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Supplementary Information for

Decision between mitophagy and apoptosis by Parkin via VDAC1 ubiquitination

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Supplementary text
Fig. S1 to S6
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Supplementary Materials

Constructs

Wild-type (WT) VDAC1 plasmid was constructed into pcDNA3.1 zeo (+) N-terminal HA-tagged vector. VDAC1 mutants (K12R, K20R, K53R, K109R, K110R and K274R) and 63 PD missense mutants of Parkin (Fig. S8*A-E*) were generated using a site-directed point mutagenesis method. N-terminal flag-tagged human ubiquitin was cloned into pcDNA3.1 vector. N-terminal GST-tagged pEBG vector and N-terminal Myc-tagged pcDNA3.1 zeo (+) vector were used to express Parkin constructs. The E3 ligase activity-dead mutant of Parkin, Parkin C431S, and other Parkin PD patient mutants were constructed by site-directed mutagenesis in either pEBG vector or Myc-tagged pcDNA3.1 zeo (+) vector. In addition, human PINK1 WT 3×Myc and human PINK1 kinase-dead (K219A, D362A, and D384A) 3×Myc were cloned into pcDNA3.1 zeo (+) vector. Porin cDNA was cloned into pCDNA3 N-terminal HA-tagged vector. *Drosophila* Parkin WT and C431S were cloned into pAC5.1 vector. Cells were treated with carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Sigma).

Antibodies

For immunoblot analyses the following antibodies were used: rabbit anti-HA (1:1,000; Cell Signaling), mouse anti-Flag (1:1,000; MBL), rabbit anti-ubiquitin (1:1,000, Cell Signaling), mouse anti-GST (1:1,000, Upstate), mouse anti-Myc (1:1,000, MBL), rabbit anti-cleaved caspase3 (1:1,000, Cell Signaling), mouse anti-VDAC1/Porin (1:1,000, Abcam), and mouse anti-tubulin antibody (1:1,000, E7 clone, DSHB). Peroxidase-conjugated secondary antibodies were purchased from Jackson Laboratory.

Immunoblotting for *Drosophila*

Adult flies (n=10) were homogenized with RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 2 mM DTT, 1 mM PMSF, 10 g/ml leupeptin, and 1 g/ml pepstatin A) at 4°C for 30 minutes. Total proteins were quantified using the BCA protein assay kit (Pierce). Lysates were subjected to SDS-PAGE analysis followed by immunoblotting according to standard procedures. The blots were developed and viewed under LAS4000 (Fujifilm).

Transmission electron microscopy

HeLa cells stably expressing GFP-Parkin were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2)-containing 0.1% $CaCl_2$ for 3 hours at RT. They were washed five times with 0.1 M cacodylate buffer at 4°C. Then, they were post-fixed with 1% OsO4 in 0.1 M cacodylate buffer-containing 0.1% $CaCl_2$ for 2 hours at 4°C. After rinsing with cold distilled water, the cells were dehydrated slowly with an ethanol series and propylene oxide at 4°C. The samples were embedded in Embed-812 (EMS). After polymerization of the resin at 60°C for 36 hours, serial sections were cut with a diamond knife on an ULTRACUT UC7 ultramicrotome (Leica) and mounted on formvar-coated slot grids. Sections were stained with 4% uranyl acetate for 10 minutes and lead citrate for 7 minutes. They were observed using a Tecnai G2 Spirit Twin transmission electron microscope (FEI).

