



# PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy

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Parkinson's disease genes *PINK1* and *parkin* encode kinase and ubiquitin ligase, respectively. The gene products PINK1 and Parkin are implicated in mitochondrial autophagy, or mitophagy. Upon the loss of mitochondrial membrane potential ( $\Delta\Psi_m$ ), cytosolic Parkin is recruited to the mitochondria by PINK1 through an uncharacterised mechanism – an initial step triggering sequential events in mitophagy. This study reports that Ser65 in the ubiquitin-like domain (Ubl) of Parkin is phosphorylated in a PINK1-dependent manner upon depolarisation of  $\Delta\Psi_m$ . The introduction of mutations at Ser65 suggests that phosphorylation of Ser65 is required not only for the efficient translocation of Parkin, but also for the degradation of mitochondrial proteins in mitophagy. Phosphorylation analysis of Parkin pathogenic mutants also suggests Ser65 phosphorylation is not sufficient for Parkin translocation. Our study partly uncovers the molecular mechanism underlying the PINK1-dependent mitochondrial translocation and activation of Parkin as an initial step of mitophagy.

Mutations of the *PINK1* gene cause selective degeneration of the midbrain dopaminergic neurons in autosomal recessive juvenile Parkinson's disease (PD)<sup>1</sup>. The *PINK1* gene encodes a serine/threonine kinase with a predicted mitochondrial target sequence and a putative transmembrane domain at the N-terminus<sup>2–5</sup>. Loss of the *PINK1* gene in *Drosophila* results in the degeneration of mitochondria in cells with high energy demands, such as muscle and sperm cells, which is suppressed by the introduction of the *parkin* gene, another gene responsible for autosomal recessive juvenile PD<sup>6–8</sup>. The gene product Parkin encodes a RING-finger type ubiquitin ligase (E3) with a Ubl domain at the N-terminus<sup>9–12</sup>.

A series of cell biological studies have provided strong evidence that there are important roles for PINK1 and Parkin in regulating mitochondrial homeostasis. PINK1 is constitutively proteolysed by the mitochondrial rhomboid protease, PARL, at the mitochondrial membrane of healthy mitochondria, resulting in processed forms of PINK1<sup>13–16</sup>. The processed PINK1 is rapidly degraded by the proteasome<sup>2,17</sup>. The reduction of  $\Delta\Psi_m$  leads to the accumulation and activation of PINK1 in the mitochondria<sup>17–19</sup> through a currently unresolved mechanism<sup>20</sup>. The accumulation of PINK1 recruits Parkin from the cytosol to the mitochondria with decreased membrane potential, which stimulates Parkin E3 activity, promoting mitochondrial degradation via an autophagic event known as mitophagy<sup>17,21–24</sup>. The recruitment of cytosolic Parkin to the mitochondria upon disruption of  $\Delta\Psi_m$  is believed to be the first step of mitophagy for the removal of damaged mitochondria. This recruitment is required for the kinase activity of PINK1<sup>17,21–25</sup>. Although two separate studies have proposed that Parkin is directly phosphorylated by PINK1<sup>26,27</sup>, others have failed to detect Parkin phosphorylation by PINK1<sup>21</sup>, suggesting that the kinase activity of PINK1 itself is relatively low. One reason biochemical analysis has been unable to obtain direct evidence is that recombinant human PINK1 purified from mammalian cultured cells or bacteria easily loses kinase activity, while insect PINK1 has significant autophosphorylation activity<sup>28,29</sup>.

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Very recently, Kondapalli, C. *et al.* reported that PINK1 directly phosphorylates Parkin at Ser65 in the Ubl domain<sup>18</sup>. However, the extent and consequences of Parkin phosphorylation by PINK1 in mitochondrial regulation are still not fully understood.

To address this issue, we attempted to independently monitor and compare the phosphorylation status of Parkin in wild-type and *PINK1*-deficient cells, thereby excluding the possibility of phosphorylations by uncharacterised kinases other than PINK1<sup>30</sup>. Here, we also report that Parkin is demonstrably phosphorylated at Ser65 in a PINK1-dependent manner. Furthermore, we show that this phosphorylation event is implicated in the regulation of mitochondrial translocation of Parkin and the subsequent degradation of mitochondrial surface proteins during mitophagy.

## Results

**Parkin is phosphorylated upon depolarisation in  $\Delta\Psi_m$ .** We used [<sup>32</sup>P] orthophosphate to metabolically label mouse embryonic fibroblasts (MEFs) derived from *PINK1* deficient mice, in which HA-tagged Parkin together with FLAG-tagged wild-type or kinase-dead forms (triple mutant with K219A, D362A and D384A) of PINK1 were virally introduced (hereafter referred to as “PINK1-FLAG WT” or “KD/HA-Parkin/*PINK1*<sup>-/-</sup>” MEFs) and then induced Parkin-mediated mitophagy via treatment with the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). As shown in Figure 1a, Parkin was specifically phosphorylated in CCCP-treated PINK1-FLAG WT/HA-Parkin/*PINK1*<sup>-/-</sup> MEFs, but not in PINK1-FLAG KD/HA-Parkin/*PINK1*<sup>-/-</sup> MEFs. Phos-tag Western blotting, in which phosphorylated proteins appear as slower migrating bands<sup>28</sup>, revealed that Parkin was phosphorylated within 10 min following CCCP treatment (Fig. 1b). Phosphorylation of Parkin reached its maximum level approximately 40 min after CCCP treatment and was sustained at least until 6 hr (Supplementary Fig. S1). Under these conditions, slower migrating bands of PINK1 also appeared, which very likely reflects the autophosphorylation of PINK1 when activated (Fig. 1b)<sup>18</sup>. The suppression of PINK1 accumulation by RNA interference suggested that  $\Delta\Psi_m$  depolarisation-dependent activation of PINK1 along with PINK1 accumulation is a key element for Parkin phosphorylation (Fig. 1c). Every PINK1 deletion and pathogenic mutant we tested failed to stimulate Parkin phosphorylation effectively, strongly suggesting that intact PINK1 is required for this action (Fig. 1d and e). Importantly, human fibroblasts from a patient with *PINK1*-linked parkinsonism also lacked the activity to phosphorylate Parkin (Fig. 1f). The phosphorylated Parkin disappeared within 30 min during the recovery of  $\Delta\Psi_m$  depolarisation by the removal of CCCP from the culture medium (Fig. 1g). Further analysis using phosphatase and proteasome inhibitors suggested that phosphorylated Parkin is at least partly degraded by proteasomal activity in the mitochondria (Supplementary Fig. S2).

