Supporting Information

The interaction between E3 ubiquitin ligase Parkin and mitophagy receptor PHB2 links inner mitochondrial membrane ubiquitination to efficient mitophagy

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Figure S1:

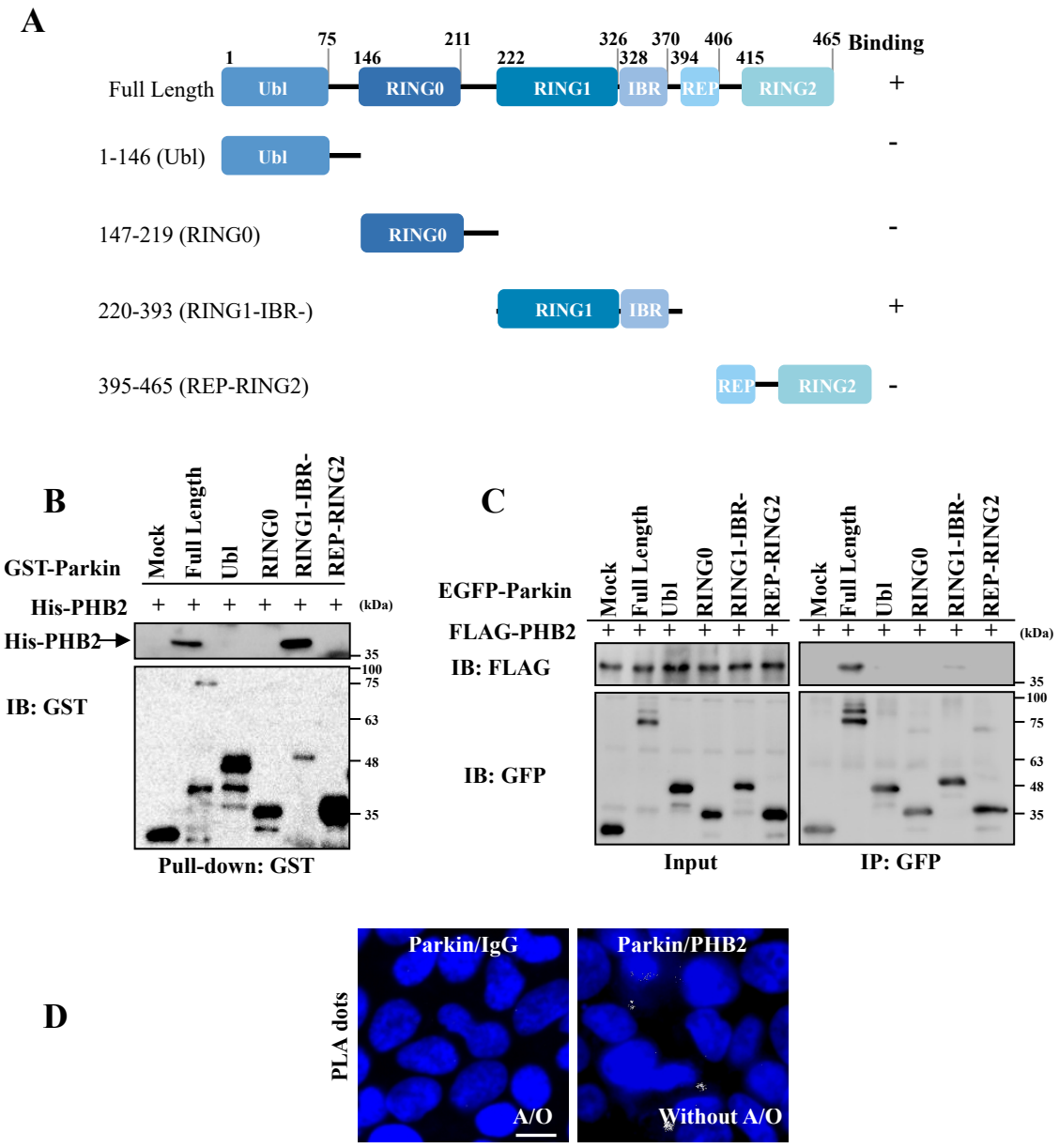

Figure S1. Identification of the domain required for Parkin-PHB2 interaction. (A) Schematic diagram of Parkin constructs used in this study. **(B)** After purification by GST binding resin, different constructs of GST-Parkin (as indicated in A) were separately mixed with His-PHB2. Bound proteins were analyzed by immunoblot analysis with anti-GST and anti-PHB2 antibodies. **(C)** Different constructs of EGFP-Parkin (as indicated in A) and FLAG-PHB2 were transfected into HEK293T cells, and then were treated with 5 μg/ml A/O for 4 h. Cell lysates were processed for immunoprecipitation analysis using anti-GFP antibody and then were analyzed with anti-GFP and anti-FLAG antibodies. **(D)** HEK293 cells expressing EGFP-Parkin were treated with or without 5 μg/ml A/O for 4 h, and then the cells were fixed and stained with anti-GFP and anti-IgG (R) or anti-PHB2 antibody in PLA experiment. The cells were visualized using Nikon microscope. Blue: nuclei (DAPI); white dots: PLA positive puncta. Scale bar, 10 μm.

Figure S2:

