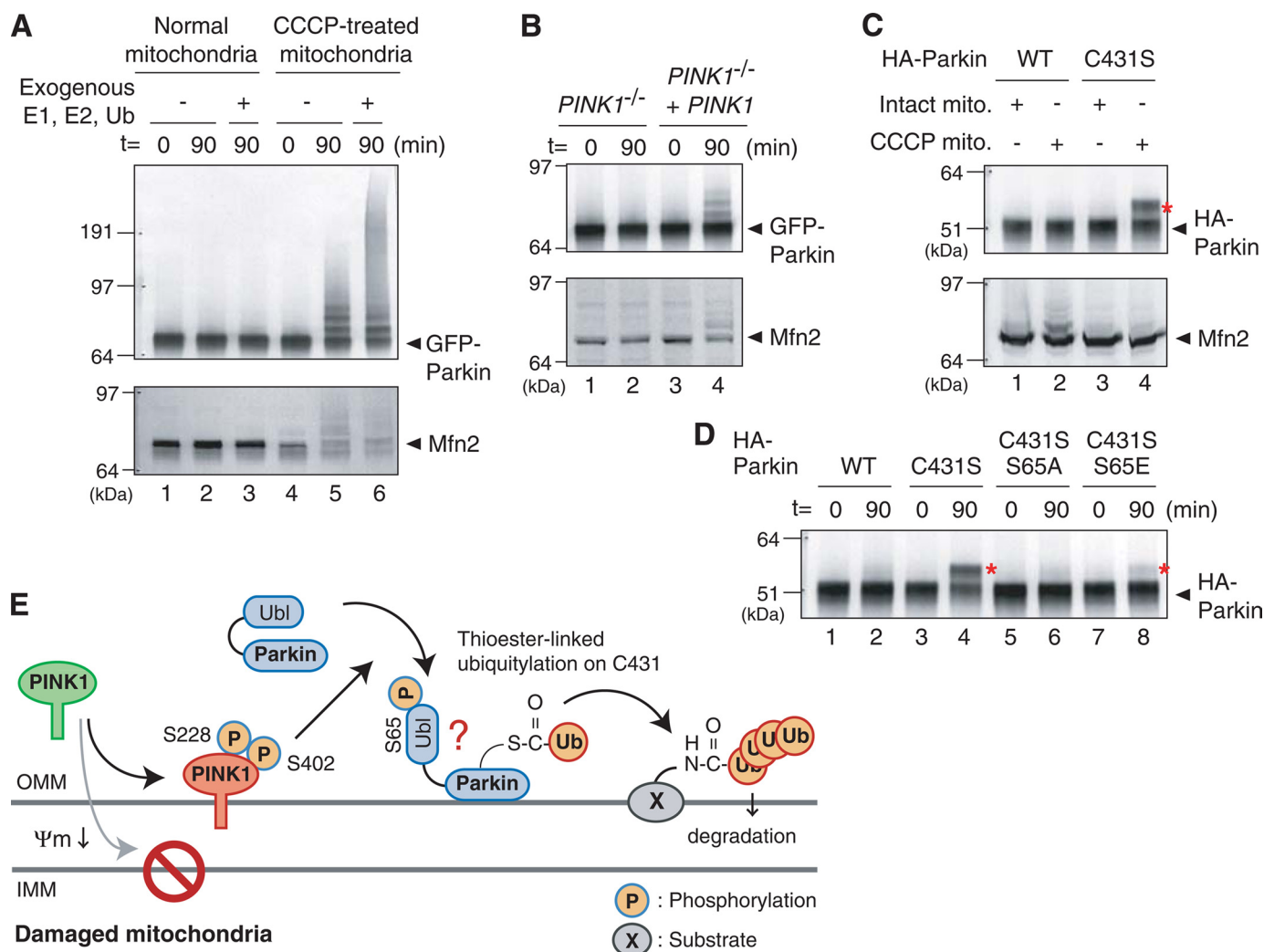


FIGURE 8. *A*, mitochondria collected from cells \pm CCCP pretreatment were incubated at 30 °C with cytosol expressing GFP-Parkin collected from cells with intact mitochondria. In this cell-free ubiquitylation assay, CCCP-treated mitochondria stimulate autoubiquitylation of GFP-Parkin and substrate ubiquitylation toward Mfn2. *B*, activation of GFP-Parkin by CCCP-pretreated mitochondria depends on PINK1. Mitochondria collected from *PINK1*^{-/-} MEFs following CCCP treatment do not activate GFP-Parkin in the cell-free ubiquitylation assay, whereas exogenous *PINK1* complements the aforementioned defect. *C*, formation of an apparent ubiquitin-oxyster adduct on the Parkin C431S mutant is dependent on the presence of CCCP-pretreated mitochondria in the cell-free assay. The red asterisks indicate ubiquitin-oxyster formation unless otherwise specified. *D*, ubiquitin-oxyster formation of Parkin harboring C431S, C431S/S65A, or C431S/S65E mutations in the cell-free ubiquitylation experiments. The S65A mutation disrupted ubiquitin-ester formation completely, whereas the C431S/S65E mutant has weak but detectable ubiquitin-oxyster formation. *E*, a model for Parkin activation on damaged mitochondria. See text for details.

thereby partially solving the aforementioned contradiction. However, even in that paper, reconstitution of ubiquitin-ester formation using recombinant Parkin protein was missing and the mechanism of how PINK1 regulates the ubiquitin-ester formation of Parkin was vague. To address this issue, we performed *in vitro* biochemical analyses using recombinant Parkin, and confirmed ubiquitin-oxyster formation of recombinant Parkin C431S mutant (Fig. 2) and an active site-directed ubiquitin probe (Ub-VS) targets Cys-431 of WT Parkin (Fig. 3).

