PINK1 drives Parkin self-association and HECT-like E3 activity upstream of mitochondrial binding

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Genetic studies indicate that the mitochondrial kinase PINK1 and the RING-between-RING E3 ubiquitin ligase Parkin function in the same pathway. In concurrence, mechanistic studies show that PINK1 can recruit Parkin from the cytosol to the mitochondria, increase the ubiquitination activity of Parkin, and induce Parkin-mediated mitophagy. Here, we used a cell-free assay to recapitulate PINK1-dependent activation of Parkin ubiquitination of a validated mitochondrial substrate, mitofusin 1. We show that PINK1 activated the formation of a Parkin–ubiquitin thioester intermediate, a hallmark of HECT E3 ligases, both in vitro and in vivo. Parkin HECT-like ubiquitin ligase activity was essential for PINK1-mediated Parkin translocation to mitochondria and mitophagy. Using an inactive Parkin mutant, we found that PINK1 stimulated Parkin self-association and complex formation upstream of mitochondrial translocation. Self-association occurred independent of ubiquitination activity through the RING-between-RING domain, providing mechanistic insight into how PINK1 activates Parkin.

Introduction

Mutations in the E3 ligase Parkin and the mitochondrial kinase PINK1 can cause familial Parkinson's disease (Kitada et al., 1998; Valente et al., 2004). Parkin functions downstream of PINK1 in the same pathway (Clark et al., 2006; Park et al., 2006; Yang et al., 2006) to ubiquitinate mitochondrial outer membrane proteins and induce autophagy of damaged mitochondria (Narendra et al., 2008). How PINK1 activates Parkin and how Parkin is recruited to mitochondria remain unclear. PINK1 is imported into mitochondria, cleaved by PARL in the inner mitochondrial membrane (IMM), then degraded to restrict its expression (Whitworth et al., 2008; Jin et al., 2010; Deas et al., 2011; Meissner et al., 2011; Greene et al., 2012). However, when mitochondria lose membrane potential, protein import into the IMM is prevented, diverting PINK1 away from PARL to accumulate on the outer mitochondrial membrane (OMM) bound to the TOM complex (Lazarou et al., 2012). On the OMM, PINK1 recruits Parkin to mitochondria via its kinase activity (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010). PINK1 not only recruits Parkin to mitochondria, it also induces Parkin ubiquitin ligase activity (Matsuda et al., 2010; Tanaka et al., 2010). Although many OMM proteins are found to be ubiquitinated in cells overexpressing Parkin (Chan et al., 2011; Yoshii et al., 2011), Mitofusin 1 and 2 (Mfn1 and Mfn2) appear to be among the most susceptible mitochondrial substrates and are ubiquitinated by endogenous Parkin (Gegg et al., 2010; Poole et al., 2010; Tanaka et al., 2010; Ziviani et al., 2010).

Parkin contains a ubiquitin-like (UBL) domain at the N terminus and a RING-between-RING (RBR) domain at the C terminus. Recent evidence indicates that the UBL domain of Parkin inhibits the RBR domain (Chaugule et al., 2011). Like HECT domain E3 ligases, RBR E3 ligases HHARI and HOIP have been recently shown to form a thioester linkage with ubiquitin, differentiating them from classical RING domain ligases that, without forming a covalent ubiquitin intermediate, juxtapose E2 enzymes with their substrates (Wenzel et al., 2011; Stieglitz et al., 2012). Parkin has a cysteine at a conserved position with the active site cysteine in HHARI that forms a thioester linkage with ubiquitin. This cysteine at position 431 is mutated in some cases of Parkinson's disease (Nuytemans et al., 2010) and is required for Parkin E3 ligase activity with the HECT-specific E2 UBE2L3, suggesting that Parkin also utilizes a HECT-like

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Abbreviations used in this paper: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DTT, DL-Dithiothreitol; FCS, fluorescence correlation spectroscopy; IP, immunoprecipitation; Mfn, mitofusin; RBR, RING-between-RING; UBL, ubiquitin-like; WT, wild type.

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thioester intermediate for ubiquitin transfer to substrates (Wenzel et al., 2011).

We developed a cell-free assay that recapitulates PINK1-dependent activation of Parkin E3 ligase activity against the established substrate Mfn1. This assay reveals that PINK1 activates a latent HECT-like activity of Parkin that is required for translocation of Parkin to depolarized mitochondria and induction of mitophagy. In addition, PINK1 induces Parkin to self-associate involving the RBR domain, which may mediate Parkin enzyme activation.

Results and discussion

To reconstitute Parkin activation in a cell-free system, we incubated cytosol isolated from HeLa cells ectopically expressing untagged Parkin with mitochondria isolated from HeLa cells not expressing Parkin but stably expressing PINK1-V5/His. Mitochondria were isolated from cells that were treated for 3 h with DMSO as a control or the uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to stabilize PINK1 on the outer membrane. A time-dependent increase in higher molecular weight bands of the Parkin substrate Mfn1 occurred only in the presence of mitochondria isolated from cells pretreated with CCCP that display stabilized PINK1 (Fig. 1 A). These higher molecular weight bands represent polyubiquitin chains based on Mfn1-denaturing immunoprecipitation (IP) and Western blotting using the polyubiquitin antibody FK1 (Fig. 1 A, right). Mfn1 ubiquitination was not detectable when the assay included mitochondria isolated from PINK1 siRNA cells (Fig. 1 B, lanes 1 and 2; Fig. S1 A). Rescue of PINK1 expression by transfection with PINK1 resistant to siRNA restored Mfn1 ubiquitination (Fig. 1 B, lanes 3 and 4), whereas siRNA-resistant kinase-dead PINK1 did not (Fig. 1 B, lanes 5 and 6). We confirmed that endogenous PINK1 activates Mfn1 ubiquitination by Parkin (Fig. S1 B), although a slightly higher amount of mitochondrial protein (50 µg) was required to produce similar activity as mitochondria from PINK1-V5/His HeLa (35 µg). To assess if the PINK1-dependent Mfn1 ubiquitination requires Parkin activity, we used cytosol from untransfected HeLa cells that lack endogenous Parkin expression (Denison et al., 2003). No polyubiquitination of Mfn1 was observed after a 120-min incubation of cytosol lacking Parkin with mitochondria (Fig. 1 A, lanes 15 and 16). Thus, this cell-free assay of Mfn1 ubiquitination depends on the presence of Parkin in the cytosol as well as PINK1 kinase activity.

