Okatsu et al., http://www.jcb.org/cgi/content/full/jcb.201410050/DC1

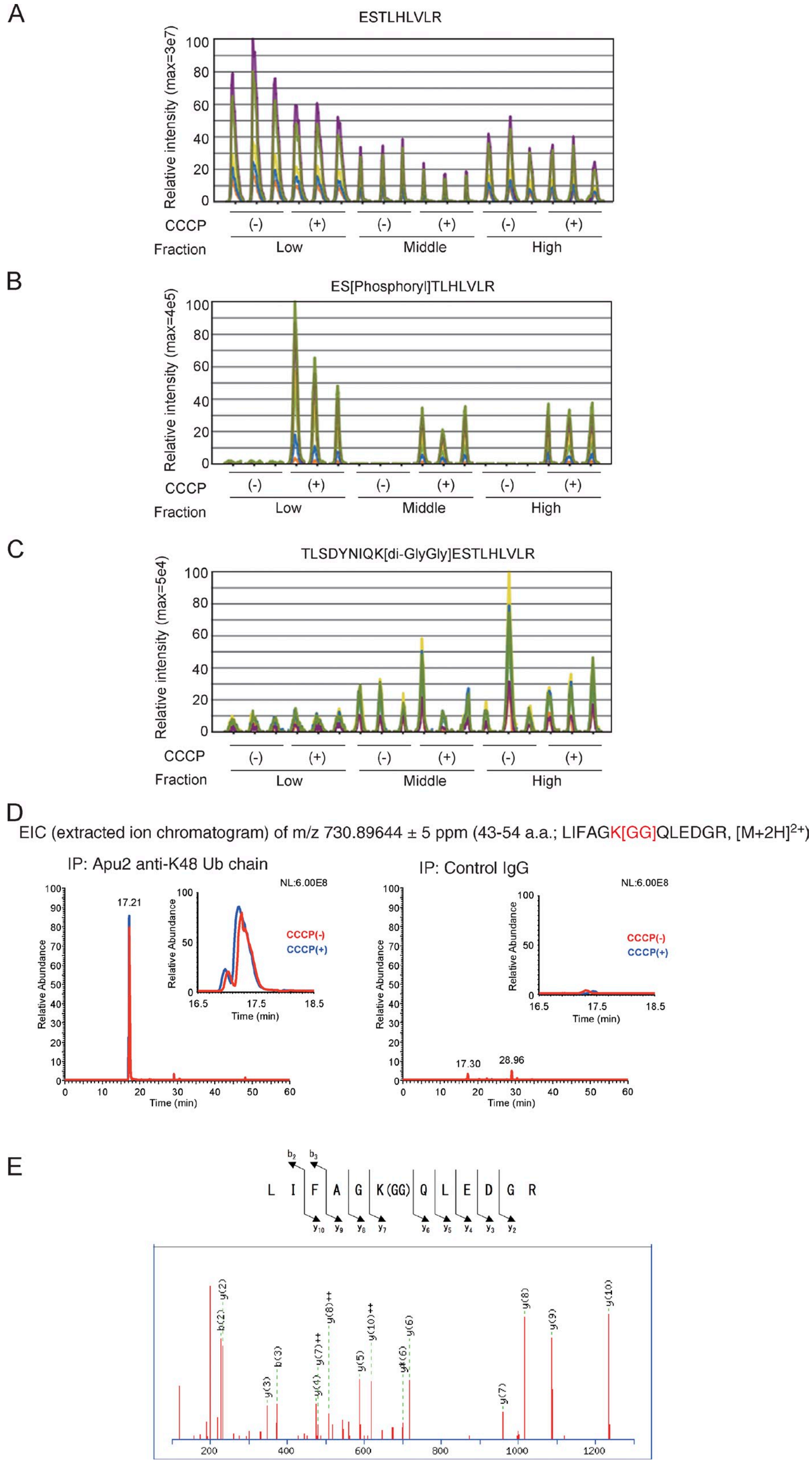

Figure S1. **Mass-spectrometry–based analysis of phosphorylated ubiquitin.** (A–C) Signals derived from the nonmodified peptide (ESTLHLVLR; A), the S65-phosphorylated peptide (ES[Phosphoryl]TLHLVLR; B), and the K63-GlyGly branch peptide (TLSDYNIQK[di-GlyGly]ESTLHLVLR) were obtained by MS-based analyses from whole cell lysates. The unmodified peptide was detected in all fractions examined (A), whereas the S65-phosphorylated peptide was observed only in CCCP-treated fractions (B), and the peptide with the K63-GlyGly branch (TLSDYNIQK[di-GlyGly]ESTLHLVLR) was detected in the middle (14,000–55,000) and the high (>55,000) molecular weight fractions (C). The data shown are from a single MS analysis of three independently prepared samples. (D) Extracted m/z 730.89644 ion chromatogram corresponding to the doubly charged ubiquitin peptide containing a diglycine branch at K48 in anti-K48–linked polyubiquitin chain antibody (Apu2) immunoprecipitates or control IgG. This experiment was completed once (*n* = 1). (E) The diglycine branch at K48 is demonstrated by the MS/MS spectrum of the m/z 730.90 ion at a retention time of 17.23 min in Apu2 immunoprecipitates from CCCP-treated cells.