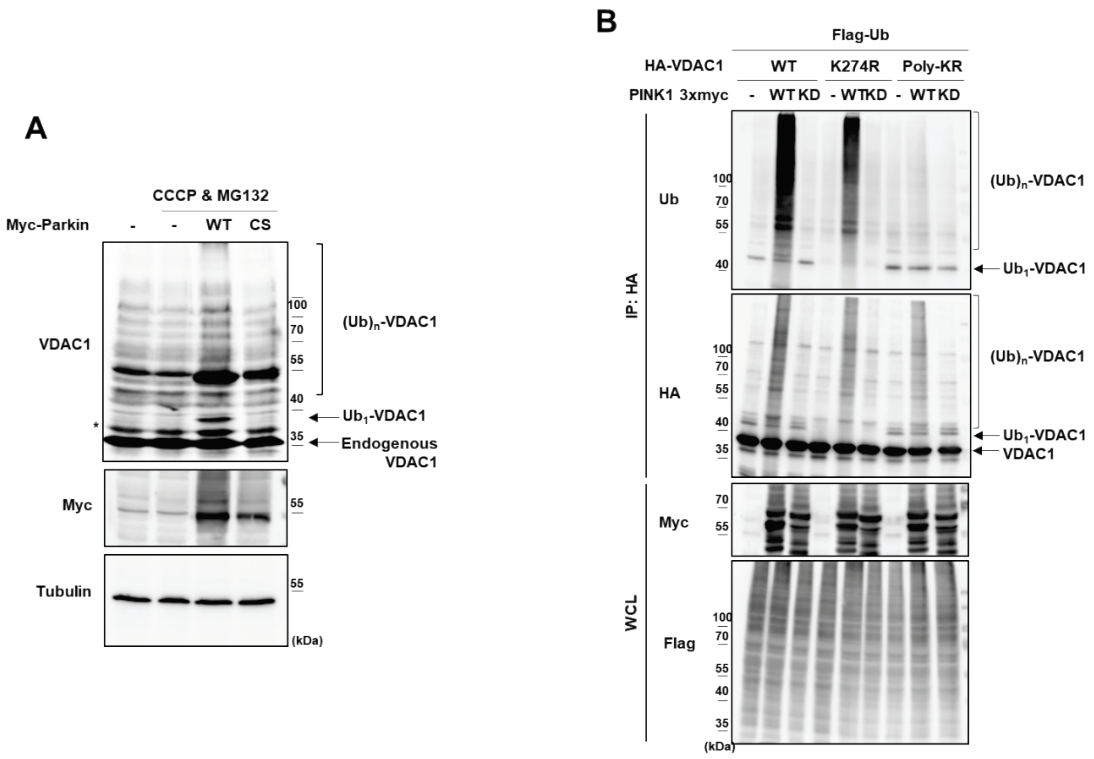


Fig. S1. VDAC1 mono- and poly-ubiquitination are dependent on PINK1 and Parkin activity.

(*A*) Ubiquitination assays for endogenous VDAC1. Myc-tagged Parkin wild type (WT) or CS (C431S; an E3 ligase-dead Parkin mutant) was expressed in HEK293T cells and treated with 20 μM CCCP and 20 μM MG132 for 4 hours. Whole cell lysates were analyzed for immunoblot analysis with indicated antibodies. The mono-ubiquitination band was marked on the right side (Ub_1-VDAC1) and the poly-ubiquitination bands were marked by a bracket. Star was marked for a non-specific protein band. (*B*) HEK293T cells expressing Flag-tagged ubiquitin and HA-tagged VDAC1 wild type (WT), K274R, or Poly-KR were co-expressed with Myc-tagged PINK1 WT or a kinase-dead mutant (KD; K219A, D362A, and D384A). Whole cell lysates (WCL) or anti-HA immunoprecipitated (IP) samples were analyzed for immunoblotting to detect indicated proteins. The mono- and poly-ubiquitinated VDAC1 were marked by arrows and square brackets, respectively.

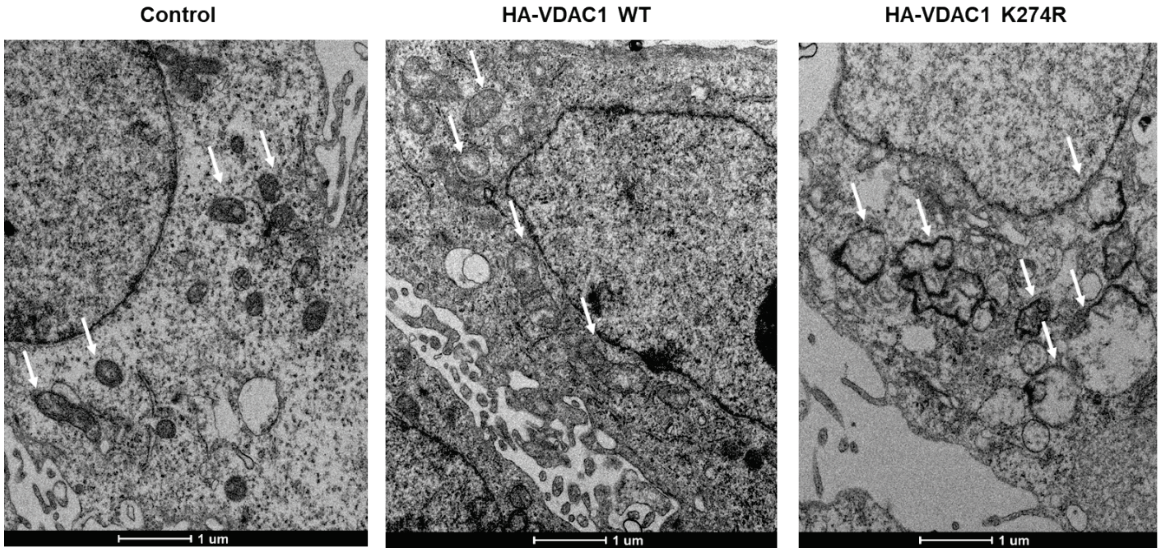


Fig. S2. Abnormal mitochondrial morphology displayed by VDAC1 K274R expression. HeLa cells stably expressing GFP-Parkin were expressed with an empty vector (left), HA-tagged VDAC1 WT (middle), or HA-tagged VDAC1 K274R mutant (right). We observed mitochondrial morphology under an electron microscopy. Scale bars indicate 1 μm. White arrows indicate mitochondria.