Previous studies have identified several putative substrates of Parkin owing to their decreased levels in CCCP-treated cells expressing Parkin (Chan et al., 2011; Yoshii et al., 2011; Narendra et al., 2012). We analyzed a selection of these proteins located on the OMM for PINK1/Parkin-stimulated ubiquitination using the cell-free assay and observed varying levels of apparent ubiquitination (Fig. S1 C). One of the substrates, MitoNEET, contains a labile [2Fe-2S] cluster and was originally identified as a target of the type II diabetes drug pioglitazone (Colca et al., 2004). Recently, MitoNEET was found to play a regulatory role in β-oxidation and membrane potential by inhibiting iron transport into the matrix (Kusminski et al., 2012), making it an important putative substrate. We assessed Parkin's direct activity as well as the chain linkage formation on both MitoNEET and Mfn1 by using recombinant Parkin in the absence of cytosol and various E2s. In the presence of purified E1, ubiquitin, select E2 enzymes, and mitochondria with or without CCCP-stabilized PINK1, recombinant Parkin mediated Mfn1 and MitoNEET ubiquitination using the E2s UBE2E1 and UBE2L3, and to a lesser extent UBE2K (Fig. 1 C). MitoNEET ubiquitination was confirmed by denaturing IP and Western blotting for polyubiquitin (Fig. 1 D). IP with linkage-specific antibodies K48 and K63 revealed that Parkin activity in the presence of the HECT-specific E2 UBE2L3 (Wenzel et al., 2011) supports both K48 and K63 linkages on Mfn1 and MitoNEET (Fig. 1, D and E). Our results confirm MiotNEET as a direct substrate of Parkin and identify UBE2E1 as an additional E2 used by Parkin.

RBR E3 ligases HHARI and HOIP have been shown to form a HECT-like thioester intermediate with ubiquitin on a conserved cysteine in RING2, distinguishing them from canonical RING E3s (Wenzel et al., 2011; Stieglitz et al., 2012). Parkin shares a conserved cysteine in RING2 that is essential for PINK1-independent Parkin E3 ligase activity in vitro (Wenzel et al., 2011). As the Parkin thioester linkage was not detected using the C431S Parkin mutant to trap an oxyester intermediate in the absence of PINK1 (Wenzel et al., 2011), we asked if PINK1 would activate Parkin–ubiquitin oxyester formation. We mutated Parkin C431 to serine and performed the PINK1-dependent cell-free ubiquitination assay (Fig. 2 A). In contrast to wild-type (WT) Parkin, C431S Parkin fails to polyubiquitinate Mfn1, although a prominent mono-ubiquitin band of Mfn1 is seen to increase upon incubation with CCCP-treated mitochondria. Parkin itself forms a prominent mono-ubiquitinated band that is dependent on CCCP (Fig. 2 A). This ubiquitin linkage on WT Parkin is resistant to NaOH treatment, indicating that it is not a thioester (or oxyester) intermediate but an amide linkage between the ubiquitin C-terminal glycine carboxyl group and an amino group of the substrate. Although C431S Parkin fails to polyubiquitinate Mfn1, it still becomes monoubiquitinated (Fig. 2 A, lane 6). In contrast to WT Parkin, the monoubiquitinated form of C431S Parkin is cleaved by NaOH treatment (Fig. 2 A, lane 8), indicating that C431S forms an oxyester intermediate when serine is substituted for the native cysteine, as does HHARI and HOIP (Wenzel et al., 2011; Stieglitz et al., 2012). This covalent intermediate of Parkin is not detectable in the absence of CCCP, indicating that PINK1 induces the HECT-like activity of Parkin (Fig. 2 A, lane 5).

We compared a number of E2s for promoting the HECT-like activity of Parkin by assessing oxyester intermediate conjugation using cytosol containing C431S Parkin that was gel filtered to remove endogenous E2s. UBE2D1, UBE2D2, UBE2D3, UBE2E1, UBE2L3, and UBE2C support HECT-like activity of Parkin in a CCCP-dependent fashion (Fig. 2 B) that is sensitive to NaOH incubation (Fig. 2 C).

We examined whether loss of HECT-like activity by mutation of C431 would affect Parkin translocation to depolarized mitochondria in cultured cells. Mutation of C431 to phenylalanine, a mutation linked to parkinsonism (Nuytemans et al., 2010), or serine prevents Parkin translocation to mitochondria (Fig. 3 A) and prevents Parkin-mediated mitophagy (Fig. 3 B).