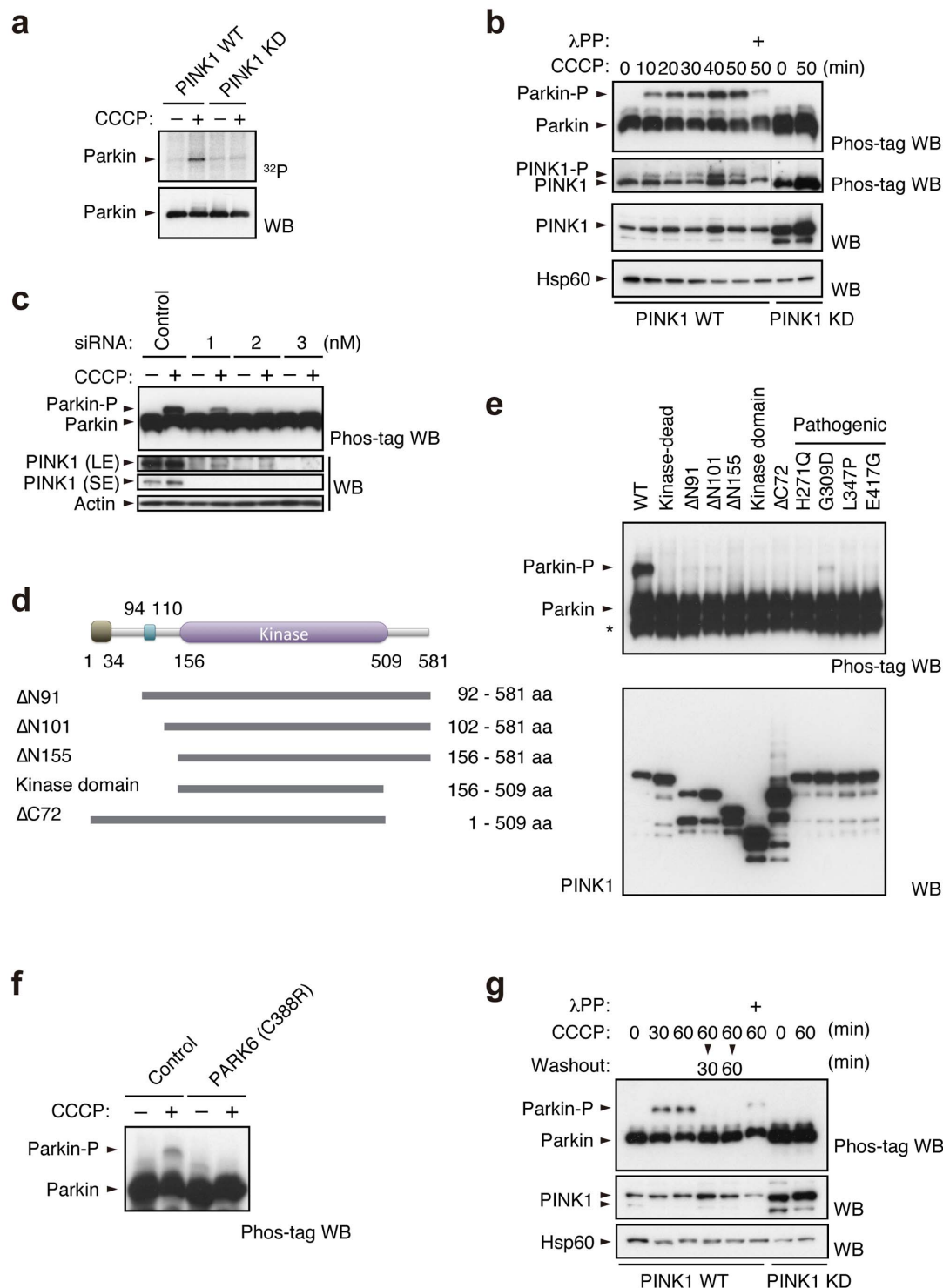
**Phosphorylation of Ser65 in the Parkin Ubl domain primes the mitochondrial translocation of Parkin.** To determine which residue(s) of Parkin are phosphorylated, we immunopurified HA-tagged Parkin from PINK1-FLAG WT or KD/HA-Parkin/*PINK1*<sup>-/-</sup> MEFs treated with or without CCCP and performed mass spectrometric analysis for phospho-peptides (Supplementary Fig. S3). Although Phos-tag Western blotting of Parkin mainly detected a single band shift, which represents a single phospho-modification, the mass spectrometric analysis identified Ser9 or Ser10 and Ser65, Ser101 and Ser198 as phosphorylated residues of Parkin. Among these residues, only Ser65 phosphorylation increased (33-fold) in CCCP-treated PINK1-FLAG WT/HA-Parkin/*PINK1*<sup>-/-</sup> MEFs (Supplementary Fig. S3). Phos-tag Western blotting with mutant forms of Parkin, in which the identified phospho-serine residues are replaced with alanine, revealed that the band shift represents Ser65 phosphorylation (Fig. 2a). An *in vitro* kinase assay with recombinant insect PINK1, which has marked kinase activity<sup>28</sup>, strongly suggested that

PINK1 directly phosphorylates Parkin at Ser65 (Supplementary Fig. S4). The Ser65 residue lies in the Ubl domain and is highly conserved from human to *Drosophila* (Fig. 2b). We next examined whether phosphorylation of Ser65 is required for Parkin-mediated mitophagy. GFP-tagged Parkin WT, which was localised both in the cytoplasm and in the nuclei of mock (DMSO)-treated cells (0 hr, Fig. 2c and d), was translocated to the mitochondria and induced the perinuclear aggregation of mitochondria 2 hr after CCCP treatment, as previously reported (2 hr, Fig. 2c and d)<sup>17,23</sup>. Replacement of Ser65 with alanine (S65A) did not affect the subcellular localisation of Parkin in mock-treated cells when compared with that of GFP-Parkin WT (0 hr, Fig. 2c and d). However, GFP-Parkin S65A almost completely inhibited the mitochondrial translocation of Parkin and the perinuclear rearrangement of mitochondria 0.5 hr after CCCP treatment (0.5 hr, Fig. 2c and d) and showed delayed translocation in 2 hr (2 hr, Fig. 2c and d). The expression of a putative phosphomimetic Parkin S65E also showed a subcellular localisation similar to that of GFP-Parkin WT in both DMSO- and CCCP-treated cells (Fig. 2c). However, GFP-Parkin S65E exhibited a mild translocation defect, suggesting that S65E does not fully mimic the phosphorylated Ser65 (Fig. 2d).