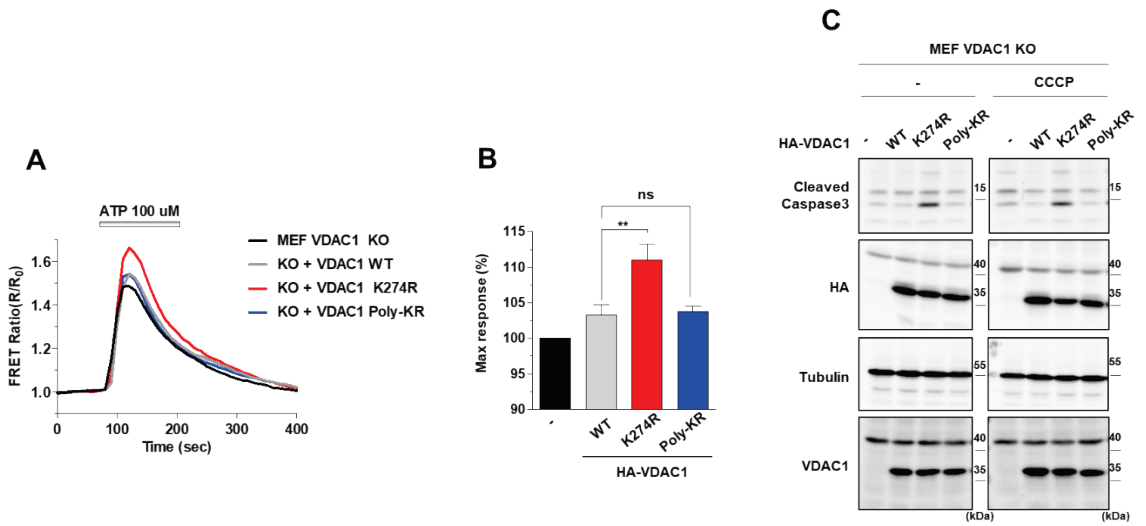


Fig. S3. VDAC1 K274R induces mitochondrial calcium uptake and apoptosis.
(*A-B*) VDAC1 KO mouse embryonic fibroblast (MEF) cells were transfected with HA-tagged VDAC1 WT, K274R, or Poly-KR and co-transfected with 4mitD3, a mitochondrial calcium indicator. Cells were then treated with 100 μM of ATP for 3 minutes and were monitored for the changes in 4mitD3 fluorescence. (*B*) Maximum mitochondrial calcium uptake for VDAC1 KO MEF cells expressing VDAC1 WT, K274R, or Poly-KR in (*A*). n=50~70 cells. Analyzed by ANOVA tukey's test, **p<0.05. (*C*) VDAC1 KO MEF cells expressing HA-tagged VDAC1 WT, K274R, or Poly-KR were compared with VDAC1 KO MEF cells (left panels). Those cells were treated with 30 μM CCCP for 6 hours (right panels). Whole cell lysates were analyzed for immunoblotting to detect indicated proteins.

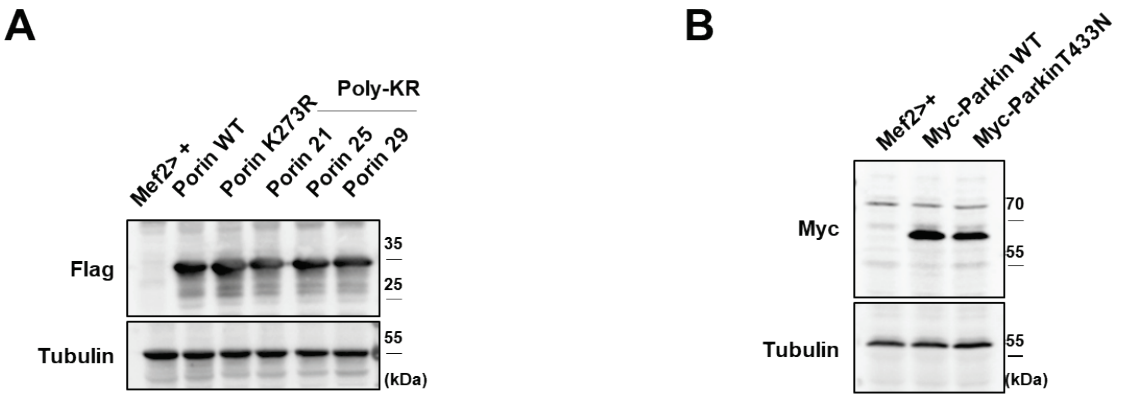


Fig. S4. Characterization of Porin or Parkin transgenic flies.
(*A*) Transgenic flies expressing Flag-tagged Porin were analyzed by immunoblotting to determine Porin protein levels. We used Porin WT, K273R, and #21 Poly-KR lines for further studies. (*B*) Transgenic flies expressing Myc-tagged Parkin WT or T433N using *Mef2-gal4* driver were analyzed for Parkin protein levels.