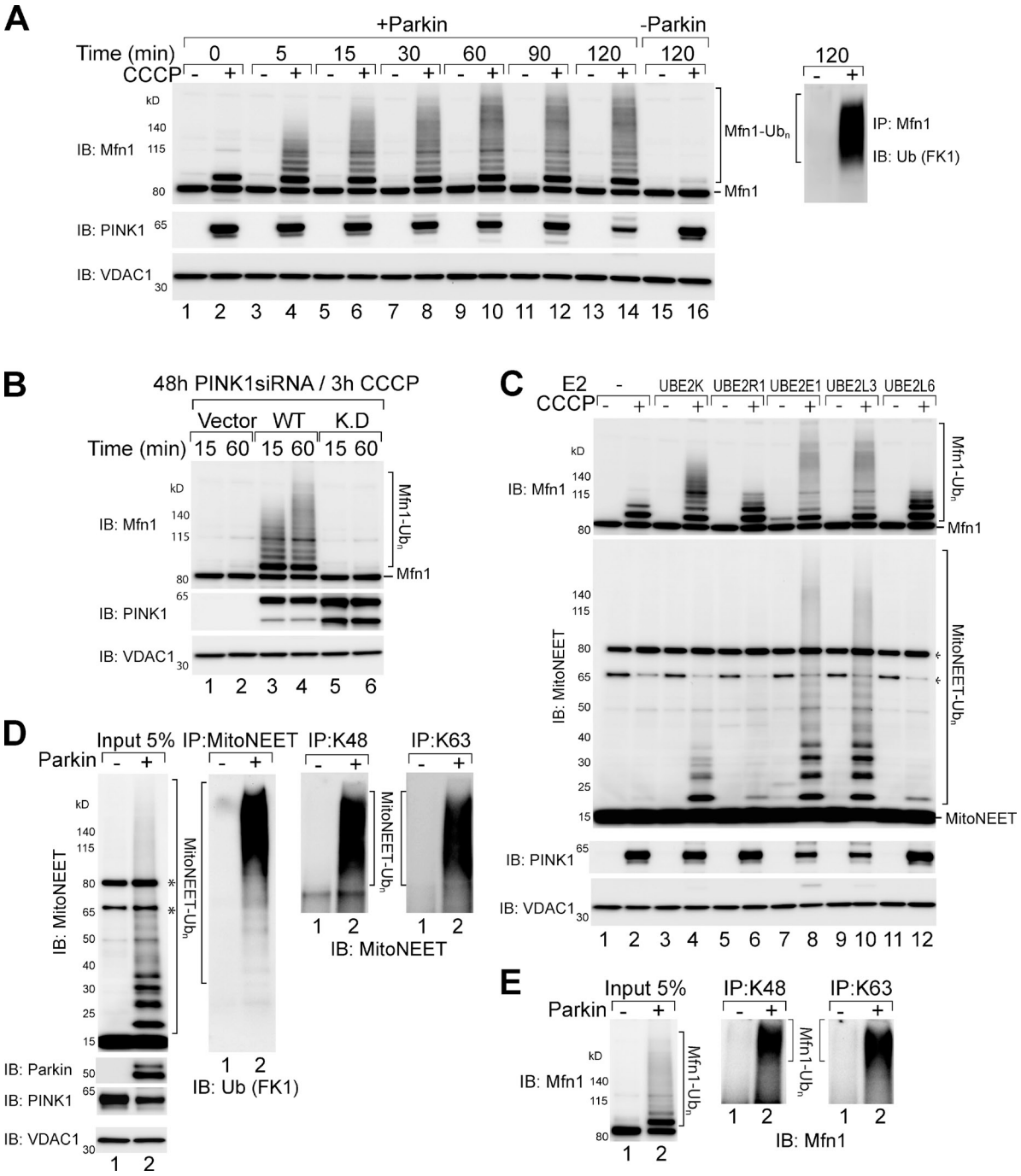

Figure 1. **Cell-free PINK1/Parkin ubiquitination assay.** (A) Mitochondria with or without CCCP-stabilized PINK1-V5/His were incubated for increasing times at 30°C with HeLa cytosol with or without ectopic Parkin. Mitochondria were analyzed by immunoblotting using antibodies as indicated. A fraction of the 120-min time point was immunoprecipitated with Mfn1 antibodies and blotted for polyubiquitin with anti-FK1. (B) HeLa cells were transfected with PINK1 siRNA for 24 h before transfection with empty vector or PINK1 siRNA-resistant constructs for 24 h as indicated. Cells were treated with 10 µM CCCP for 3 h before mitochondrial isolation and incubation with Parkin cytosol as in A. (C) Mitochondria with or without stabilized PINK1-V5/His were incubated for 60 min at 30°C with 100 nM Parkin, 100 nM E1, 2 µM ubiquitin, and 100 nM of various E2 enzymes as indicated. Mitochondria were analyzed by immunoblotting using antibodies as indicated. (D and E) PINK1-V5/His-stabilized mitochondria were incubated with ubiquitin conjugation components as in C in the presence or absence of 100 nM Parkin for 60 min at 30°C followed by IP using either MitoNEET, K48, or K63 antibodies. 5% of the input and IP eluates were analyzed by immunoblotting using antibodies as indicated. *, nonspecific band.

To test if Parkin HECT-like activity is activated by PINK1 in vivo, untagged C431S Parkin or C431F Parkin were expressed in cells treated with or without CCCP. Only in the presence of CCCP was a prominent higher molecular weight band of C431S Parkin (Fig. 3 C, lane 4) detected. Parkin C431F, which cannot form an oxyester linkage, failed to form the higher molecular weight band after CCCP treatment (Fig. 3 C, lane 2). This higher band of C431S Parkin was labile to NaOH treatment, indicating that the stabilized ubiquitin oxyester enzyme intermediate observed in the cell-free assay (Fig. 2 A) occurs in cells (Fig. 3 C).