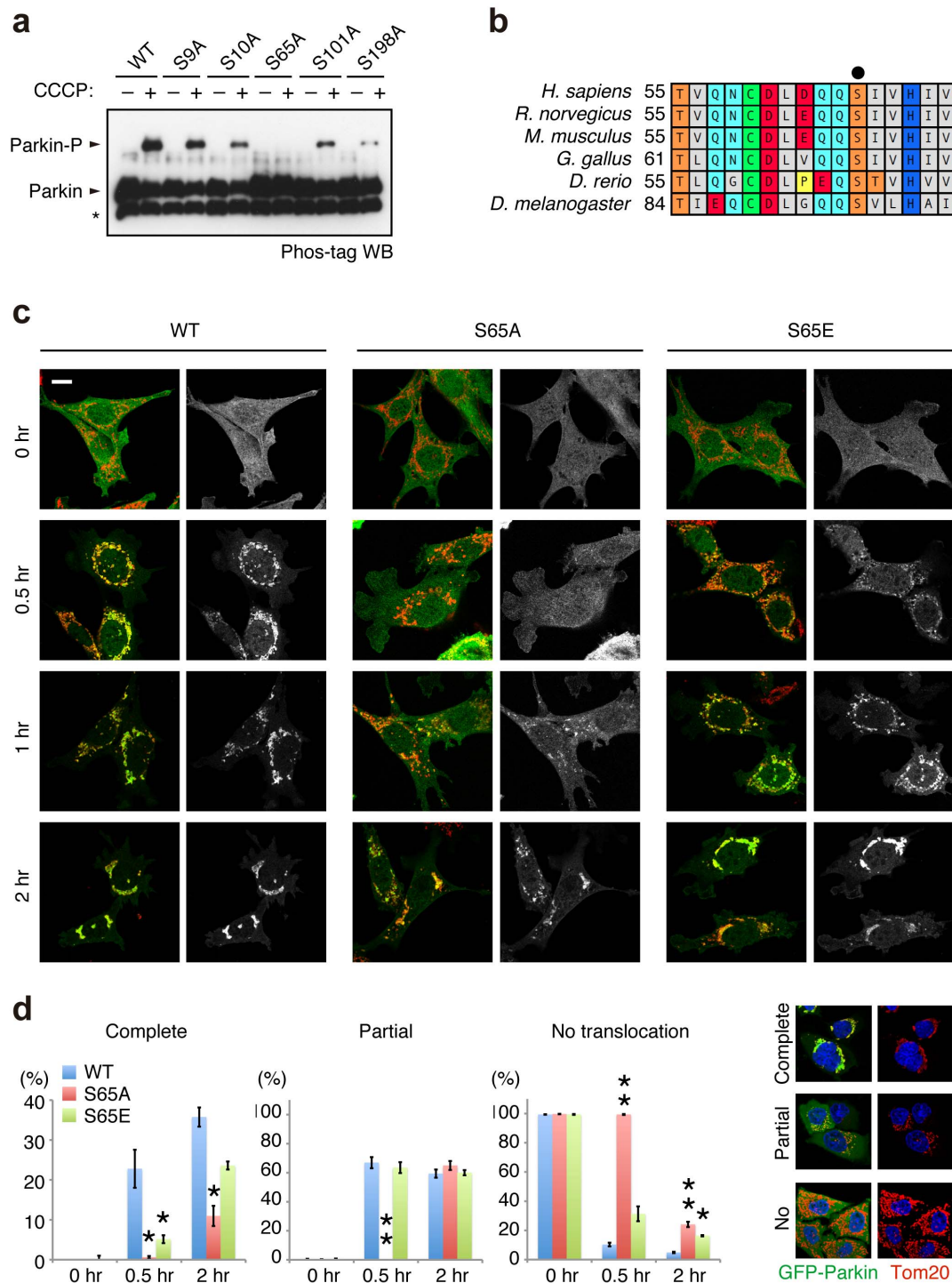
**Parkin Ser65 phosphorylation is not sufficient for mitochondrial translocation upon depolarisation of  $\Delta\Psi_m$ .** As PINK1-mediated Ser65 phosphorylation appeared to be required for efficient translocation of Parkin, we next examined whether well-characterised pathogenic Parkin mutants were subjected to phosphorylation upon CCCP treatment. In this experiment, we used three kinds of Parkin mutants based on the previous and current studies (Supplementary Fig. S5)<sup>17,22,23</sup>. The first group, V15M, P37L, R42P and A46P, had intact or weakly impaired mitochondrial translocation activity. The second group, T415N and G430D, had mildly impaired translocation activity. The third group, K161N, K221N and T240R, almost completely lacked translocation activity (Fig. 3a). Surprisingly, all of the mutants possessed comparable phosphorylation efficiencies to those of WT (Fig. 3b). This result suggests that Ser65 phosphorylation is not sufficient for the mitochondrial translocation of Parkin.

Biochemical fractionation of endogenous Parkin from SH-SY5Y cells detected only the phosphorylated form of Parkin in the mitochondrial fraction upon CCCP treatment (Fig. 3c), which strongly suggests that phosphorylation of Parkin is required for mitochondrial translocation. There was a slight difference in the gel mobility of phosphorylated Parkin between the cytosolic and the mitochondrial fractions and between CCCP-treated periods of time. These differences very likely reflect differences in the complexity of the contents of each fraction rather than in the phosphorylation status of Parkin because a single shifted band appears in the mixed fractions (Mito + Cyto in Fig. 3c; CCCP 30 min + 60 min in Supplementary Fig. S6).