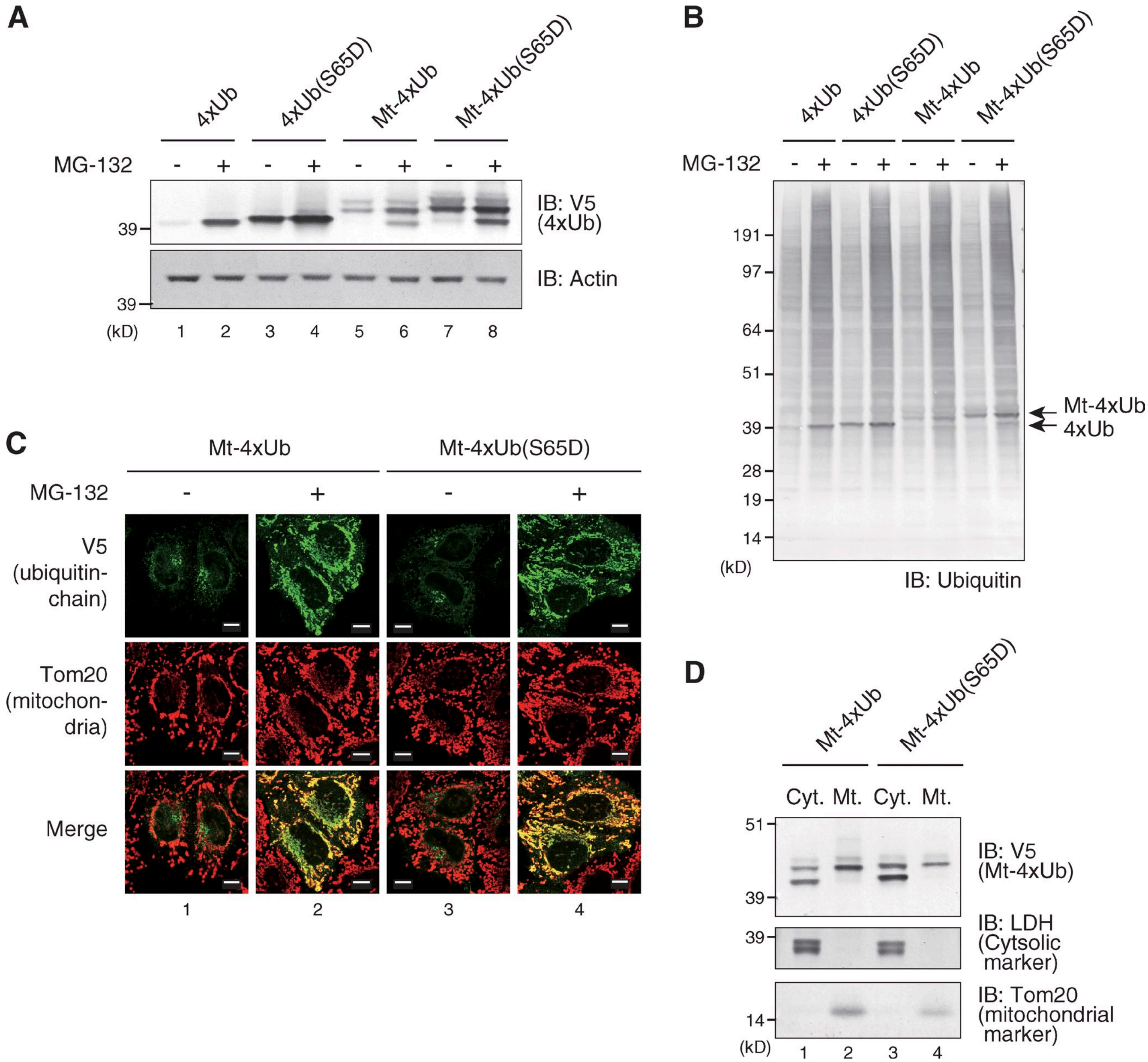

Figure S2. **Tandem tetra-ubiquitin targeting to mitochondria is stabilized by treatment with the proteasome inhibitor MG-132.** (A) HeLa cells expressing 4×Ub, 4×Ub(S65D), Mt-4×Ub, or Mt-4×Ub(S65D) were treated with MG-132 (10 µM, 3 h) and immunoblotted using the indicated antibodies. In the tandem repeat ubiquitin proteins, a G76V mutation was introduced to impede ubiquitin processing enzymes. 4×Ub and Mt-4×Ub were clearly stabilized by MG-132 treatment. (B) The total cell lysates from A were immunoblotted using an anti-ubiquitin antibody P4D1. (C) Immunocytochemistry of HeLa cells expressing Mt-4×Ub and Mt-4×Ub(S65D) after MG-132 treatment (10 µM, 3 h) confirmed that both Mt-4×Ub and Mt-4×Ub(S65D) localized on mitochondria. Bars, 10 µm. (D) HeLa cells expressing Mt-4×Ub and Mt-4×Ub(S65D) were fractionated to obtain cytosolic (Cyt) and mitochondria-enriched (Mt) fractions, and immunoblotted with the indicated antibodies.