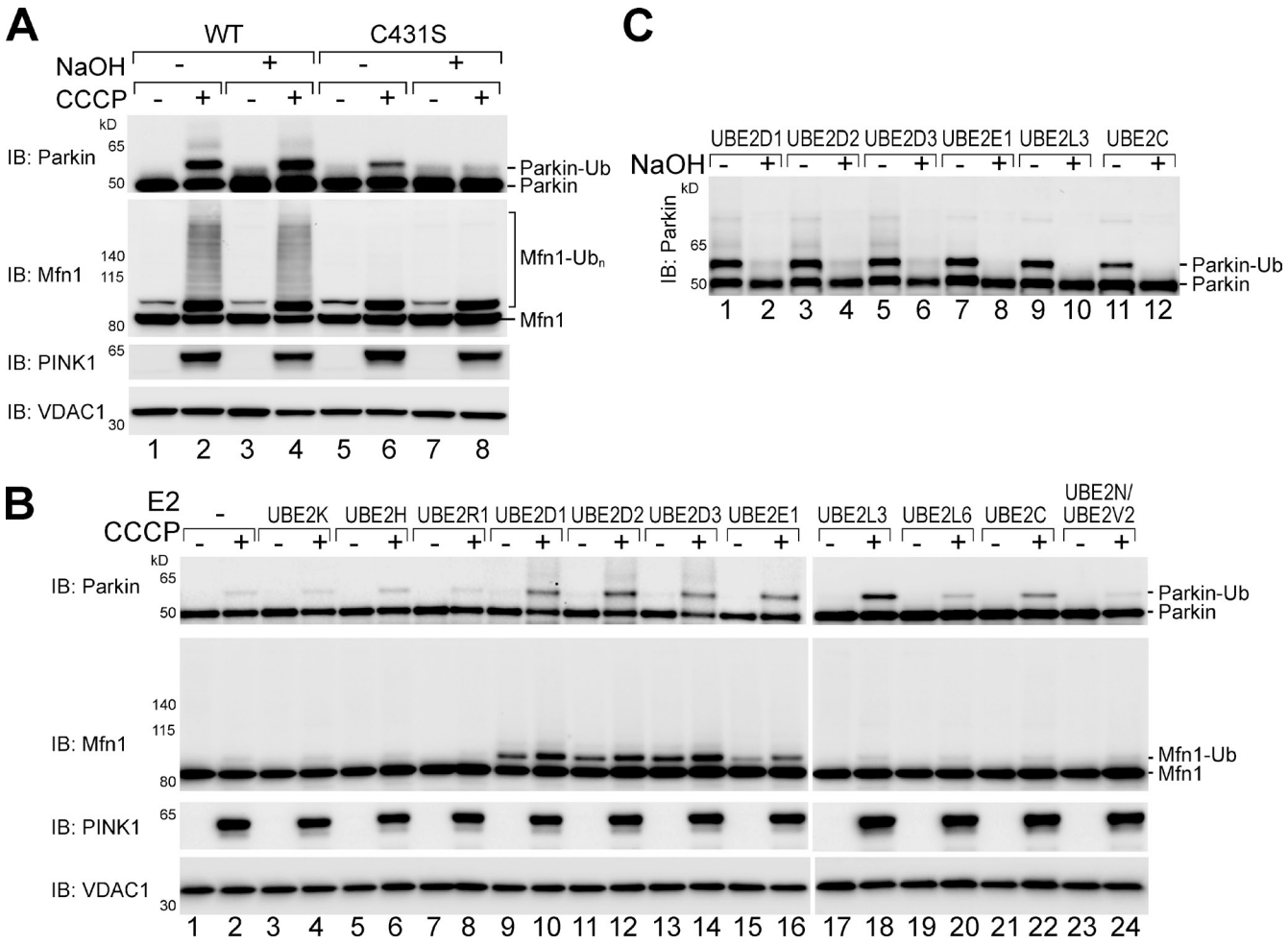

Figure 2. **C431S Parkin oxyester analysis.** (A) Mitochondria with or without stabilized PINK1-V5/His were incubated for 60 min at 30°C with HeLa cytosol expressing either WT or C431S Parkin. Total fractions were heat denatured using 1% SDS at 90°C and then split and treated with or without 100 mM NaOH at 37°C for 1 h. Samples were immunoblotted using various antibodies as indicated. (B) Gel-filtered C431S Parkin cytosol was incubated with mitochondria as in A with or without E2 enzymes (100 nM), and total lysates were subjected to immunoblotting using antibodies as indicated. (C) Mitochondria and C431S Parkin cytosol were incubated with E2s as in B and NaOH treatment was performed as in A.

C431S Parkin oxyester formation was not observed after PINK1 siRNA (Fig. 3 C, lane 8), indicating that PINK1 activates a latent HECT-like ubiquitin ligase activity in Parkin.

Previously examined Parkin mutations that occur in PD patients display a range of mitochondrial translocation activities but most fail to induce mitophagy (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010). R275W Parkin, mutated in the RING1 domain, is recruited to mitochondria by PINK1 but fails to induce mitophagy (Geisler et al., 2010; Narendra et al., 2010). Mutations in the Parkin UBL domain display an intermediate translocation phenotype, with a delay or defect in translocation but not a complete block and, like R275W Parkin, fail to induce mitophagy. Although other mutations in Parkin, such as C289G in RING1 and C441R in RING2, completely fail to translocate to depolarized mitochondria, these mutations in zinc-coordinating cysteines appear to cause folding defects (Hampe et al., 2006). Parkin C431S attaches ubiquitin to the active site serine to form an oxyester intermediate, indicating that C431S Parkin is functionally folded. However, C431S Parkin fails to polyubiquitinate the mitochondrial substrate Mfn1 and fails to induce mitophagy, indicating that HECT-like ubiquitination by Parkin is essential for these processes. Surprisingly, C431S Parkin also fails to translocate to depolarized mitochondria, suggesting that HECT-like ubiquitin ligase activity of Parkin is required for the mitochondrial translocation step apparently upstream of the lesion observed with other mutations such as R275W. Our results indicate that Parkin's HECT-like activity is latent in cells until activated by PINK1. Furthermore, given that PINK1 can activate Parkin without obvious mitochondrial translocation, one can mechanistically separate Parkin E3 activation and translocation into two distinct steps.

As Parkin C431S can still be activated by PINK1, we asked whether the R275W Parkin mutant in RING1 could complement C431S and C431F Parkin in trans to rescue their translocation defect and enzymatic function. We assessed the mitochondrial translocation activity of untagged R275W Parkin alone and found that it translocated in ∼60% of cells that were treated with CCCP for 6 h (Fig. S2 A). Little to no translocation of C431S and C431F YFP–Parkin was observed under the same condition (Fig. 4, A [top half] and B). However, when cotransfected with untagged R275W Parkin, both C431S and C431F YFP–Parkin efficiently translocated to mitochondria (Fig. 4, A [bottom half] and B) to an