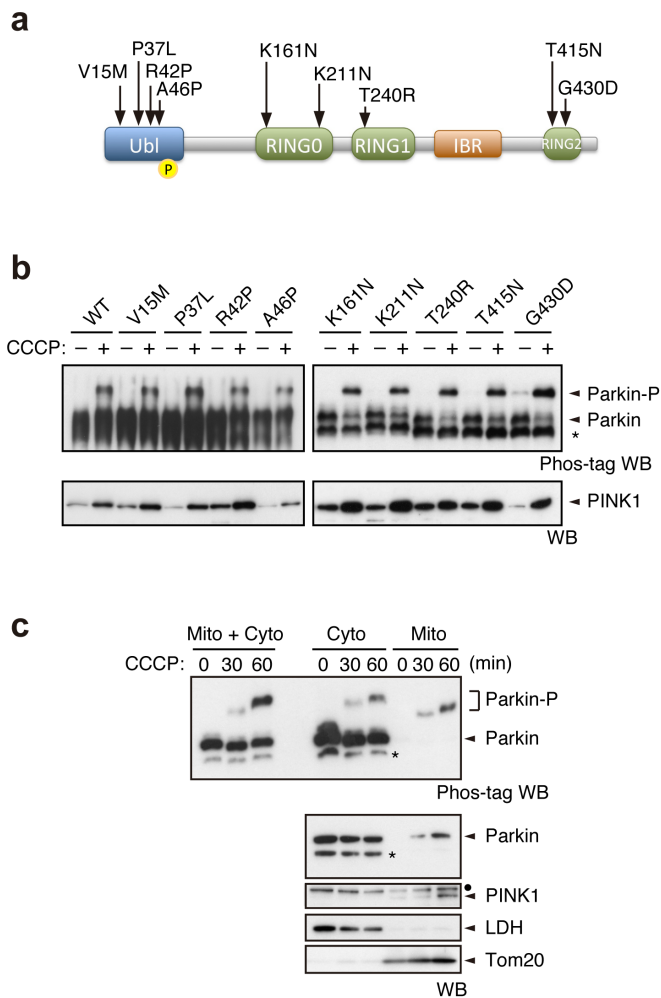
**Effect of Parkin Ser65 phosphorylation on the autophagic reaction.** We next examined whether Ser65 phosphorylation is required for the subsequent autophagic reaction, in which various ubiquitin-proteasome- and autophagy-related proteins are involved, including the 26S proteasome, p97/VCP, p62/SQSTM1, LC3, ATG5 and ATG7<sup>22,23,31–35</sup>. Parkin has been reported to be involved in the ubiquitin-proteasome-dependent degradation of a variety of mitochondrial outer membrane proteins, including Mitofusin1 (Mfn1)<sup>32</sup>, Mfn2<sup>32</sup>, Miro1<sup>36,37</sup>, Miro2<sup>37</sup>, VDAC1<sup>22</sup> and Tom20<sup>31</sup>. Degradation of Mfn1, VDAC1 and Tom20 at the mitochondrial outer membrane was observed in PINK1 WT/GFP-Parkin/*PINK1*<sup>-/-</sup> MEFs 1 to 4 hr after CCCP treatment (Fig. 4a). While GFP-Parkin harbouring S65A or S65E mutations was also capable of inducing Mfn1, VDAC1 and Tom20 degradation, the efficiency was impaired, especially in Mfn1 and VDAC1 (Fig. 4a). Long-term time course analysis revealed that in cells expressing Parkin with S65A or S65E mutations, Mfn1 and VDAC1 cannot be degraded effectively, and the mitochondrial outer membrane was likely more intact as indicated by the sustained



**Figure 1** | PINK1-dependent phosphorylation of Parkin *in vivo*. (a) PINK1-FLAG WT or KD/HA-Parkin/*PINK1*<sup>-/-</sup> MEFs were labelled with [<sup>32</sup>P] orthophosphate and treated with 30  $\mu\text{M}$  CCCP for 1.5 hr. Phosphorylated Parkin was detected by autoradiography (<sup>32</sup>P). Immunoprecipitated HA-Parkin was detected by Western blotting (WB) with anti-Parkin. (b) PINK1-FLAG WT or KD/HA-Parkin/*PINK1*<sup>-/-</sup> MEFs were treated with or without 30  $\mu\text{M}$  CCCP for the indicated periods of time. Cell lysate was subsequently separated on a Phos-tag gel, followed by WB with anti-PINK1 or anti-Parkin antibodies (Phos-tag WB). Phosphorylated bands of Parkin and PINK1 were confirmed by their disappearance with lambda protein phosphatase ( $\lambda\text{PP}$ ) treatment. Mitochondrial Hsp60 was used as a loading control. (c) Suppression of endogenous PINK1 expression inhibits Parkin phosphorylation. HeLa cells stably expressing non-tagged Parkin were treated with the indicated concentrations of stealth siRNA duplex against PINK1 (Invitrogen) with or without 10  $\mu\text{M}$  CCCP for 1 hr. Long- (LE) and short-exposure (SE) blot signals for PINK1 were shown. Actin was used as a loading control. (d) Truncated PINK1 mutants used in this study. Putative mitochondria-targeting sequence, 1–34 aa; transmembrane domain, 94–110 aa; kinase domain, 156–509 aa. (e) Full-length PINK1 is required for Parkin phosphorylation. *PINK1*<sup>-/-</sup> MEFs stably expressing non-tagged Parkin were transfected with various PINK1 constructs with C-terminal FLAG-tags. PINK1 expression was confirmed with anti-FLAG-HRP. (f) Human fibroblasts from a normal control and a *PARK6* case with a homozygous C388R mutation<sup>44</sup> were transfected with Parkin and were treated with or without 30  $\mu\text{M}$  CCCP for 1 hr. (g) Cells treated with CCCP up to 60 min as in (b) were further incubated with fresh culture medium without CCCP for the indicated periods of time (Washout).