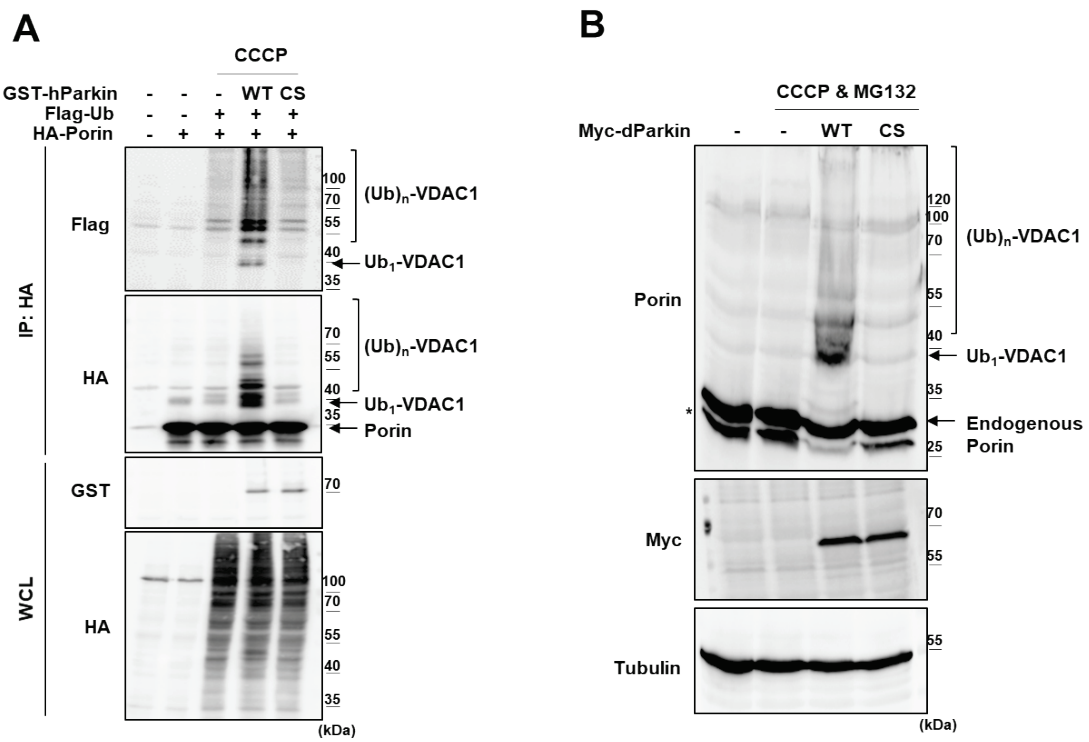


Fig. S5. Parkin-dependent ubiquitination of Porin in HEK293T cells and S2 cells.
(*A*) Ubiquitination assays for Porin (the *Drosophila* orthologue of human VDAC1) by Parkin. HEK293T cells were transfected with indicated constructs and treated with or without 20 μM CCCP for 4 hours. Whole cell lysates (WCL) were immunoprecipitated (IP) and analyzed for immunoblot (IB) analyses with indicated antibodies. Human Parkin wild type (WT) and CS (C431S) mutants were expressed as indicated. (*B*) Ubiquitination assays for Porin in Schneider 2 (S2) cells. S2 cells were transfected with Myc-tagged *Drosophila* Parkin (dParkin) WT or CS (C431S) and were analyzed for ubiquitination of endogenous Porin protein after treatment of 40 μM MG132 and 40 μM CCCP for 6 hours. Whole cell lysates were analyzed for immunoblot analyses with indicated antibodies.