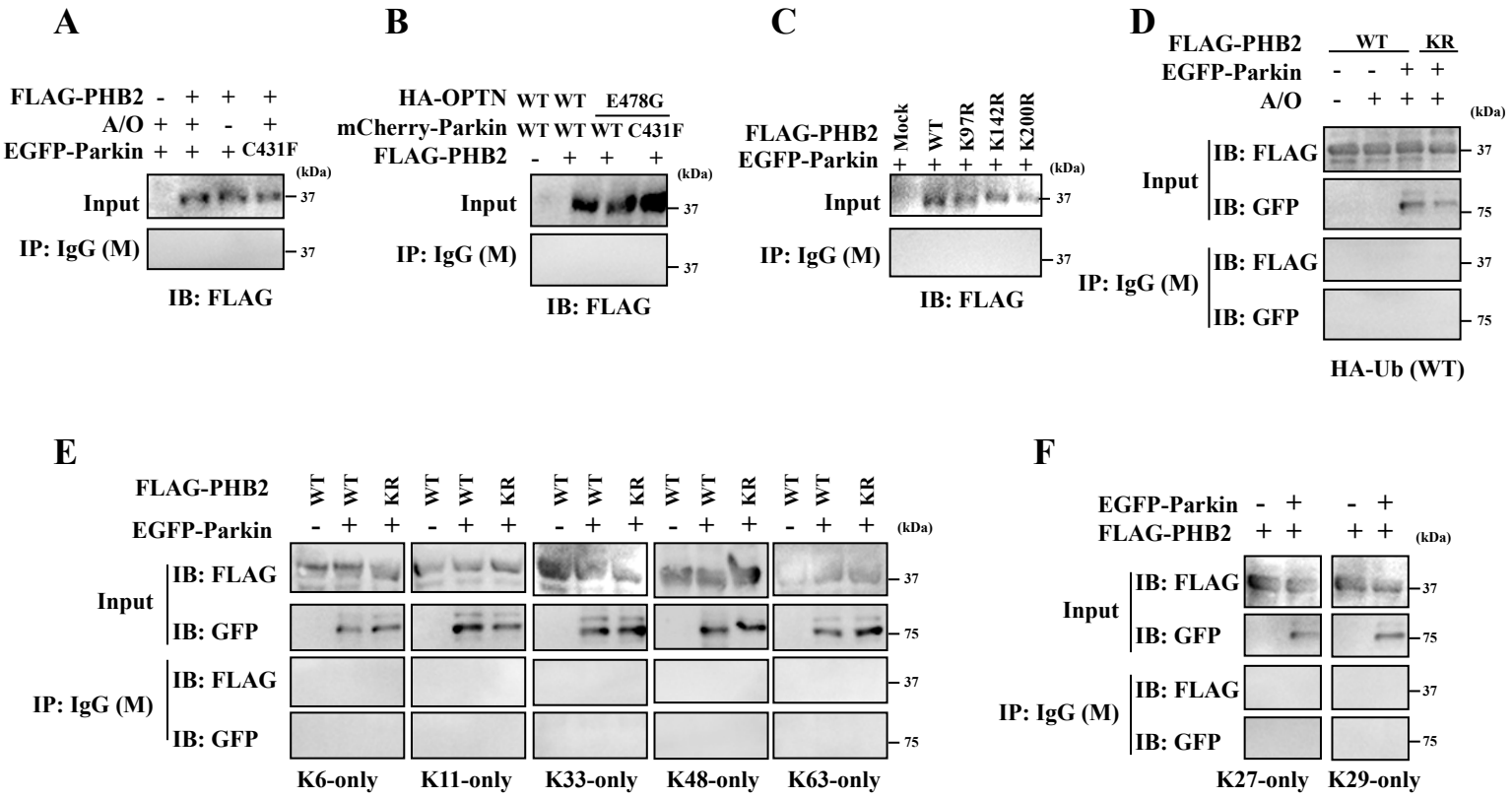

Figure S2. IgG control experiments related to Figure 3. (A) FLAG vector or FLAG-PHB2 were transfected into HEK293T cells with WT or C431F mutant EGFP-Parkin respectively, followed by treatment with or without 5 μg/ml A/O for 4 h. Cell lysates were subjected to immunoprecipitation analysis using anti-IgG (M) antibody, and then were detected using anti-FLAG antibody. **(B)** WT or E478G mutant HA-OPTN, WT or C431F mutant mCherry-Parkin, and FLAG vector or FLAG-PHB2 were transfected into si-OPTN HEK293T cells respectively, followed by treatment with 5 μg/ml A/O for 4 h. Cell lysates were subjected to immunoprecipitation analysis using anti-IgG (M) antibody, and then were detected using anti-FLAG antibody. **(C)** FLAG vector or WT, K97R, K142R, K200R mutant FLAG-PHB2 were transfected into HEK293T cells, along with EGFP-Parkin respectively, followed by treatment with 5 μg/ml A/O for 4 h. Cell lysates were subjected to immunoprecipitation analysis using anti-IgG (M) antibody, and then were detected using anti-FLAG antibody. **(D)** EGFP vector or EGFP-Parkin, and WT or KR mutant FLAG-PHB2 were transfected into HEK293T cells respectively, along with WT HA-Ub, and then the cells were treated with or without 5 μg/ml A/O for 4 h. Cell lysates were subjected to immunoprecipitation analysis using anti-IgG (M) antibody, and then were detected using indicated antibodies. Here and hereafter KR mutant FLAG-PHB2 indicates K142R/K200R mutant FLAG-PHB2. **(E)** EGFP vector or EGFP-Parkin, along with WT or KR FLAG-PHB2 were transfected into HEK293T cells with HA-Ub (K6, K11, K33, K48 or K63-only) respectively, followed by treatment with 5 μg/ml A/O for 4 h. Cell lysates were subjected to immunoprecipitation analysis using anti-IgG (M) antibody, and then were detected using indicated antibodies. **(F)** The similar experiment (without KR mutant FLAG-PHB2) were separately performed in HA-Ub (K27 or K29-only) as **(E)**.

Figure S3:

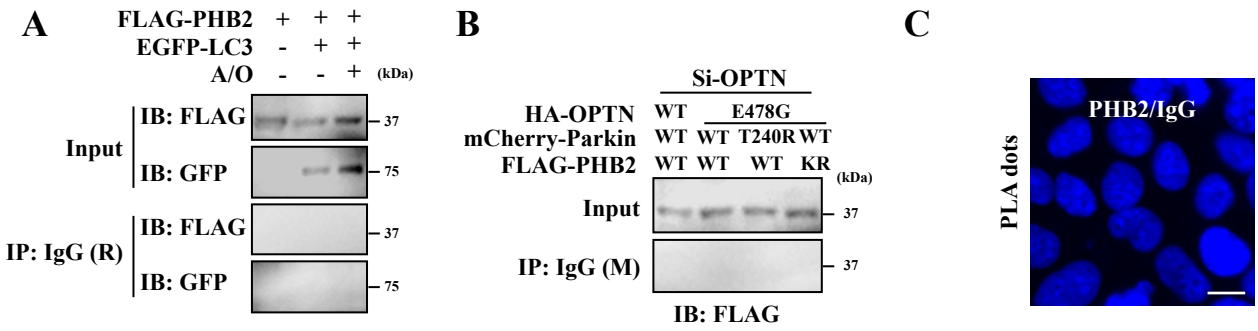

Figure S3. IgG control experiments related to Figure 4. (A) HEK293T cells expressing EGFP vector or EGFP-LC3 with FLAG-PHB2 were treated with DMSO or 5 μg/ml A/O for 4 h. Cell lysates were subjected to immunoprecipitation analysis using anti-IgG (R) antibody, and then were detected using indicated antibodies. **(B)** WT or E478G mutant HA-OPTN, WT or T240R mutant mCherry-Parkin, WT or KR mutant FLAG-PHB2 with EGFP-LC3 were transfected into si-OPTN HEK293T cells, followed by treatment with 5 μg/ml A/O for 4 h. Cell lysates were subjected to immunoprecipitation analysis using anti-IgG (M) antibody, and then were detected using anti-FLAG antibody. **(C)** WT FLAG-PHB2 and HA-Parkin were transfected into HEK293 cells, followed by treatment with 5 μg/ml A/O for 4 h. Then the cells were fixed and stained with anti-FLAG and anti-IgG (R) antibody in PLA experiment. The cells were visualized using Nikon microscope. Blue: nuclei (DAPI); white dots: PLA puncta. Scale bar, 10 μm.

Figure S4:

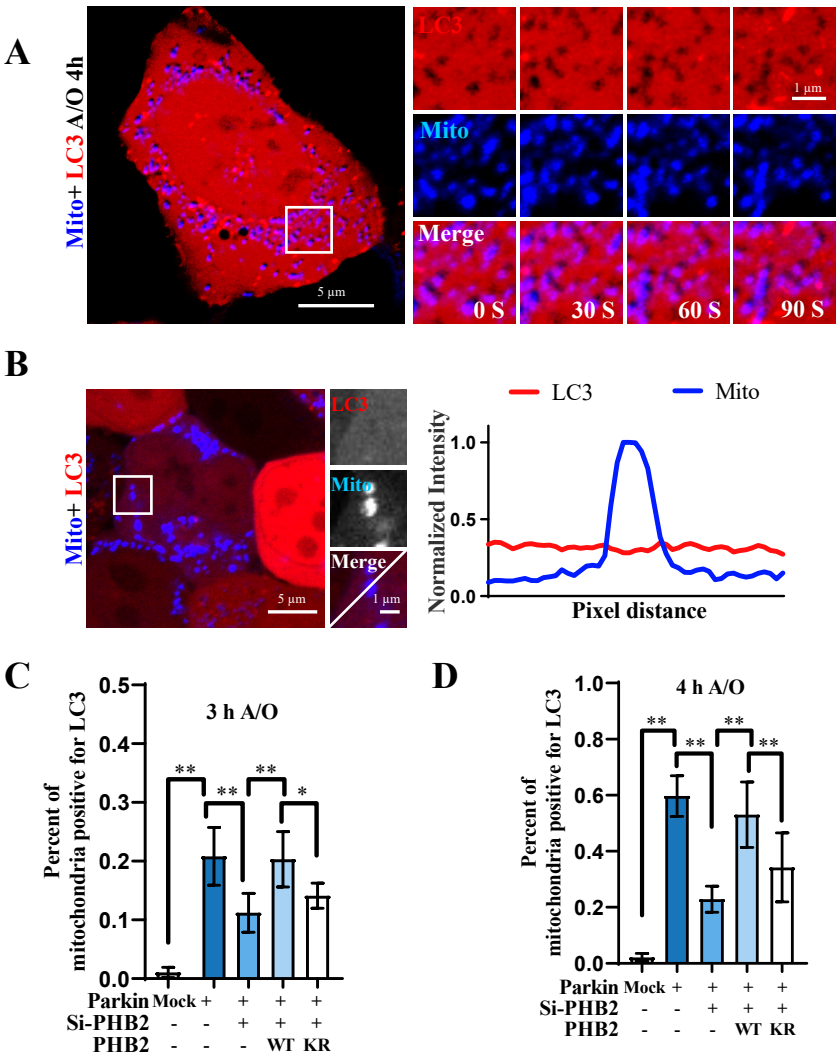

Figure S4. Control experiments and quantification data related to Figure 5. (A-B) HEK293 cells expressing BFP-Mito, EGFP-OPTN and mCherry-LC3 were treated with 5 μg/ml A/O for 6 h. The cells were analyzed by multi-channel live cell imaging using confocal microscopy. **(A)** Regions in white box was magnified on the right side. **(B)** The fluorescence intensity of the paint line (white line) was measured. **(C-D)** FLAG vector or FLAG-Parkin, BFP-Mito, EGFP-OPTN and mCherry-LC3 (with or without WT or KR mutant FLAG-PHB2) were transfected into control or si-PHB2 HEK293 cells. The cells were treated with 5 μg/mL A/O for 3 h (C) or 4 h (D), and then LC3 "rings" co-localization with mitochondria was qualified. Data were collected from three independent experiments and were represented as means ± S.D., **, P<0.01; *, P<0.05, *One-way ANOVA* followed by post hoc Tukey's tests.