**Figure 2 | Ser65 in the Ubl domain of Parkin is phosphorylated upon depolarisation of  $\Delta\Psi_m$ .** (a) Phos-tag Western blotting detected phosphorylation of Ser65. HeLa cells were transiently transfected with Parkin WT and a series of alanine mutants for the candidate phospho-residues followed by treatment with or without 20  $\mu\text{M}$  CCCP for 1 hr. Cell lysates were analysed by Phos-tag Western blotting. An asterisk indicates degraded Parkin. (b) Alignment of the amino acid sequences surrounding Ser65 (marked by a black dot) from a variety of animal species. The numbers on the left correspond to the residue numbers of Parkin proteins. (c) Introduction of the S65A mutation delayed Parkin translocation to the depolarised mitochondria in PINK1 WT/GFP-Parkin/*PINK1*<sup>-/-</sup> MEFs. Cells retrovirally introduced with GFP-Parkin WT or its phospho-mutants (S65A and S65E) were treated with or without 30  $\mu\text{M}$  CCCP for the indicated periods of time. GFP-Parkin and mitochondria were visualised with anti-GFP (green) and anti-Tom20 (red), respectively. Parkin signals are also shown as monochrome images. Scale bar = 10  $\mu\text{m}$ . (d) Mitochondrial translocation efficiency of Parkin mutants. PINK1 WT/*PINK1*<sup>-/-</sup> MEFs stably expressing GFP-Parkin WT, S65A or S65E were treated as in (c). Cells expressing GFP-Parkin perfectly overlapped (Complete, examples are shown on the right), partially overlapped (Partial) or non-overlapped (No) with the Tom20 signal were counted. The data represent means  $\pm$  SE from three experiments ( $n = 99$ –143 cells in each). \*\*  $p < 0.01$ , \*  $p < 0.05$  vs. WT at each time point.



**Figure 3 | Pathogenic mutants of Parkin are subjected to Ser65 phosphorylation.** (a) Diagram of Parkin protein illustrating the pathogenic mutants used in this study. The Ser65 residue in the Ubl domain is shown as a yellow circle. RING, Ring-finger motif; IBR, in-between-Ring fingers domain. (b) Phos-tag Western blotting for Parkin and Western blotting for PINK1 were performed using Parkin WT and a series of pathogenic mutants as shown in Figure 2a. (c) Endogenous Parkin was also phosphorylated in SH-SY5Y cells after CCCP treatment. Post-nuclear cell lysates from SH-SY5Y cells treated with or without 10  $\mu$ M CCCP for 30 and 60 min were fractionated into mitochondria-rich (Mito) and cytosolic (Cyto) fractions. These two fractions and their combination (Mito + Cyto) were subjected to Phos-tag or normal Western blotting analyses. Endogenous PINK1 was fractionated in the Mito fraction, as previously reported<sup>45</sup>. Lactate dehydrogenase (LDH) and Tom20 were used as cytosolic and mitochondrial marker proteins, respectively. Asterisks: putative cleaved Parkin; dots: non-specific bands.

accumulation of PINK1 (Fig. 4b). The impaired degradation cannot be explained simply by the delayed translocation of Parkin mutants because both mutants completed the mitochondrial translocation by the 6 hr time-point (data not shown and see Fig. 4c). In contrast, the profiles of Parkin expression and autoubiquitination in Parkin S65A- or S65E-expressing cells were comparable with those of WT (Fig. 4b). We also examined whether Ser65 mutations affect the accumulation of proteasome (Fig. 4c) and p62 (Supplementary Fig. S7) at the mitochondria during mitophagy via the immunostaining of the proteasome subunit alpha type 7 ( $\alpha$ 7) and p62. However, there was no evidence that Ser65 mutations inhibit or delay the recruitment of proteasome and p62 to the mitochondria. Finally, we tested whether the Parkin Ubl domain itself is indispensable for the mitochondrial

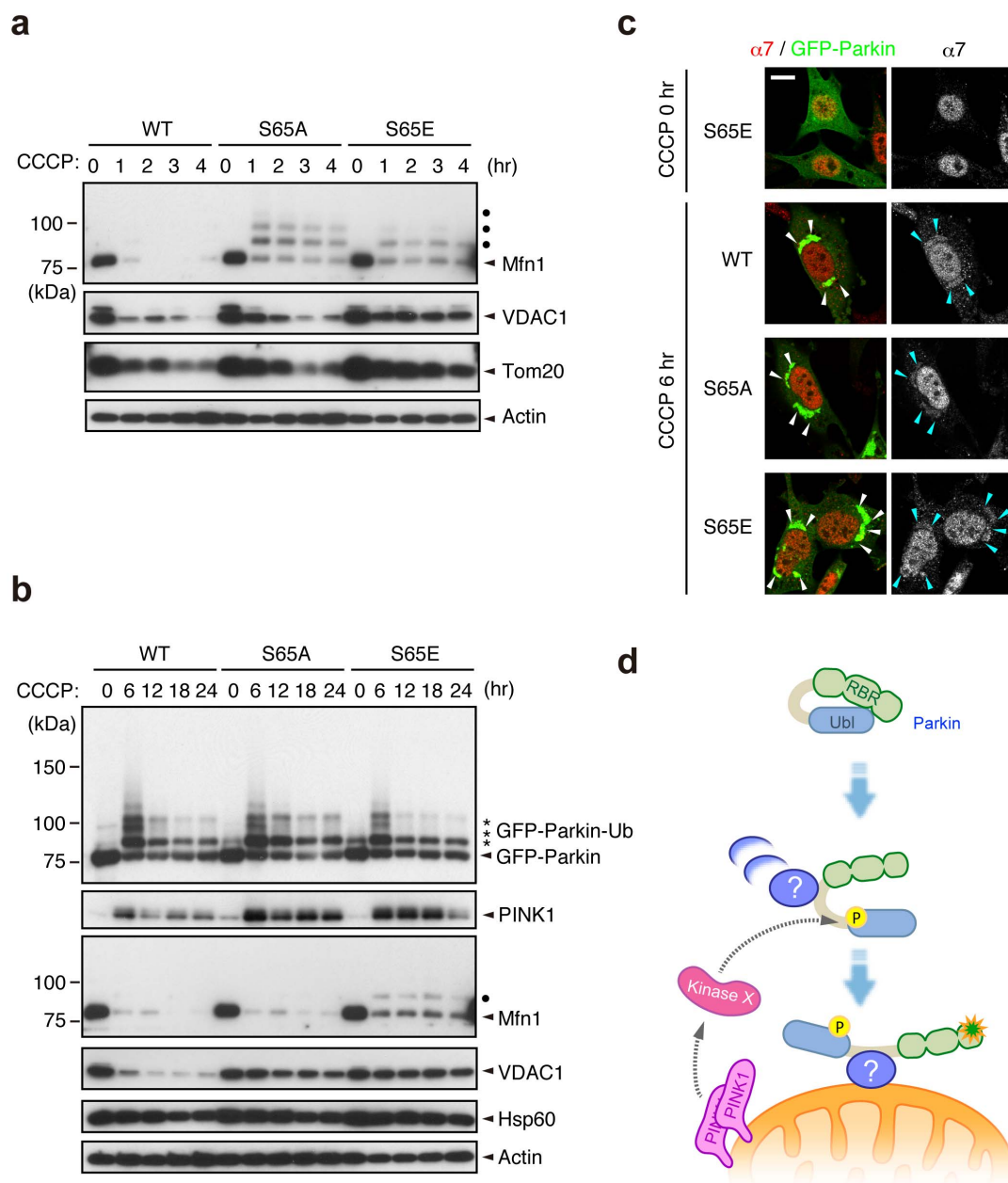
translocation and the substrate degradation (Supplementary Fig. S8). Interestingly, Parkin mutant lacking the Ubl domain ( $\Delta$ Ubl) showed a mild delay in the mitochondrial translocation, slowed the mitochondrial reorganization to the perinuclear region (Supplementary Fig. S8b and c) and impaired the degradation of mitochondrial outer membrane proteins (Supplementary Fig. S8d). These results suggest that proper regulation of the Parkin Ubl domain through the Ser65 phosphorylation is required not only for efficient translocation to mitochondria as an initial step of mitophagy, but also for the degradation of mitochondrial outer membrane proteins during mitophagy through an as yet unknown mechanism.