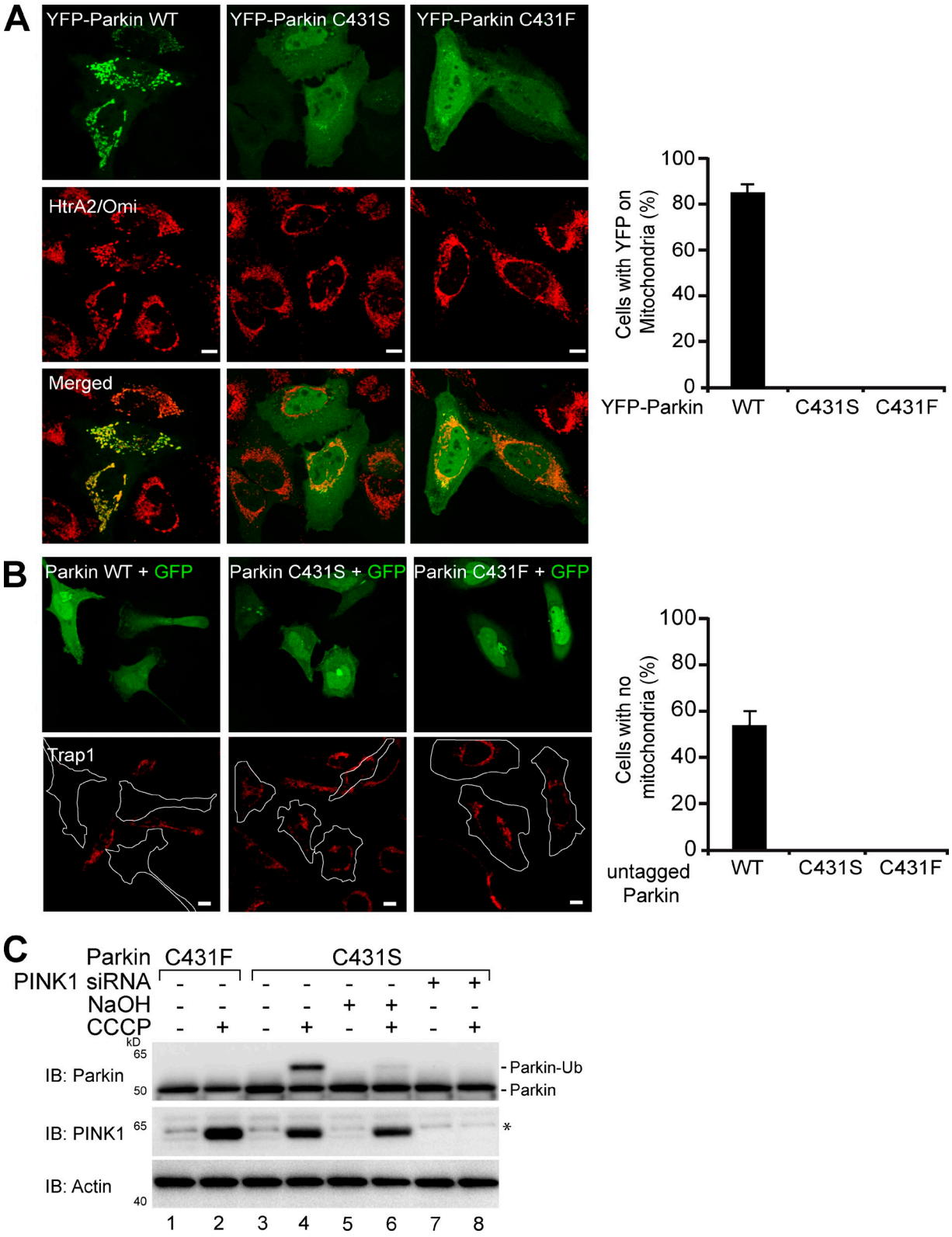

Figure 3. **C431S Parkin activity in cells.** (A) HeLa cells expressing either WT, C431S, or C431F YFP–Parkin were treated with 10 µM CCCP for 3 h. Cells were immunostained using antibodies against the mitochondrial marker HtrA2/Omi. The graph represents means ± SD of counts in >150 cells per sample in three independent experiments of YFP–Parkin colocalizing with mitochondria. Bars, 10 µm. (B) HeLa cells were cotransfected with untagged Parkin variants as in A and EGFP-N1 in a 3:1 ratio. Cells were treated with 10 µM CCCP for 24 h and immunostained for the matrix protein Trap1. The graph represents scoring of cells positive for GFP and negative for mitochondria by lack of Trap1 signal. More than 150 cells were counted per sample in three replicates examined on at least two separate occasions. Error bars represent means ± SD. Bars, 10 µm. (C) HeLa cells were transfected with or without PINK1 siRNA for 24 h before transfection with untagged C431F or C431S Parkin for 24 h followed by treatment with DMSO or 10 µM CCCP for 3 h. Cytosol fractions were heat denatured with 1% SDS at 90°C and then split and treated with or without 100 mM NaOH at 37°C for 1 h. Samples were analyzed by immunoblotting using Parkin, PINK1, and β-actin antibodies. *, nonspecific band.

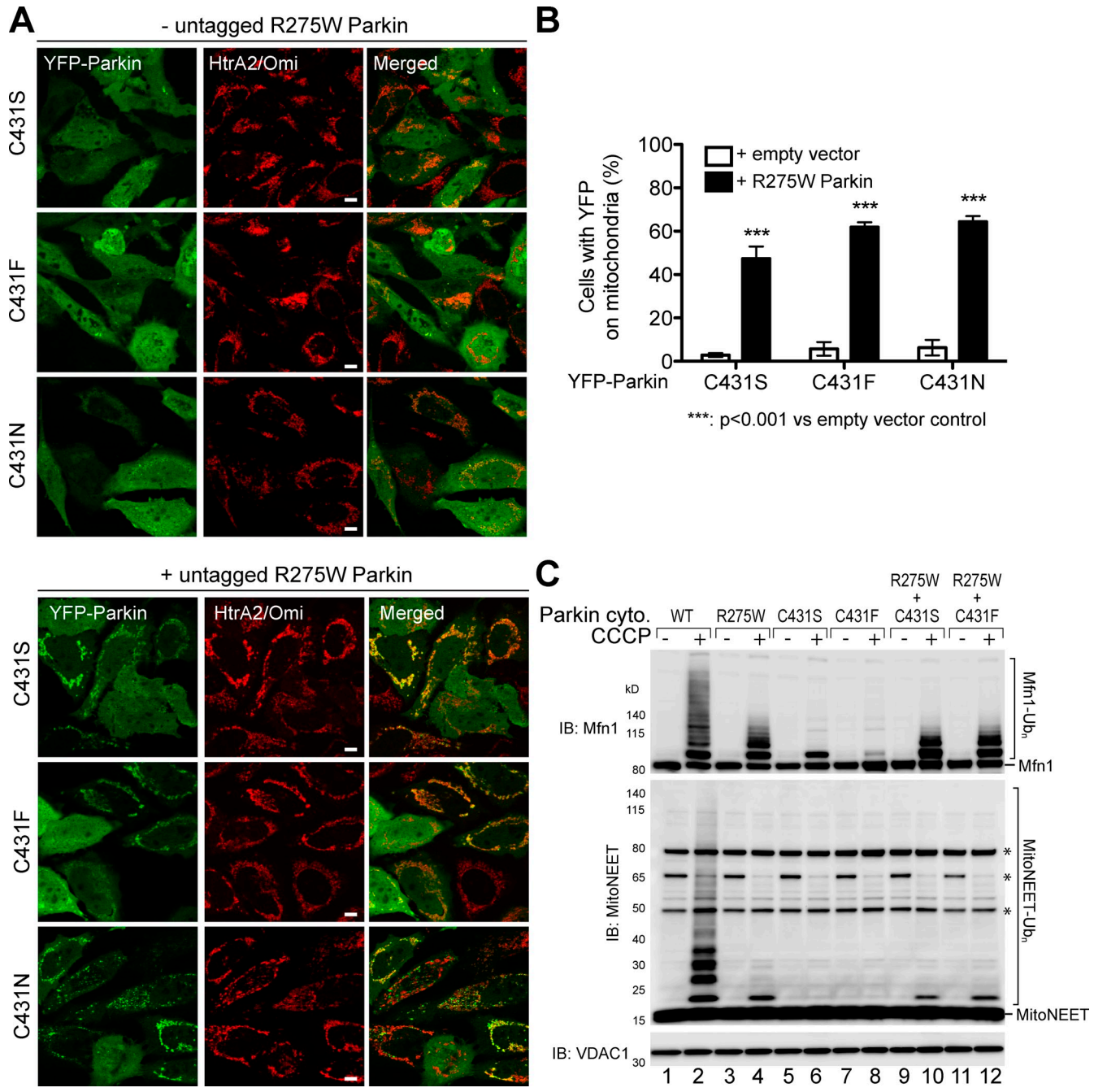

Figure 4. **Parkin mutant complementation analysis.** (A) HeLa cells were transfected with either a YFP–Parkin C431 mutant construct alone (top panels) or with untagged R275W Parkin (bottom panels) for 24 h as indicated, followed by treatment with 10 µM CCCP for 6 h. Cells were fixed and immunostained with the mitochondrial-specific marker HtrA2/Omi. Bars, 10 µm. (B) Cells from A were counted for YFP–Parkin colocalizing with mitochondria. The graph represents means ± SD of counts in >150 cells per sample in three independent experiments and analyzed with one-way ANOVA. (C) Mitochondria with or without stabilized PINK1-V5/His were incubated for 60 min at 30°C with cytosol expressing various untagged Parkin constructs as indicated. Mitochondria were analyzed by immunoblotting using antibodies against Mfn1, MitoNEET, and VDAC1. *, nonspecific band.

extent similar to that of R275W Parkin alone (Fig. S2 A). Using the cell-free Parkin assay, we analyzed C431S and C431F Parkin ubiquitination activity toward Mfn1 and MitoNEET in the presence or absence of R275W Parkin. Although R275W Parkin induced some minor ubiquitination of Mfn1 and MitoNEET (Fig. 4 C, lane 4), we observed no trans complementation of E3 enzyme activity when R275W was mixed with C431S or C431F Parkin (Fig. 4 C, lanes 10 and 12). This raised the question of how R275W Parkin rescued the translocation defect of the C431 Parkin mutants. We generated a mutant C431N YFP–Parkin that is more conservative than C431F yet does not form oxyester bonds as does C431S, and found a similar mitochondrial translocation complementation efficiency with untagged R275W Parkin as seen with C431F and C431S Parkin (Fig. 4, A and B).