Molecular Mechanism Underlying Parkin Catalysis of Ubiquitylation—We found in the current study that the IBR-RING2 domain of Parkin catalyzes *in vitro* ubiquitin-oxyster formation with ATP, ubiquitin, and E1, even in the absence of E2 (Fig. 2E). The ubiquitin-oxyster formation of IBR-RING2 was dependent on E1, suggesting that IBR-RING2 is unable to form the ubiquitin-ester bond *de novo* but is able to discharge the ubiquitin-thioester from E1 and then transfer it to Parkin

Cys-431. In the recently proposed model, the RING1 domain functions as an “ubiquitin-charged E2” binding domain. Because the IBR-RING2 domain functions in conjunction with the neighboring RING0 and RING1 domains in cells, experiments incorporating only IBR-RING2 might be artificial. Nevertheless, the aforementioned results clearly showed that the IBR-RING2 domain catalyzes transthiolation and/or acyl transfer rather than E2-recruitment. Another interesting result is that the IBR-RING2 domain catalyzes ubiquitylation irrespective of dissipation of $\Delta\Psi_m$ and mitochondrial localization in cells (Fig. 3, F–H), suggesting that IBR-RING2 is converted to a constitutively active form.

While this manuscript was in preparation, the Parkin structure was resolved (50). Interestingly, structural analysis revealed that RING2 domain topology is distinct from other typical RING fingers, consistent with our anticipation that the IBR-RING2 is not a conventional RING finger E2-recruitment

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domain (67). Moreover, the RING0 domain occludes the Cys-431 ubiquitin acceptor site in RING2, suggesting that deletion of the RING0 domain de-repressed the ester-transfer (trans-thioesterification) activity of the RING2 domain, and thus GFP-IBR-RING2 exhibited constitutive activity (Fig. 3, *G*, and *H*).

PINK1-dependent Phosphorylation of Parkin Ser-65 Is Important for Formation of the Ubiquitin-Thioester—Genetic analyses of PINK1 using *Drosophila melanogaster* mutants have shown that it acts as an upstream factor of Parkin (68–70). We and other groups have since reported that PINK1 is essential for translocation of Parkin to damaged mitochondria, revealing that PINK1 regulates the subcellular localization of Parkin (6, 7, 43, 55, 71). In addition, we have demonstrated that the E3 activity of Parkin is also up-regulated by a decrease in $\Delta\Psi_m$ and PINK1 (6), although the molecular details remain obscure.

We confirmed in this study that Parkin is phosphorylated at Ser-65 following a decrease in $\Delta\Psi_m$ as reported (56, 61), and showed that ubiquitin-thioester formation of Parkin is regulated by this PINK1-dependent phosphorylation event. Although the lack of a detectable ubiquitin adduct in the absence of CCCP (Fig. 7*B*, lanes 10 and 13) suggests that the putative phosphomimic mutations (S65D/S65E) do not bypass the decrease in $\Delta\Psi_m$ requirement for ubiquitin-oxyester formation, we speculate that Parkin is not a unique PINK1 substrate and that phosphorylation of other PINK1 substrate(s) is important for full Parkin activation. Alternatively, Glu-65 and Asp-65 might be incomplete mimics of phosphor-Ser-65. We showed that the ubiquitin-oxyester adduct was not formed on unphosphorylated Parkin (Fig. 4*A*) and that the phosphorylation-deficient S65A mutation inhibited ubiquitin-oxyester formation of Parkin (Figs. 7*B* and 8*D*). To our knowledge, this is the first report that connects PINK1-dependent phosphorylation and ubiquitin-ester formation of Parkin.

Conclusion—In this study, we have shown that PINK1-dependent phosphorylation of Parkin Ser-65 plays an important role in ubiquitin-ester formation on Cys-431 following a decrease in $\Delta\Psi_m$. Moreover, biochemical analyses indicate that RING2 is not an E2-recruiting domain but is rather an ubiquitin-ester-transferring domain. Although other mode(s) of activation (e.g. Ser-65 phosphorylation strengthens the interaction of Parkin with mitochondria) cannot be ruled out completely, our model for Parkin activation is that phosphorylation of Ser-65 in the Parkin Ubl domain by PINK1 de-represses its autoinhibitory activity. This allows the RING2 domain to transfer the ubiquitin-thioester to Cys-431 and thus catalyze substrate ubiquitylation (depicted in Fig. 8*E*). The results presented in this work provide insights into the molecular details of Parkin activation, and are potentially beneficial for disease treatment by ameliorating the E3 activity of Parkin.

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Note Added in Proof—While our manuscript was under review, four groups independently published results that are related to those described herein (72–75).

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