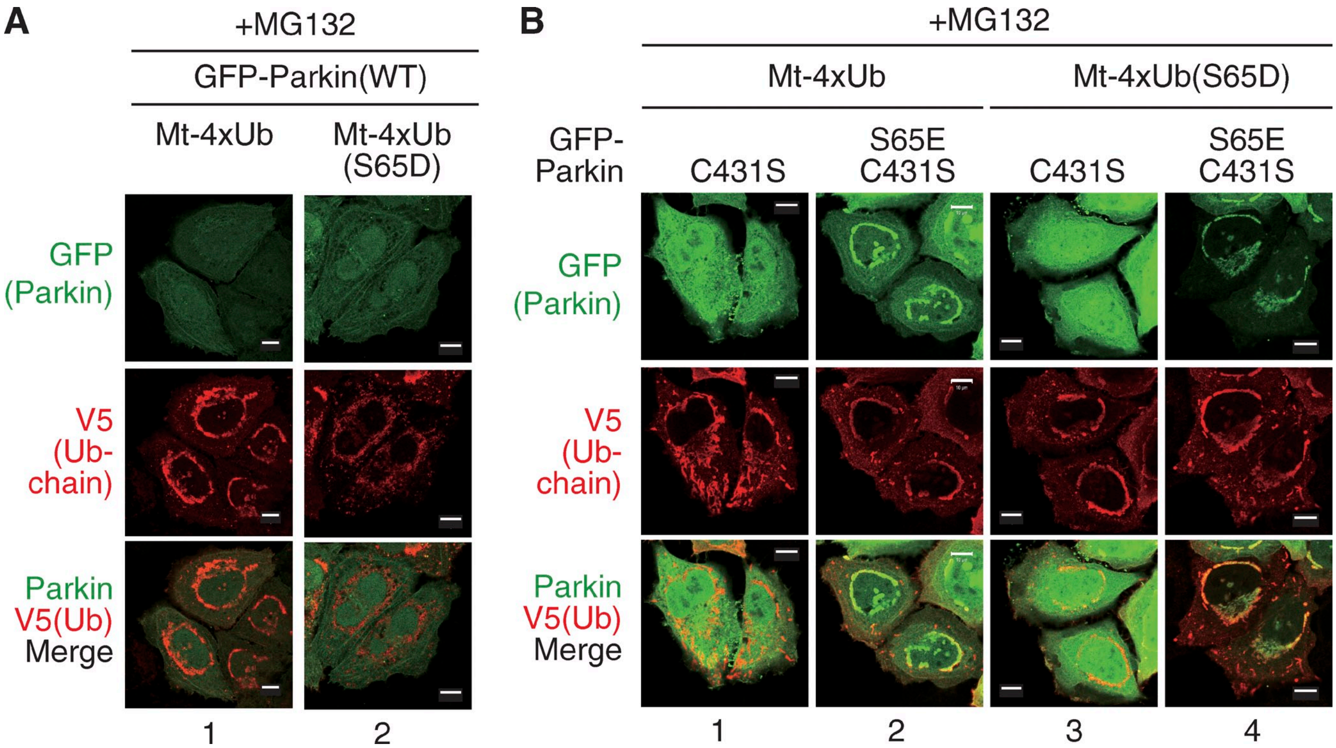

Figure S3. **Colocalization between Parkin and tetra-ubiquitin chain.** (A and B) HeLa cells expressing WT GFP-Parkin (A) or the indicated GFP-Parkin mutants (B) were coexpressed with Mt-4×Ub or Mt-4×Ub(S65D), treated with MG-132 (10 µM, 3 h), and subjected to immunocytochemistry using an anti-GFP antibody and an anti-V5 antibody that detects Mt-4×Ub. Bars, 10 µm.