## Discussion

A series of *Drosophila* genetic and cell biological studies have clearly demonstrated that PINK1 is required for Parkin-mediated mitochondrial maintenance. The mitophagy of damaged mitochondria is a well-characterised event in which PINK1 and Parkin are involved. However, how PINK1 regulates Parkin is largely unclear. This study has shown that Ser65 in the Ubl domain of endogenous Parkin is phosphorylated in an activated PINK1-dependent manner. In addition to mitochondrial accumulation of PINK1,  $\Delta\Psi$ m depolarisation-dependent PINK1 autophosphorylation has been reported to be an important element for PINK1 activation and Parkin recruitment<sup>19,29</sup>. Consistent with these observations, our investigation of PINK1 siRNA suggests that a lower level of PINK1 is able to phosphorylate Parkin after  $\Delta\Psi$ m depolarisation (Fig. 1c, compare lanes 1 and 4). Our domain analysis of PINK1 demonstrates that intact PINK1 is required for CCCP-dependent Parkin phosphorylation, and the lack of phosphorylation in fibroblasts from a *PARK6* patient implies relevance to the pathogenesis of PD.

The biological significance of this phosphorylation event is suggested by the fact that replacement of Ser65 with alanine or glutamic acid impairs the mitochondrial translocation of Parkin and/or the subsequent mitophagy process. Our observation that maximal phosphorylation of Parkin occurs within 1 hr of CCCP treatment supports the idea that Ser65 phosphorylation is required for the early step of Parkin translocation. In contrast, PINK1 accumulation appears to last at least 6 hr (Fig. 4c and Supplementary Fig. S1b). The difference in time course between PINK1 accumulation and Parkin phosphorylation could be explained by the observation that phosphorylated Parkin is degraded by proteasomal activity. The biochemical evidence that only the phosphorylated form of endogenous Parkin is present in the mitochondrial fraction also implies that Parkin phosphorylation is an essential event for its mitochondrial translocation and subsequent activation (Fig. 3c and Supplementary Fig. S6). Overexpression of PINK1 and Parkin itself leads to mitochondrial translocation of Parkin independently of  $\Delta\Psi$ m depolarization, which suggests that excessive amounts of PINK1 and Parkin do not faithfully reflect endogenous reactions. Our study using *PINK1*<sup>-/-</sup> MEFs stably co-expressing PINK1 and GFP-Parkin might also be saddled with such a problem. We believe that the endogenous observation in which phosphorylated Parkin is accumulated in mitochondria is a more reliable proposal as a molecular model. The delay of exogenous GFP-Parkin S65A in the mitochondrial translocation would indicate that modification of Ser65 is important for Parkin translocation at least. At the same time, another important finding is that pathogenic mutants that lose their translocation activity are also phosphorylated (Fig. 3b), raising the possibility that phosphorylation of Parkin at Ser65 is insufficient for translocation. Thus, Ser65 phosphorylation likely leads to other events in mitochondrial translocation, such as the association or dissociation of protein(s) involved in the mitochondrial translocation of Parkin or the modification of Parkin itself for activation at a different site(s).

Both the S65A and S65E Parkin mutants cannot undergo efficient mitophagy, as indicated by the incomplete degradation of



**Figure 4 | Ser65 phosphorylation affects the subsequent autophagy reaction.** (a) CCCP-dependent degradation of mitochondrial outer membrane proteins in PINK1 WT/PINK1<sup>-/-</sup> MEFs expressing WT or mutant forms of GFP-Parkin. Mfn1, VDAC1 and Tom20 were used as markers of mitochondrial outer membrane proteins. Actin: a loading control. Dots: ubiquitinated Mfn1. (b) Long-term time-course analysis of CCCP-dependent mitochondrial protein degradation. The degradation of outer membrane proteins was impaired in cells expressing GFP-Parkin S65A or S65E mutations. Hsp60 was used as a marker of mitochondrial matrix proteins. (c) S65A and S65E mutations do not affect proteasome recruitment to the mitochondria during mitophagy. PINK1 WT/PINK1<sup>-/-</sup> MEFs expressing WT or mutant forms of GFP-Parkin (green) were treated with 30  $\mu$ M CCCP for 3 or 6 hr. Cells were stained with anti-proteasome subunit alpha type 7 ( $\alpha$ 7, red).  $\alpha$ 7-immunoreactivity was enriched in the nuclei of all three cell genotypes under normal conditions, as displayed in the representative image of S65E (CCCP 0 hr), and overlapped with the aggregated mitochondria (arrowheads) 6 hr after CCCP treatment irrespective of genotype. Similar results were obtained 3 hr after CCCP treatment. Scale bar = 10  $\mu$ m. (d) Model for Parkin translocation and activation. The Parkin Ubl domain masks C-terminal RING-IBR-RING (RBR) domains for E3 activity<sup>46</sup>. A Parkin phosphorylation event at Ser65 (P), combined with unknown factor(s) (?), stimulates the mitochondrial translocation of Parkin, releasing the RBR domains from autoinhibition by the Ubl domain.