Figure S5:

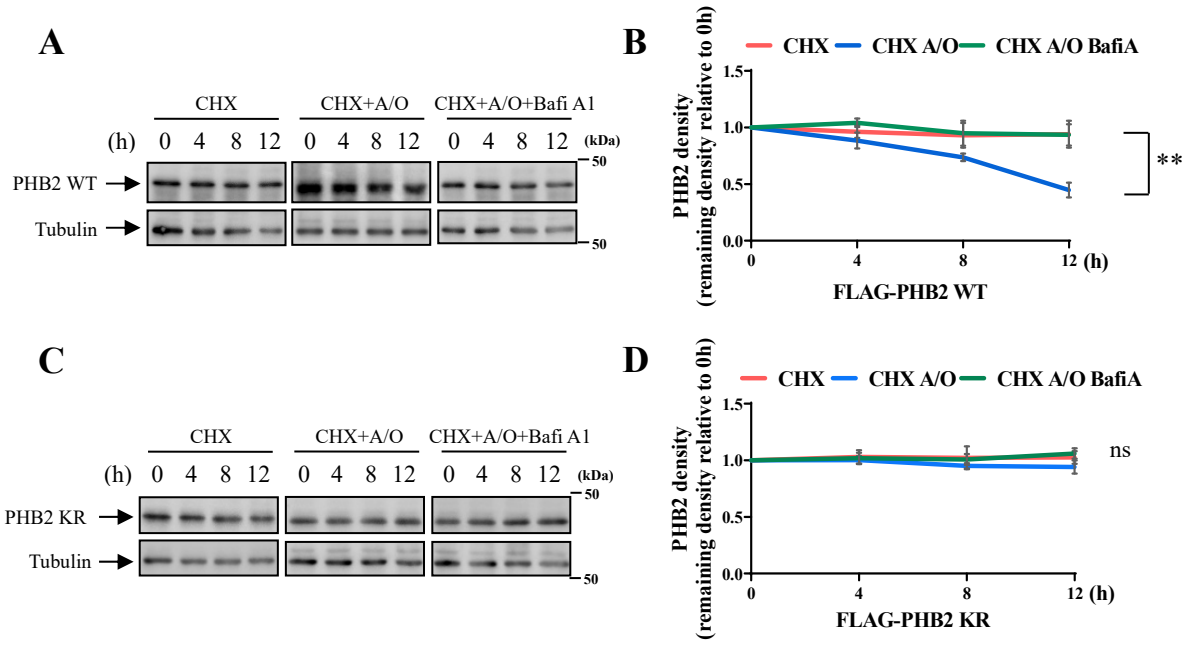

Figure S5. PHB2 is degraded by lysosomal pathway. (A-D) HEK293T cells expressing WT or KR mutant FLAG-PHB2 with mCherry-Parkin were treated with cycloheximide (CHX, 100 μg/ml), along with or without A/O (5 μg/ml) or bafilomycin A_1 (Bafi A1, 100 nM) for 0 h, 4 h, 8 h, 12 h. Then the cell lysates were analyzed by immunoblot analysis with anti-FLAG and anti-Tubulin antibodies. **(B)** and **(D)** separately showed the quantification of protein level about FLAG-PHB2-WT **(A)** and FLAG-PHB2-KR **(C)** at the indicated times. Data from three independent experiments were represented as means ± S.D., **, $p<0.01$; ns, not significantly, *One-way ANOVA* followed by post hoc Tukey's tests..