We hypothesized that mitochondrial translocation rescue of C431S, C431F, and YFP–Parkin C431N by R275W Parkin

may occur through Parkin self-association, allowing the C431 mutants to bind to and track with R275W Parkin to mitochondria. We tested this model by tethering mCherry-FKBP-Parkin C431N onto mitochondria via FRB-Fis1 using an inducible heterodimerization system as described previously (Lazarou et al., 2012). Inactive mCherry-FKBP-Parkin C431N was tethered to mitochondria after rapalog-induced heterodimerization of the FKBP domain to the FRB domain localized to mitochondria by the Fis1 membrane anchor. Under this condition YFP–Parkin C431N remained in the cytosol (Fig. 5 A, top row). However, after PINK1 stabilization by CCCP treatment, YFP–Parkin C431N efficiently translocated to mitochondria in cells where mCherry-FKBP-Parkin C431N also localized on mitochondria (Fig. 5 A, bottom row), supporting the self-association hypothesis. YFP–Parkin C431N mitochondrial translocation was not observed in rapalog- and CCCP-treated cells subjected to PINK1 siRNA, confirming that PINK1 drives Parkin self-association (Fig. S2 B). WT-Parkin tethered to mitochondria also recruited C431N Parkin to mitochondria only after CCCP treatment (Fig. S2 C). Further analysis to identify the domains of Parkin that contribute to self-association revealed that the RBR domain bound to mitochondria is required and sufficient to recruit C431N Parkin to mitochondria upon PINK1 stabilization (Fig. S3, A and B).

Parkin self-association would suggest a change in its molecular mass after activation by PINK1. Given that C431N YFP–Parkin remains diffuse in the cytosol after CCCP treatment (Figs. 3 and 4), we used fluorescence correlation spectroscopy (FCS) to assess complex formation in live cells (Fig. 5 B, left graph). FCS measurements were collected in cytosolic regions of cells expressing C431N YFP–Parkin. Cells were then treated with 10 µM CCCP for 6 h and FCS data were collected on the same cells. In untreated cells, the mean diffusion coefficient of C431N YFP–Parkin was 15.8 ± 0.82 $\mu m^2/s$, consistent with a relative molecular mass estimate in the monomeric range (~80 kD). PINK1 stabilization by CCCP resulted in a reduction in the mean diffusion coefficient of C431N YFP–Parkin to 7.5 ± 0.51 $\mu m^2/s$. Assuming a globular structure, the observed ~2.1-fold reduction in the diffusion coefficient indicates that after CCCP treatment, C431N YFP–Parkin forms a complex at least sixfold larger upstream of mitochondrial translocation and independent of HECT-like E3 activity. Consistent with previous reports using a GFP variant (Wang et al., 2004), the diffusion coefficient of YFP alone in cells was 22.5 ± 0.78 $\mu m^2/s$ (Fig. 5 B, right graph) and did not significantly change after CCCP treatment, confirming that CCCP does not detectably alter the intracellular milieu. Thus, formation of a higher molecular weight C431N YFP–Parkin complex is consistent with the self-association observed by co-mitochondrial recruitment (Figs. 4 and 5 A), although the large increase in size suggests other factors such as E2s may also be bound.

Previous reports have shown that RING or U-box domain-containing E3s can form homodimers that are required for their E3 ligase activity (Vander Kooi et al., 2006; Yin et al., 2009), and certain RING domains have been shown to self-assemble into supramolecular structures (Kentsis et al., 2002). Because the HECT-like activity of Parkin is prevented by mutation at C431, it follows that PINK1 drives self-association of Parkin upstream of HECT-like activity. This suggests that Parkin self-association induced by PINK1 may mediate activation of its ubiquitin ligase activity and subsequent mitochondrial translocation. Thus, our study supports a model where Parkin is locally activated by PINK1. PINK1 kinase activity induces Parkin self-association, potentially through phosphorylation at S65 (Kondapalli et al., 2012) and subsequent ubiquitination activity through RING2, followed by attachment to substrates on mitochondria (Fig. 5 C). In the absence of HECT-like activity, Parkin can still be activated by PINK1 to self-associate and to form an oxyester ubiquitin enzyme intermediate but, without ubiquitination activity, does not stably attach to mitochondria.

Materials and methods

Cell culture, immunocytochemistry, and confocal microscopy

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% (vol/vol) FCS at 37°C under an atmosphere of 5% CO_2. Cells transfected for confocal microscopy were plated on LabTek 1.5 borosilicate chambered slides (Thermo Fisher Scientific). Constructs, as indicated in figure legends, were mixed with Fugene 6 at a 1:6 ratio in Opti-MEM (Gibco). After 15 min, the mixture was added to the culture and incubated for 24 or 48 h before 10 µM CCCP treatment for 1 h to assess Parkin translocation or 24 h to assess mitophagy. For immunostaining, cells were fixed with 4% (wt/vol) paraformaldehyde in PBS, permeabilized with 0.25% (vol/vol) Triton X-100 in PBS, and blocked with 10% (wt/vol) BSA for 30 min. Cells were incubated with primary antibodies as indicated followed by incubation with anti–rabbit or anti–mouse Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647 (Invitrogen) secondary antibodies. The fixed, immunolabeled cells were imaged in PBS at room temperature using an inverted confocal microscope (LSM510 Meta; Carl Zeiss) with a 63× 1.4 NA oil immersion Plan-Apo objective.

PINK1 siRNA

To generate siRNA-resistant WT PINK1 and kinase-dead PINK1 (Beilina et al., 2005), point mutations in the siRNA-targeted region were introduced using overlap PCR. The PINK1 sequence targeted by siRNA, 5′-CCTCGTTATGAAGAACTAT-3′, was mutated to 5′-CCTGGTAATGAAAAATTAT-3′. HeLa cells were transfected for 48 h with either PINK1 siRNA or non-targeting siRNA (Ambion) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturers' instructions. For introduction of siRNA-resistant WT PINK1 or kinase-dead PINK1, HeLa cells were transfected with PINK1 siRNA for 24 h before further transfection with PINK1 siRNA-resistant constructs for 24 h followed by treatment with 10 µM CCCP for 3 h.