the mitochondrial outer membrane proteins. Because inhibition of the degradation of the mitochondrial outer membrane proteins by proteasome inhibitors is reported to block mitophagy<sup>32,35</sup>, it may be that the modification of Parkin Ser65 has a greater than expected impact on the mitophagy process. Although our study does not demonstrate that the S65E mutant behaves exactly like the phosphorylated form of Parkin, the S65E mutant does

translocate to the mitochondria in a similar way to WT, although with slightly impaired efficiency, suggesting that S65E has at least some properties that are similar to phosphorylated Parkin. Currently, it is unknown why S65E also inhibits the later processes of mitophagy. One possible explanation is that rapid degradation of phosphorylated Parkin is required for the proper progression of mitophagy, and S65E may not be degraded effectively. However,



there is no evidence that S65E is more stable than WT, as shown in Figure 4c.

Very recently, Kondapalli *et al.* proposed a model to explain the biological significance of Ser65 phosphorylation, in which Ser65 phosphorylation relieves autoinhibition of Parkin E3 activity by the Ubl domain<sup>18</sup>. This model may explain the depolarised  $\Delta\Psi_m$ -dependent activation of Parkin. However, our data indicated that the Parkin S65A mutant is also autoubiquitinated (Fig. 4b) and that the  $\Delta$ Ubl mutant showed mild translocation defect and impaired substrate degradation (Supplementary Fig. S8). Moreover, if this is the case, the E3 activity of Parkin pathogenic mutants lacking mitochondrial translocational activity but harbouring intact E3 activity *in vitro* (such as K161N and K211N, which are subjected to the Ser65 phosphorylation) should be activated in the cytosol<sup>38</sup>. However, our previous data indicate that K161N and K211N are not activated by CCCP treatment<sup>23</sup>. Thus, it is conceivable that another step is required for depolarised  $\Delta\Psi_m$ -dependent activation of Parkin E3. In addition, the Ubl domain might not only autoinhibit its E3 activity but also contribute to the mitochondrial translocation and the substrate degradation through an as yet unknown mechanism. We believe that an appropriate way to estimate Parkin E3 activity in the context of mitophagy is to evaluate the ubiquitination and degradation of substrates in cells with depolarised  $\Delta\Psi_m$ . Mfn1 is a well-characterised direct substrate of Parkin<sup>32</sup>, and Parkin-dependent poly-ubiquitination modification of Mfn1 can be detected by Western blotting upon  $\Delta\Psi_m$  depolarisation<sup>32,39,40</sup>. Parkin S65A and S65E appear to ubiquitinate Mfn1, as poly-ubiquitinated forms of Mfn1 were observed (Fig. 4b). However, they cannot degrade it effectively, which suggests that the process of substrate degradation is also impaired in these mutants.

Kondapalli *et al.* have also shown that *T. castaneum* PINK1 (TcPINK1) directly phosphorylates human Parkin at Ser65<sup>18</sup>. We confirmed their finding using recombinant TcPINK1 produced from the same construct (Supplementary Fig. S4). The replacement of MBP-Parkin Ser65 with alanine completely abolished PINK1-mediated phosphorylation, indicating that Ser65 is the sole phosphorylation site *in vitro*. However, experiments in cultured cells showed that the replacement of Ser9, Ser10, Ser101 and Ser198 with alanine affects the Ser65 phosphorylation efficiency (Ser9, ~35% reduction; Ser10, ~76% reduction; Ser101, ~65% reduction; Ser198, ~92% reduction) (Fig. 2a). These residues might be priming phosphorylation sites for Ser65 phosphorylation.

Because PINK1 is believed to be activated in the mitochondria, a topological inconsistency arises from our cell-based data that cytosolic Parkin lacking the mitochondrial translocation activity is phosphorylated. Therefore, it is possible that PINK1 indirectly regulates Parkin phosphorylation. One possible explanation for this is the presence of another cytosolic kinase(s) regulated by PINK1 (Fig. 4d). Alternatively, because mitochondria are a dynamic organelle, cytosolic Parkin adjacent to the moving and fragmented mitochondria with depolarised  $\Delta\Psi_m$  might be phosphorylated incidentally. The issue as to whether or not PINK1 directly phosphorylates Parkin in cells remains to be solved.

In conclusion, this study has suggested that PINK1-dependent Parkin phosphorylation at Ser65 accelerates the mitochondrial translocation of Parkin and showed that the introduction of mutations at this site also affects subsequent mitophagy processes. Concurrently, our data provide the possibility that there is an elaborate multi-step mechanism for the mitochondrial translocation of Parkin upon the loss of  $\Delta\Psi_m$  (Fig. 4d), the clarification of which awaits further study.

## Methods

**Antibodies, plasmids and cell lines.** Antibodies used in Western blot analysis were as follows: anti-Parkin (1 : 1,000 and 1 : 5,000 dilution for endogenous and exogenous Parkin, respectively; Cell Signaling Technology, clone PRK8), anti-PINK1 (1 : 1,000 dilution; Novus, BC100-494 or 1 : 1,000 dilution; Cell Signaling Technology, clone D8G3), anti-Mfn1 (1 : 1,000 dilution; Abnova, clone 3C9), anti-VDAC1 (1 : 1,000