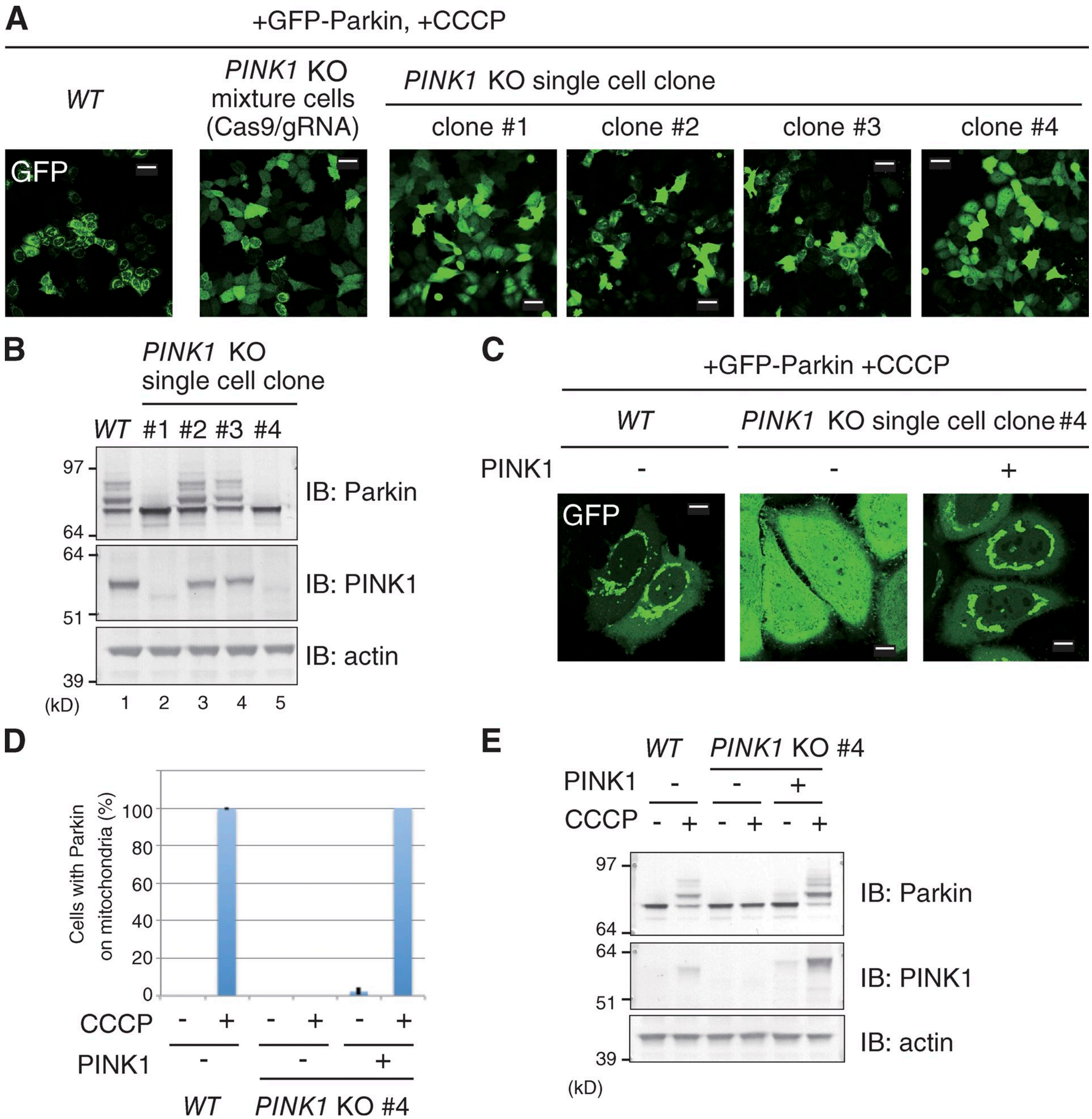

Figure S4. **Generation of PINK1 KO HeLa cell line.** (A and B) WT HeLa cells, HeLa cells treated with the CRISPR/Cas9 system for PINK1 KO, and the resulting candidate clonal cells (#1–4) were transfected with GFP-Parkin and treated with CCCP (10 µM, 6 h). Mitochondrial localization of GFP-Parkin in these cells was observed with a fluorescence microscope (A), and PINK1 expression and Parkin E3 activity were detected by immunoblotting with the indicated antibodies (B). Bars, 10 µm. (C and D) Mislocalization of GFP-Parkin in clone #4 was complemented by reintroduction of PINK1. (C) The subcellular localization of GFP-Parkin in the indicated cells was observed with a fluorescence microscope after CCCP treatment (10 µM, 6 h). Bars, 10 µm. (D) The rate of Parkin mitochondrial localization in 100 cells was calculated in three independent experiments. Error bar represents the mean ± SD (error bars). (E) Restoration of Parkin E3 activity by exogenous PINK1. PINK1 KO HeLa cells expressing PINK1 and GFP-Parkin were treated with 10 µM CCCP for 6 h, and then immunoblotted with the indicated antibodies.