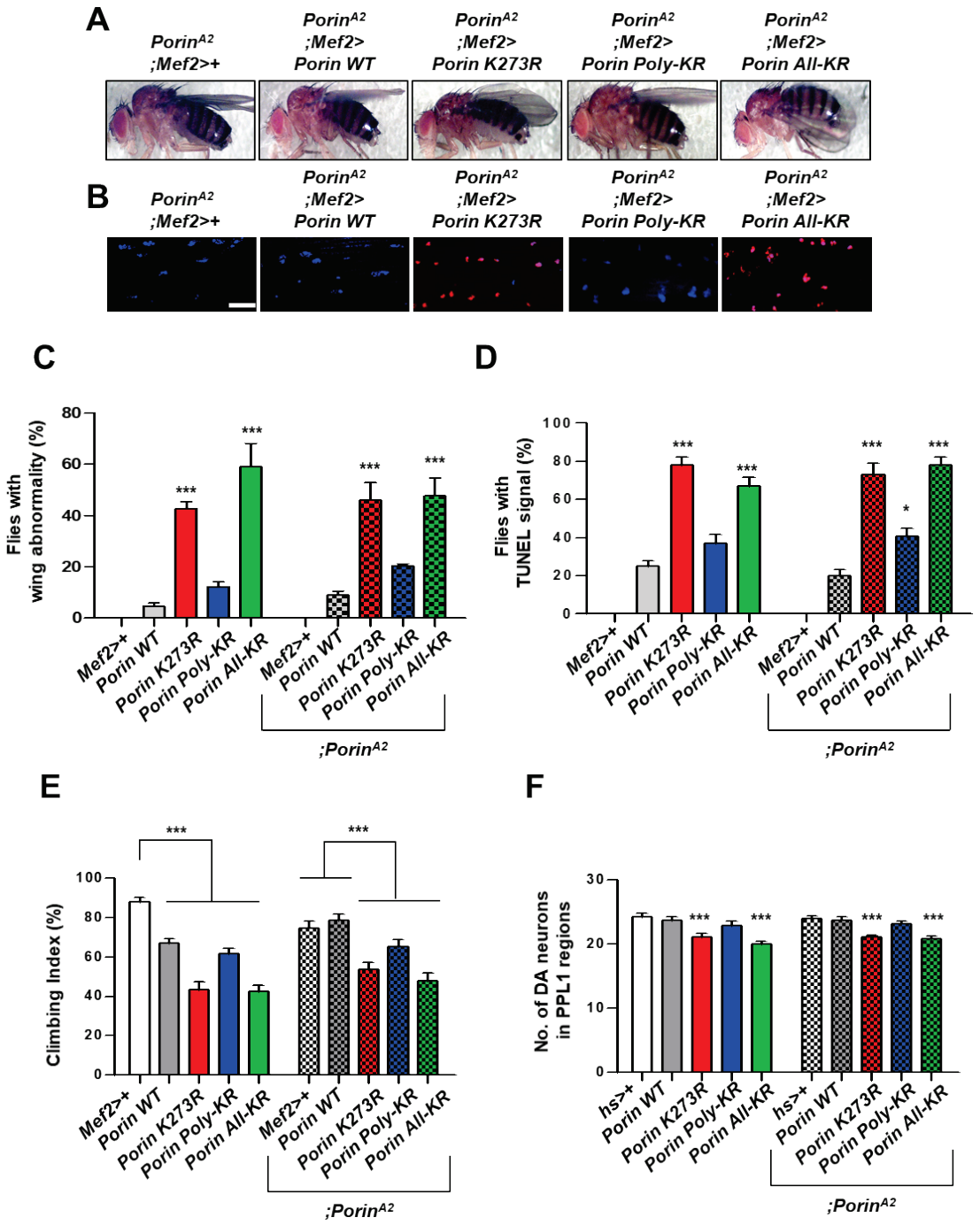


Fig. S6. Porin K273R and All-KR flies display phenotypes related to PD.
(*A*, *C*) Defects in the wing postures of Porin flies. Transgenic flies expressing Porin WT, K273R, Poly-KR, or All KR using *Mef2-gal4* driver in Proin (*Porin*^{A2}) null background. (*C*) Percentage of the flies with defective wing postures in (*A*). n=300 from three independent experiments. (*B*, *D*) TUNEL signals for apoptosis (red) and DAPI staining for the nucleus (blue) in the thoraces of transgenic flies. White bar indicates 5 μm. (*D*) Percentage of the flies with TUNEL signals in (*B*). n=100 from ten independent experiments. (*E*) Percentage of the transgenic flies with defective climbing ability. n=100 from ten independent experiments. (*F*) Numbers of DA neurons in the brain protocerebral posterior lateral 1 (PPL1) region. n=10. All quantifications were analyzed by

One-way ANOVA with tukey's multiple comparison test and are presented as mean ± SD, ***$p<0.001$, *$p<0.05$.