dilution; Abcam, Ab15895), anti-Tom20 (1 : 500 dilution; Santa Cruz Biotechnology, FL-145), anti-FLAG-HRP (1 : 2,000 dilution; Sigma-Aldrich, clone M2), anti-GFP (1 : 5,000 dilution; Abcam, ab290), anti-Actin (1 : 10,000 dilution; Millipore, MAb1501), anti-LDH (1 : 1,000 dilution; Abcam, ab7639-1), anti-phospho-GSK3 $\beta$  (1 : 1,000 dilution; Cell Signaling Technology, clone 5B3), anti-GSK3 $\beta$  (1 : 1,000 dilution; Cell Signaling Technology, clone 27C10), and anti-Hsp60 (1 : 10,000 dilution; BD Biosciences, clone 24/Hsp60). Antibodies used in immunocytochemistry were as follows: FITC-conjugated anti-GFP (1 : 1,000 dilution; Abcam, ab6662), anti-Tom20 (1 : 1,000 dilution; Santa Cruz Biotechnology, FL-145), anti-Myc (1 : 500 dilution; Millipore, clone 4A6), anti-p62 (1 : 500 dilution; Progen Biotechnik, GP62-C), anti-Parkin (1 : 1,000 dilution; Cell Signaling Technology, clone PRK8) and anti-proteasome  $\alpha$ 7 (1 : 250; a kind gift of Dr S. Murata at the University of Tokyo). cDNAs for human Parkin, PINK1 and its pathogenic and engineered mutants are as described in previous studies<sup>23,41</sup>. Parkin phospho-mutants were generated by PCR-based mutagenesis followed by sequencing confirmation of the entire gene. PINK1<sup>-/-</sup> MEFs, cultured as previously described<sup>23</sup>, were retrovirally transfected with pMXs-puro harbouring non-tagged PINK1, PINK1-FLAG, non-tagged Parkin, HA-Parkin, GFP-Parkin and related cDNA; transfected cells were then selected with 1  $\mu$ g/ml puromycin. HeLa cells maintained at 37°C in a 5% CO<sub>2</sub> atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and 1x non-essential amino acids (GIBCO) were retrovirally transfected with pMXs-puro harbouring non-tagged Parkin along with pcDNA3Hyg-mSlc7a1-VSVG and pcDNA3Hyg-mSlc7a1-FLAG (a kind gift of Dr N. Fujita at UCSD). Stable cell lines were selected with 1  $\mu$ g/ml puromycin and cloned. Transient transfections of cultured cells were performed using Lipofectamine 2000 (Invitrogen) for plasmids and Lipofectamine RNAiMAX (Invitrogen) for stealth siRNA duplexes (Invitrogen), which were used according to the manufacturer's instructions.

**Tissue culture.** Skin biopsies were obtained from a PARK6 case and a control without mutations in any known PD genes. The study was approved by the ethics committee of Juntendo University, and all participants gave written, informed consent. Dermal primary fibroblasts established from biopsies were cultured in high glucose DMEM supplemented with 10% foetal bovine serum, 1x non-essential amino acids, 1 mM sodium pyruvate (GIBCO), 100  $\mu$ M 2-mercaptoethanol, and 1% penicillin-streptomycin at 37°C in a 5% CO<sub>2</sub> atmosphere.

**Mapping of Parkin phosphorylation sites.** PINK1<sup>-/-</sup> MEFs (6.0 x 10<sup>7</sup>) expressing HA-Parkin and PINK1-FLAG were treated with or without 30  $\mu$ M CCCP for 30 min. HA-Parkin (~500 ng in each) immunopurified with anti-HA-conjugated agarose beads was eluted with 8 M urea buffered with 50 mM Tris-HCl at pH 9.0. Samples from two independent experiments were digested with trypsin or chymotrypsin and analysed by nano-scale liquid chromatography-tandem mass spectrometry (Dionex Ultimate3000 RSLCnano and ABSciex TripleTOF 5600) followed by MASCOT searching and Mass Navigator/PhosPepAnalyzer processing for identification and label-free quantitation, respectively<sup>42</sup>. Determination of phosphosite localisation was performed based on the presence of site-determining ions<sup>43</sup>.

**Phosphorylation assay and mitochondrial fractionation.** PINK1<sup>-/-</sup> MEFs harbouring HA-Parkin along with wild-type or a kinase-dead form of PINK1-FLAG were metabolically labelled with 175  $\mu$ Ci/ml of [<sup>32</sup>P] orthophosphate in phosphate-free DMEM (GIBCO) with 10% FBS at 37°C for 3 hr. The medium was then replaced with fresh DMEM containing 10% FBS. Cells were treated with CCCP for 1.5 hr and were lysed on ice with lysis buffer containing 0.2% NP-40, 50 mM Tris (pH 7.4), 150 mM NaCl and 10% glycerol supplemented with protease inhibitor (Roche Diagnostics) and phosphatase inhibitor (Pierce) cocktails, and HA-Parkin and PINK1-FLAG were immunoprecipitated with anti-HA (Wako Pure Chemical, clone 4B2)- or anti-FLAG (Sigma-Aldrich, clone M2)-conjugated agarose beads. Immunoprecipitates were separated by SDS-PAGE and transferred onto a PVDF membrane. Autoradiography and Western blotting were performed to visualise proteins. Phos-tag Western blotting was performed as previously described<sup>28</sup>. Briefly, phospho-Parkin and phospho-PINK1 were separated on 8% gels containing 50  $\mu$ M Phos-tag. Mitochondrial and cytosolic fractionations were performed as previously described, with some modifications<sup>29</sup>. The cytosolic fractions were further clarified by a second centrifugation at 105,000 g for 60 min to remove residual organelle membranes.

**Immunocytochemical analysis.** Cells plated on 3.5 mm glass-bottom dishes (MatTek) were fixed with 4% paraformaldehyde in PBS and permeabilised with 50  $\mu$ g/ml digitonin for anti-Tom20 and anti-p62 staining or with 0.1% NP-40 for anti- $\alpha$ 7 staining in PBS. Cells were stained with anti-Tom20 or anti- $\alpha$ 7 antibodies in combination with FITC-conjugated anti-GFP antibody and were counterstained with DAPI for nuclei. Cells were imaged using laser-scanning microscope systems (TCS-SP5, Leica or LSM510 META, Carl Zeiss).

**Statistical analysis.** A one-way repeated measures ANOVA was used to determine significant differences between multiple groups unless otherwise indicated. If a significant result was achieved ( $p < 0.05$ ), the means of the control and the specific test group were analysed using the Tukey-Kramer test.

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## Author contributions

K.S., Y. Imai and N.H. designed the research; K.S., Y. Imai, S.Y., T.K. and Y. Ishihama performed the experiments; S.S. contributed new reagents/analytic tools; K.S. and Y. Imai analysed the data; and Y. Imai and N.H. wrote the paper. K.S. and Y. Imai contributed equally to this work.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

**Competing financial interests:** The authors declare no competing financial interests.

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