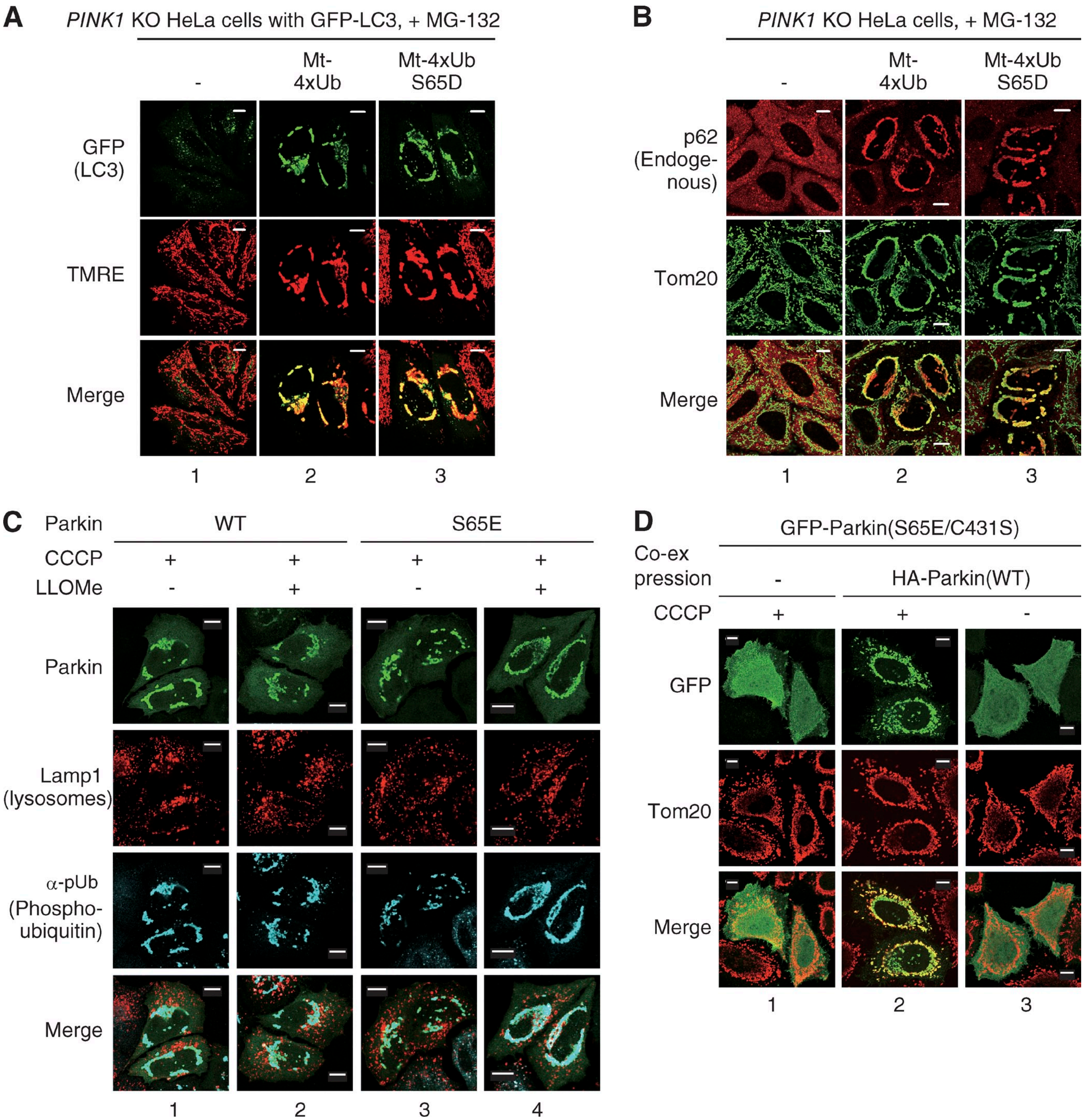

Figure S5. **Immunocytochemical analyses suggest recruitment of Parkin by mitochondrial phosphorylated ubiquitin and recruitment of LC3 and p62 by a mitochondrial ubiquitin chain.** (A) In PINK1 KO HeLa cells, GFP-LC3 does not merge with mitochondria under steady-state conditions (1), whereas Mt-4×Ub and Mt-4×Ub(S65D) trigger obvious accumulation of LC3 on energized TMRE-stainable mitochondria (2 and 3). (B) Endogenous p62 is dispersed throughout the cytosol (1), whereas it accumulates on mitochondria in Mt-4×Ub– or Mt-4×Ub(S65D)–expressing cells (2 and 3). (C) HeLa cells stably expressing PINK1 were transfected with WT GFP-Parkin or phosphomimetic GFP-Parkin (S65E mutant), treated with CCCP and LLOMe, and then immunostained with the indicated antibodies. WT and phosphomimetic Parkin were transported to mitochondria (see also Fig. 5 C) regardless of LLOMe treatment. (D) HeLa cells expressing GFP-Parkin(S65E/C431S) with or without WT HA-Parkin were treated with CCCP (10 µM, 1.5 h), and immunostained with anti-GFP and anti-Tom20 antibodies. Mislocalization of the GFP-Parkin(S65E/C431S) mutant after CCCP treatment was complemented by coexpressing WT Parkin in trans. Bars, 10 µm.

Table S1. **List of plasmids used**

Vector	Description	Source
pGEX-6P-1-*Tc*PINK1	For expression of *Tc*PINK1 in *E. coli*	Koyano et al., 2014
pEGFP-C1-Parkin WT	For expression of GFP-Parkin WT	Matsuda et al., 2010
pEGFP-C1-Parkin S65E	For expression of GFP-Parkin S65E	Iguchi et al., 2013