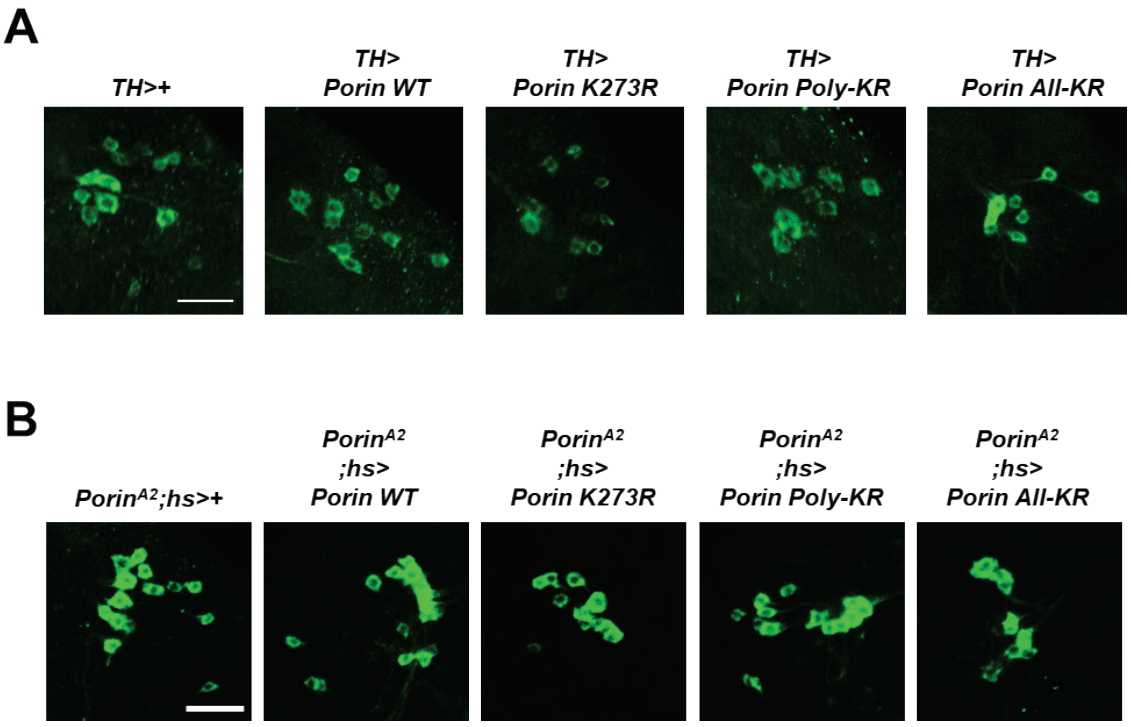
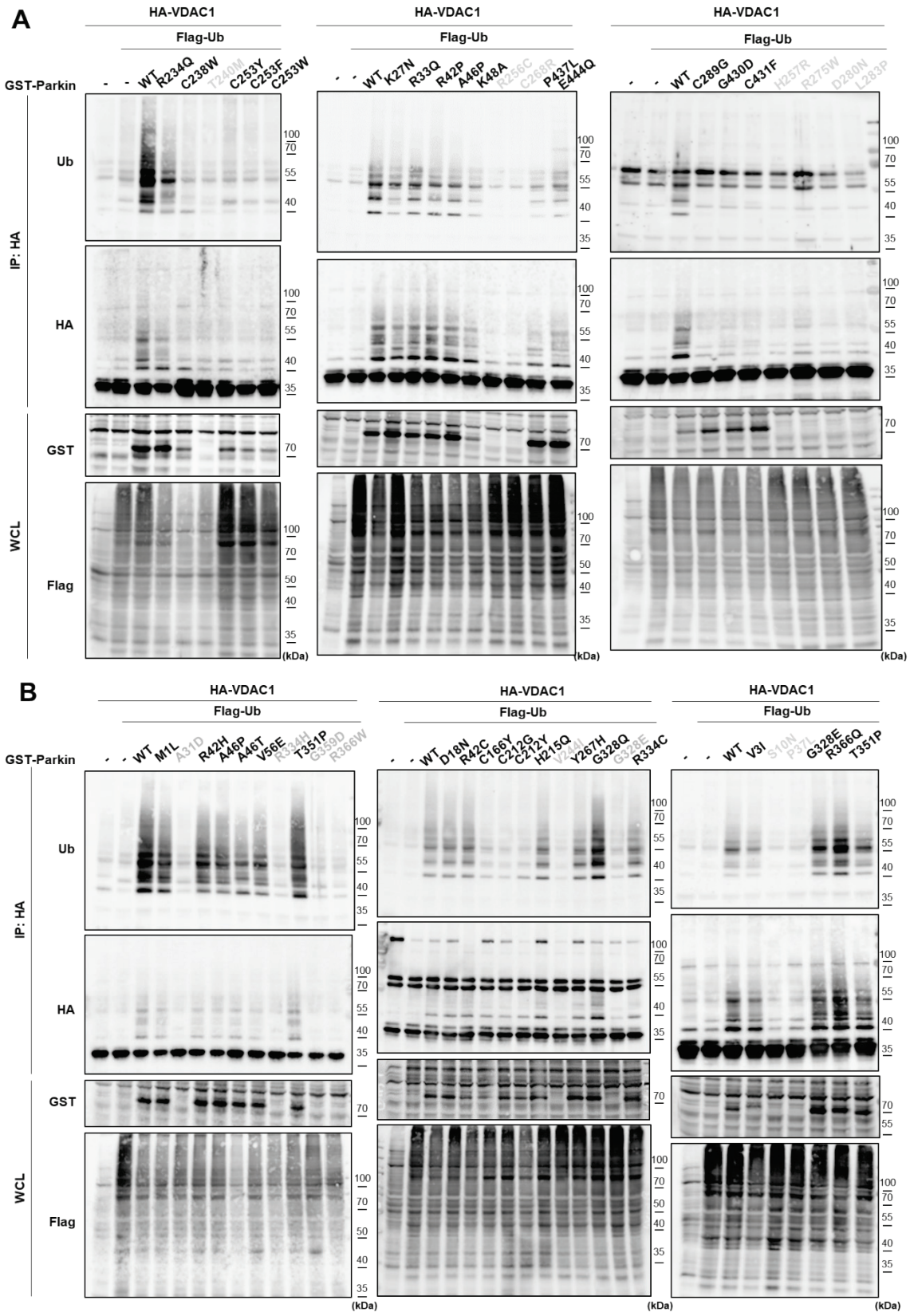