Cell fractionation

To obtain cytosol fractions, HeLa cells that were either untransfected or transfected with Parkin or C431S Parkin as indicated in figure legends were homogenized in Solution B (20 mM Hepes-KOH, pH 7.6, 220 mM mannitol, 70 mM sucrose, and 10 mM KAc) supplemented with complete protease inhibitor cocktail minus EDTA (Roche). Cell homogenates were centrifuged at 800 *g* for 10 min at 4°C to obtain a post-nuclear supernatant and then cytosol fractions were obtained by further centrifugation at 100,000 *g* for 30 min at 4°C. Cytosol fraction protein concentrations were typically 2.5 mg/ml. Cytosol samples subjected to gel filtration before use in ubiquitination assays were separated using a Superdex 200 10/30 column (GE Healthcare) and fractions 27–30 were collected, pooled, and concentrated back to the original volume injected onto the gel filtration column.

For mitochondrial isolation, HeLa cells either expressing endogenous PINK1 or stably expressing PINK1-V5/His were treated with either DMSO vehicle control or 10 µM CCCP for 3 h followed by homogenization in Solution B. Post-nuclear supernatants were obtained by centrifugation as above and mitochondria were pelleted by further centrifugation at 10,000 *g* for 20 min at 4°C.

Cell-free ubiquitination assays

HeLa cytosols with or without ectopic Parkin were supplemented with 1 mM DL-Dithiothreitol (DTT) and ATP-regenerating buffer from a 10× stock solution (20 mM Hepes-KOH, pH 7.6, 10 mM ATP, 300 mM phosphocreatine, 10 mM $MgCl_2$, 10% glycerol, and 1.5 mg/ml creatine phosphokinase). Mitochondria with or without stabilized PINK1 isolated from HeLa cells (50 µg) or PINK1-V5/His HeLa cells (35 µg), were resuspended in 10 µl of

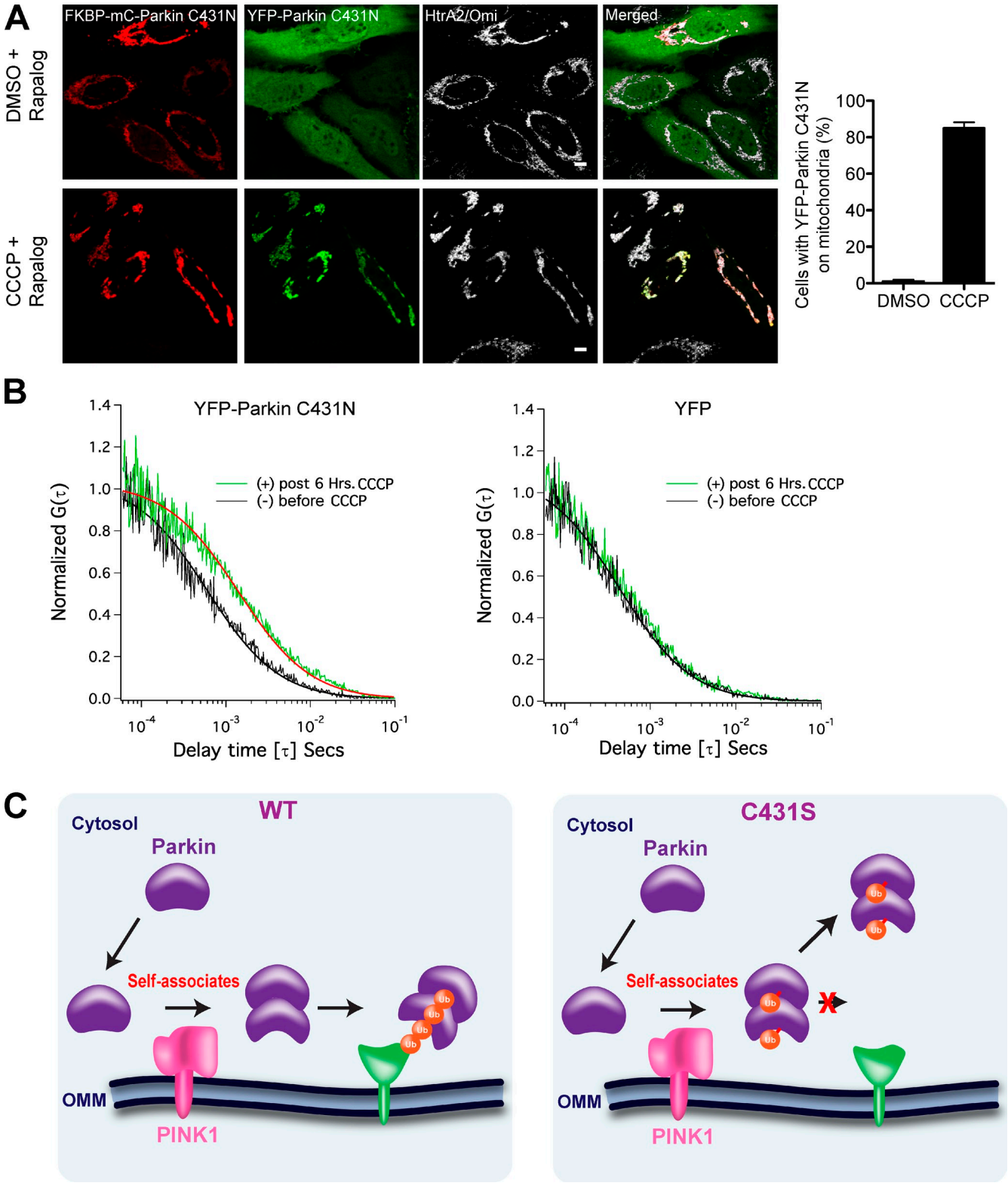

Figure 5. **Parkin self-association.** (A) HeLa cells expressing FRB-Fis1, C431N FKBP-mCherry-Parkin, and C431N YFP–Parkin were treated with 500 nM rapalog for 1 h followed by the addition of either DMSO or 10 µM CCCP for a further 6 h. Cells were fixed and immunostained with the mitochondrial-specific marker HtrA2/Omi. Cells were counted for C431N YFP–Parkin colocalizing with both mitochondria and C431N FKBP-mCherry-Parkin. The graph represents means ± SD of counts in >150 cells per sample in three independent experiments. Bar, 10 µm. (B) Normalized autocorrelation curves for cytosolic C431N YFP–Parkin (left) and YFP alone (right) before and after 10 µM CCCP treatment for 6 h. The solid lines are fits of the dataset to a single species diffusion model. FCS measurements were repeated in six cells in three independent experiments. (C) Model of PINK1 activation of Parkin and mitochondrial translocation.

energized cytosol and incubated at 30°C for various times as indicated in the figure legends. After ubiquitination, the reactions were stopped with 10 µl of 2× LDS (Invitrogen) supplemented with 200 mM DTT. For mitochondrial re-isolation, samples were subjected to centrifugation at 16,000 *g* for 5 min and mitochondrial pellets were then resuspended in 1× LDS (Invitrogen) containing 100 mM DTT.