pEGFP-C1-Parkin C431S	For expression of GFP-Parkin C431S	Iguchi et al., 2013
pEGFP-C1-Parkin S65E/C431S	For expression of GFP-Parkin S65E/C431S	Iguchi et al., 2013
pcDNA3.1-HA-Parkin WT	For expression of HA-Parkin WT	Matsuda et al., 2010
pcDNA3.1-Mt-Parkin WT	For expression of Mt-HA-Parkin WT	This study
pcDNA3-4×Ub	For expression of V5-Ub G76V	This study
pcDNA3-4×Ub S65D	For expression of V5-Ub S65D/G76V	This study
pcDNA3-Mt-4×Ub	For expression of Tom20 N33-V5-Ub G76V	This study
pcDNA3-Mt-4×Ub S65D	For expression of Tom20 N33-V5-Ub S65D/G76V	This study
pcDNA3-Mt-4×Ub S65A	For expression of Tom20 N33-V5-Ub S65A/G76V	This study
pCMVTNT(d1)-PINK1 WT -3HA	For weekly expression of PINK1 WT -3HA	Okatsu et al., 2012
pMXs-puro-PINK1 WT -3FLAG	For expression of PINK1 WT -3FLAG	Matsuda et al., 2010
pMXs-puro-PINK1 KD -3FLAG	For expression of PINK1 KD -3FLAG	Matsuda et al., 2010
pMXs-puro-PINK1 ΔN155 -3FLAG	For expression of PINK1 ΔN155 -3FLAG	Matsuda et al., 2010
pGEX-6P-1-GST-Parkin WT	For expression of GST-*Rn*Parkin WT in *E.coli*	Trempe et al., 2013
pGEX-6P-1-GST-Parkin S65E	For expression of GST-*Rn*Parkin S65E in *E.coli*	Koyano et al., 2014
pcDNA3-Ub^r-L40-IRES-HA-Ub^r-S27a	For inducible expression of Ub^r-L40 and HA-Ub^r-S27a	Xu et al., 2009
pcDNA3-Ub^r S65A-L40-IRES- HA-Ub^r S65A-S27a	For inducible expression of Ub^r S65A-L40 and HA-Ub^r S65A-S27a	This study

KD, kinase dead; Ub^r, siRNA-resistant ubiquitin; IRES, ribosomal entry site

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