Fig. S7. DA neurons in the PPL1 region of Porin transgenic flies.
(*A*) Confocal images of DA neurons from the transgenic flies expressing Porin WT, K273R or Poly-KR by *TH-gal4* driver. The DA cells were stained by anti-TH antibody as described in the Materials and Methods section. The scale bar indicates 20 μm. (*B*) Confocal images of DA neurons from the transgenic flies expressing Porin WT, K273R, Poly-KR, or All KR in Porin null background (Porin^{A2}) by *hs-gal4* driver. The DA cells were stained by anti-TH antibody. The scale bar indicates 20 μm.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HA-VDAC1
Flag-Ub
GST-Parkin
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- - WT K27N R33Q R42P A46P K48A R256C C268R P437L E444Q
- - WT C289G G430D C431F H257R R275W D280N L283P
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Ub
HA
WCL
GST
Flag
100
70
55
50
40
35
(kDa)
B
HA-VDAC1
Flag-Ub
GST-Parkin
- - WT M1L A31D R42H A46P A46T V56E R334H T351P G359D R366W
- - WT D18N R42C C166Y C212G C212Y H215Q V248I Y267H G328Q G328E R334C
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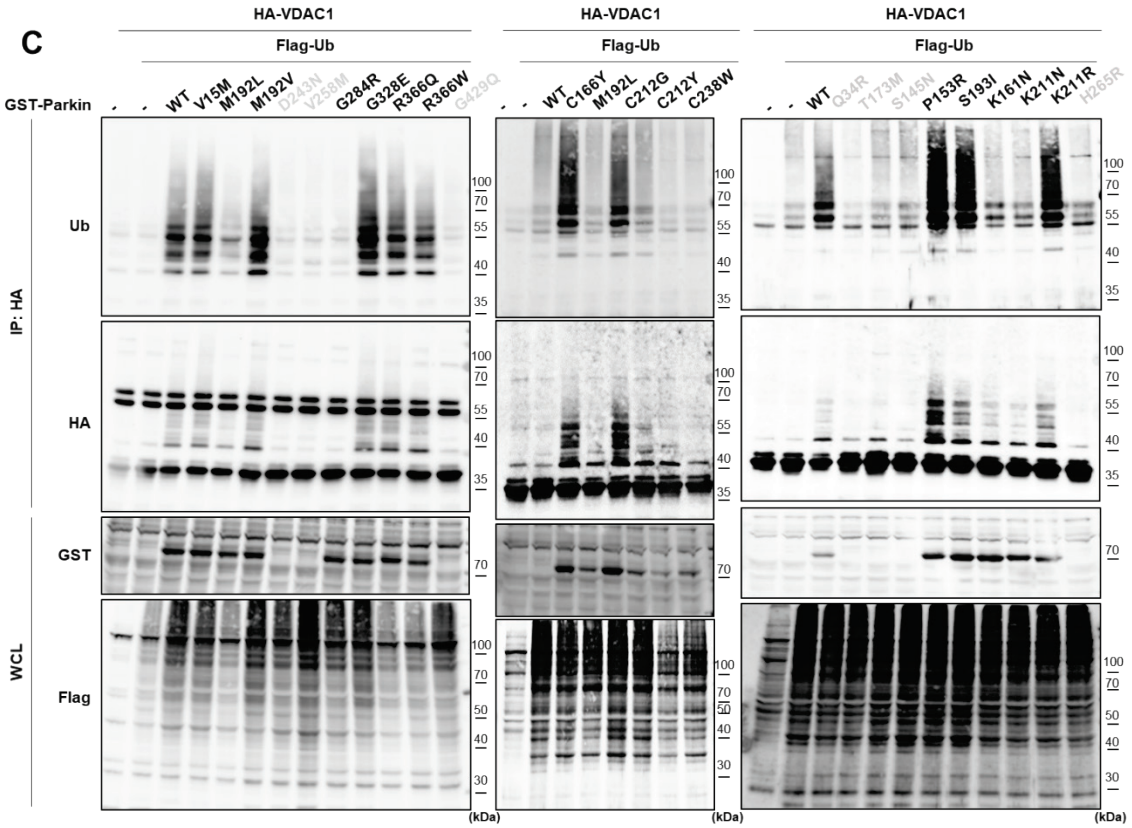
(kDa)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HA-VDAC1
Flag-Ub
GST-Parkin
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- - WT Q34R T173M S145N P153R S193I K161N K211N K211R H265R
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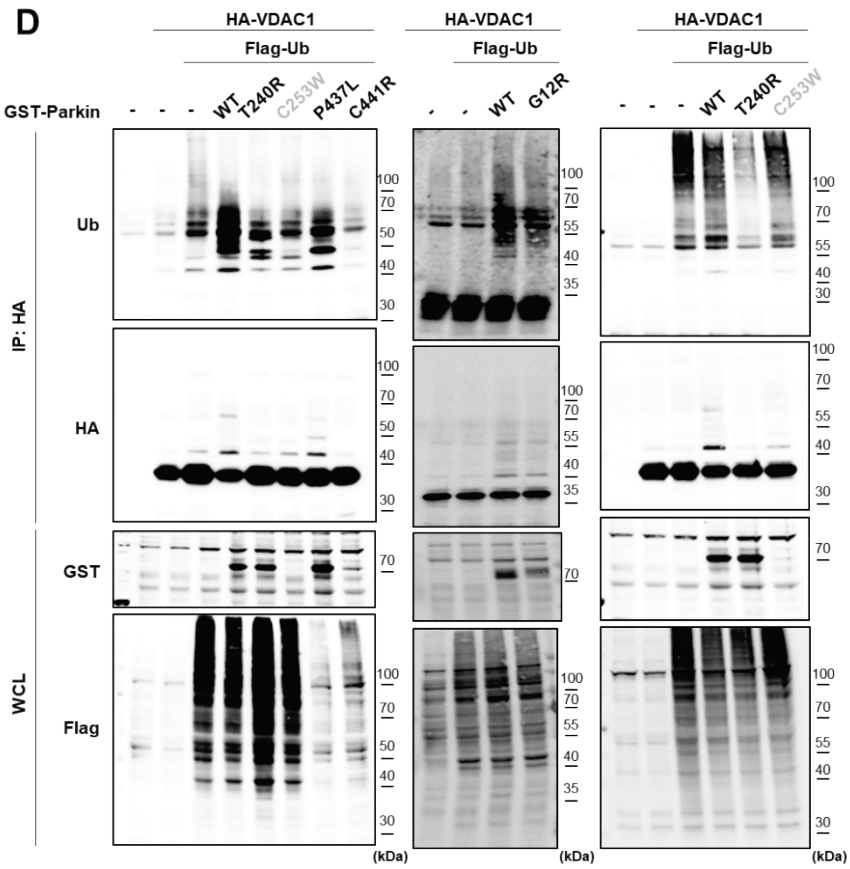
100 70 50 40 30
(kDa)