Figure S6:

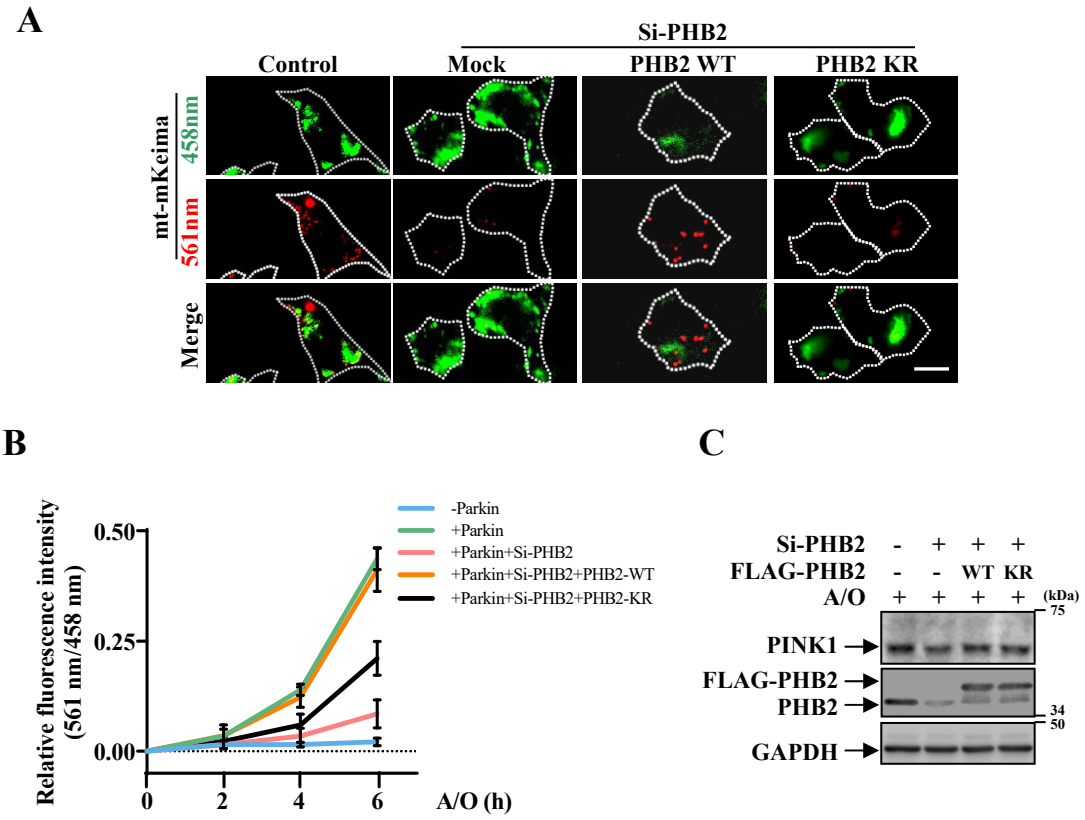

Figure S6. Parkin-mediated ubiquitination of PHB2 is required for efficient mitophagy. (A) SH-SY5Y cells were transfected with control or si-PHB2 and then were re-transfected with WT or KR mutant FLAG-PHB2 with EGFP-Parkin and mt-mKeima. The cells were treated with 5 μg/mL A/O for 4 h, and then were analyzed by confocal microscopy. Scale bars, 10 μm. **(B)** HEK293 cells were transfected with control or si-PHB2 and then were re-transfected with WT or KR mutant FLAG-PHB2 and mt-mKeima with or without EGFP-Parkin. The cells were treatment with 5 μg/ml A/O for 0, 2, 4 or 6 h, and then relative fluorescence intensity (561 nm/458 nm) was quantified. Data from three independent experiments were represented as means ± S.D. **(C)** FLAG vector, WT or KR mutant FLAG-PHB2 were transfected into control or si-PHB2 HEK293T cells, and then the cells were treated with 5 μg/mL A/O for 4 h. Cell lysates were analyzed by immunoblot analysis with anti-PINK1, anti-PHB2 and anti-GAPDH antibodies.