For in vitro assays using recombinant Parkin, 100 nM ubiquitin-activating enzyme 1 (E1), 125 nM of E2 (as indicated in the figures), 2 µM ubiquitin, and 100 nM Parkin were made up in Solution B supplemented with 1 mM DTT and 1× ATP-regenerating buffer, and 10 µl of this mix was used to resuspend 35 µg of mitochondria with or without CCCP-stabilized PINK1-V5/His and incubated at 30°C for 60 min as indicated in the figure legends. After incubation, mitochondria were re-isolated by centrifugation at 16,000 *g* for 5 min at 4°C and lysed in 1× LDS (Invitrogen) containing 100 mM DTT.

Coimmunoprecipitations and chain linkage analysis

Mitochondrial fractions from ubiquitination reactions were lysed in 1% (wt/vol) SDS, 20 mM Tris-Cl, pH 7.4, and 150 mM NaCl and heat denatured at 99°C for 3 min. Lysates were diluted 1:20 with IP buffer (0.5% [vol/vol] Triton X-100, 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 10% [vol/vol] glycerol) and incubated overnight with either Mfn1 or MitoNEET antibodies followed by incubation with Protein A–Agarose beads (Thermo Fisher Scientific) at room temperature for 2 h. Beads were washed three times with IP buffer before elution with 2× LDS (Invitrogen) supplemented with 200 mM DTT. For K48 and K63 chain linkage analysis, mitochondria from ubiquitination reactions using recombinant Parkin and the E2 UBCH7 were lysed and coimmunoprecipitations were conducted as described previously (Newton et al., 2012). In brief, mitochondria were lysed in lysis buffer containing 8 M urea, 20 mM Tris-Cl, pH 7.5, 135 mM NaCl, 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 1× EDTA-free protease inhibitor cocktail (Roche), and 2 mM *N*-ethylmalemide (Thermo Fisher Scientific). Mitochondrial lysates were then diluted to 4 M urea using lysis buffer lacking urea and were precleared using Protein A–Agarose beads (Thermo Fisher Scientific) for 2 h at room temperature. The beads were collected by centrifugation and supernatants were incubated overnight at room temperature with either K48 or K63 chain antibodies (EMD Millipore). Immunoprecipitates were then collected and subjected to centrifugation at 20,000 *g* for 5 min at room temperature and supernatants were incubated with Protein A–Agarose beads (Thermo Fisher Scientific) for 2 h at room temperature followed by five washes with lysis buffer containing 4 M urea and five washes with PBS before elution with 2× LDS (Invitrogen) supplemented with 200 mM DTT.

NaOH treatment for oxyester detection

For oxyester detection, ubiquitination reactions using cytosol expressing WT Parkin or C431S Parkin were treated with 1% (wt/vol) SDS and boiled for 3 min before treatment with 100 mM NaOH at 37°C for 1 h.

Fluorescence correlation spectroscopy

Molecular association of monomeric YFP–Parkin C431N was assessed in vivo using fluorescence correlation spectroscopy (Haustein and Schwille, 2007; Kim et al., 2007). FCS measurements were performed on a home-built spectrometer based on a modified LSM 5 (Carl Zeiss) confocal microscope. The instrument was calibrated using known diffusion coefficient of Rhodamine 6G and the focal point optical parameters determined. The YFP-protein fluorophore was excited with a 488-nm argon ion laser (<200 µW) via a 40× 1.3 NA oil immersion Plan-NeoFluar objective. The pinhole was set to 1 Airy unit, ∼74 µm. Emission signals were collected through a 505–600-nm bandpass filter onto an avalanche photo diode detector (Micro Photon Devices). TTL photon data were sent to a two-channel correlator (PicoHarp 300; PicoQuant GmbH). The raw and time-correlated ASCII data were streamed to a PC via a high-speed USB 2 interface. Data analysis and fitting procedures were performed using Igor pro 5.02 (Wavemetrics).

HeLa cells transfected on a two-well chambered cover glass slide (LabTek) with YFP–Parkin C431N and YFP alone were transferred to a microscope heated chamber (37°C) for imaging and FCS experiments. Cells were maintained in CO_2-independent medium (Invitrogen). FCS measurements (30-s scans repeated at least 7 times) were collected on a cytosolic region in six cells each from YFP–Parkin C431N and YFP-only–expressing cells. Cells were then treated with 10 µM CCCP and kept in the microscope chamber for 6 h at 37°C, after which FCS data were collected on the same cells. Data were collected over three independent experiments. Individual cell data were averaged for analysis. The data were best fit to a single species diffusion model according to Haustein and Schwille (2007).

Miscellaneous, antibodies, and recombinant proteins

All purified recombinant ubiquitin-conjugating proteins were obtained from Enzo Life Sciences. Recombinant Parkin was a kind gift from Kalle Gehring (McGill University, Montreal, Canada). All chemical reagents were purchased from Sigma-Aldrich unless stated otherwise. Antibodies used in this study were Mfn1 (rabbit polyclonal antibodies made in house), HtrA2/Omi (R&D Systems), VDAC1 (EMD Millipore), MitoNEET (Proteintech), Parkin (Santa Cruz Biotechnology, Inc.), TRAP1 (Abcam), PINK1 (Novus Biologicals), Fis1 (Enxo Life Sciences), Tom70 (Abcam), Bak (Abcam), K48 and K63 linkage specific (EMD Millipore), polyubiquitin FK1 (Enzo Life Sciences), and Mfn2 (a kind gift from Mike Ryan, La Trobe University, Melbourne, Australia). Western blotting was performed by wet transfer method. Horseradish peroxidase–coupled secondary antibodies and ECL chemiluminescent substrate (GE Healthcare) were used to detect immunoreactive proteins in blots. Images were captured with an MP gel documentation system (Bio-Rad Laboratories).

Online supplemental material

Fig. S1 shows PINK1 knockdown levels, in vitro ubiquitination using cells expressing endogenous PINK1, and a screen of apparent ubiquitination of various mitochondrial outer membrane substrates. In Fig. S2 the translocation of Parkin R275W is shown, as well as the requirement of PINK1 for Parkin self-association and the use of WT Parkin to show self-association. Fig. S3 shows which Parkin domains are involved in self-association. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201210111/DC1.

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