D
HA-VDAC1
Flag-Ub
GST-Parkin
- - - WT T240R C253W P437L C441R
- - WT G12R
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IP: HA
Ub
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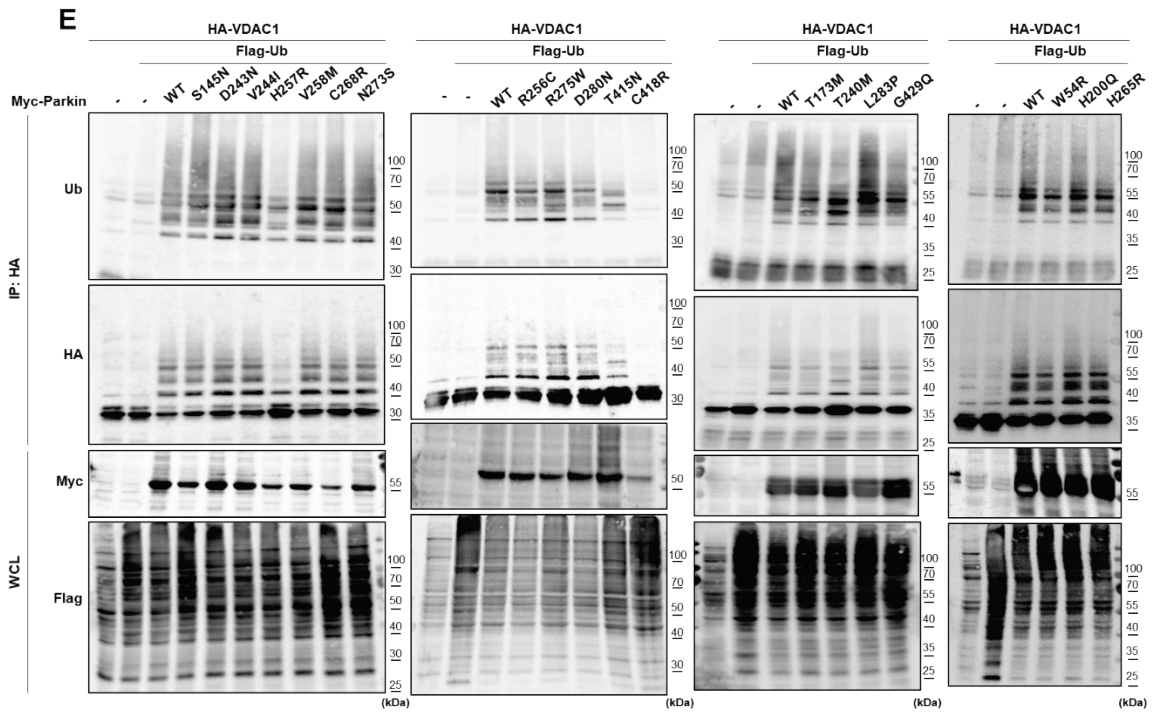

Fig. S8. Assays for VDAC1 ubiquitination by Parkin with 63 different PD patient mutations. (*A*-*E*) Parkin WT or each of 63 Parkin mutants was expressed in HEK293T cells expressing Flag-tagged ubiquitin and HA-tagged VDAC1. Whole cell lysates (WCL) or anti-HA immunoprecipitated (IP) samples were analyzed for immunoblotting to detect indicated proteins. Parkin mutants in grey color were not expressed. Each gel shows Parkin WT as a control and for comparison.

References

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