SUPPLEMENTARY INFORMATION

Plasmids. Untagged and HA-tagged PINK1 plasmids used for *in vivo* expression and *in vitro* transcription/translation were described previously [12]. The $PINK1_{\Delta 1\text{-}103}$ construct was generated from $PINK1_{\Delta 1\text{-}111}$ by PCR and corresponding primers. The Flag-tagged PINK1 constructs were generated as follows: Full-length human PINK1 cDNA (OriGene) was cloned into a pshuttle-1–3×Flag-IRES-GFP vector (Stratagene) to generate *C*-terminally Flag-tagged PINK1 (PINK1-Flag). Flag-tagged PINK1 fragment constructs (PINK1 156-507-Flag, PINK1 1-155-Flag, PINK1 156-309-Flag, PINK1 310-428-Flag and PINK1 429-581-Flag) were generated by PCR. All constructs were confirmed by sequencing.

Antibodies. Antibodies used in the study were as follows: PINK1 and TOM70 (Novus), SMAC (Santa Cruz), TOM20 and TIM23 (BD transduction laboratories), Flag (Sigma), Parkin (Cell Signaling), MPP (=PMPCA) (Sigma), RISP (Molecular Probes).

Cell culture, transfection and treatment. HeLa and HEK293T cells were cultured in DMEM (Gibco) supplemented with 10% heat-inactivated FBS (Gibco) supplemented with 100 Units/ml of penicillin and 100 µg/ml of streptomycin (Gibco). Transient transfection was performed with Lipofectamine LTX (Invitrogen) or Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. HeLa cells stably expressing YFP-Parkin were a generous gift from Dr. R.J. Youle, NIH, Bethesda, MD, USA. Valinomycin was used at a final concentration of 1 µM and MG132 at a final concentration of 10 µM.

Immunofluorescence and imaging. 24 h post-transfection, HeLa cells or HeLa cells stably expressing YFP-Parkin were treated with 1 µM Valinomycin, 10 µM MG132 or vehicle DMSO as indicated. After fixation with 4% paraformaldehyde cells were incubated with primary antibodies and fluorescent secondary Cy3 and Cy5 antibodies (Jackson ImmunoResearch) according to

the instructions of manufacturer. Images were taken using Leica TCS SP5 II confocal microscope and quantified manually as indicated in the figure legend.

Cell fractionation. HeLa cells were harvested by trypsinization and washed 3 times with ice-cold PBS (5 min, 200 × g, 4°C). Cells were disrupted on ice in a glass-glass homogenizer with 30 strokes in 210 mM Mannitol, 70 mM Sucrose, 1 mM EGTA, 5 mM Hepes, pH 7.2 supplemented with protease inhibitor cocktail (Roche). Unbroken cells were pelleted (10 min 1,500×g, 4°C) and subjected to homogenization for a total of 3 times. The supernatants were combined and spun (10 min 1,500×g, 4°C). An aliquot of the supernatant was collected (total extract) and the rest was subjected to centrifugation (15 min 15,000×, g, 4°C) to collect the cytoplasmic fraction in the supernatant and the crude mitochondria in the pellet. The pellet was then resuspended in wash buffer (250 mM Sucrose, 1 mM EGTA, 10 mM Tris, pH 7.5, supplemented with protease inhibitor cocktail (Roche) and centrifuged again (15 min 15,000×g, 4°C) to pellet crude mitochondria.

Western blotting. Samples (15-30 µg of protein) were run on precast 4-12% Bis-Tris gels (Invitrogen) with MOPS buffer according to manufacturer's protocol. Proteins were transferred onto a 0.45 µm nitrocellulose membrane (or PVDF when probed with the anti-SMAC antibody). Membranes were blocked with blocking buffer (Rockland), incubated with primary antibodies as indicated and fluorescent-conjugated secondary antibodies 1:5,000 (Odyssey). Signals were then scanned with the Odyssey infrared imager (Odyssey Detection System; Li-COR).

Proteinase K assay. HeLa cells were transiently transfected with PINK1 and harvested 24h post transfection. Crude mitochondrial fraction was isolated as described above. Half of the fraction was diluted in isotonic buffer (250 mM Sucrose, 10 mM Hepes, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.4) supplemented with 0.5% Triton X-100; the other half was diluted

without Triton X-100. After 10 min of incubation on ice, samples were aliquoted and treated with 0-200 µg/ml of Proteinase K for 30 min on ice, followed by addition of 5 mM of PMSF to terminate the digestion.

Alkaline extraction of ^{35}S-labeled PINK1. Import of radiolabeled PINK1 into isolated HeLa mitochondria was performed as described in [12]. After import, mitochondria were washed once in HMS buffer and then resuspended in Na_2CO_3 at pH 7.3, 10, 11.5 or 12, respectively. After 30 min incubation on ice, samples were subjected to ultra-centrifugation (1h, 100,000xg, 4°C). Pellets were resuspended in SDS sample buffer and supernatants TCA-precipitated before SDS-PAGE and Western blotting.

Figure S1. Protease resistance of cleaved PINK1 is not due to its sheltering inside the mitochondria of HeLa cells. (**A**) Upper panel: Immunoblot of proteinase K (PK) protection assay on mitochondria from PINK1-HA overexpressing HeLa cells without membranes permeabilization (intact mitochondria) or with membranes permeabilization (0.5% Triton X-100). Lower panels: Immunoblots for TOM70 and SMAC. These proteins are used as outer and inner mitochondrial protein markers, respectively. (**B**) Autoradiogram of PK protection assay on intact and permeabilized mitochondria after *in vitro* import of translated [^{35}S]-labeled PINK1. The asterisk indicates non-specific translation product which is also observed in the input (PINK1 translation mixture).

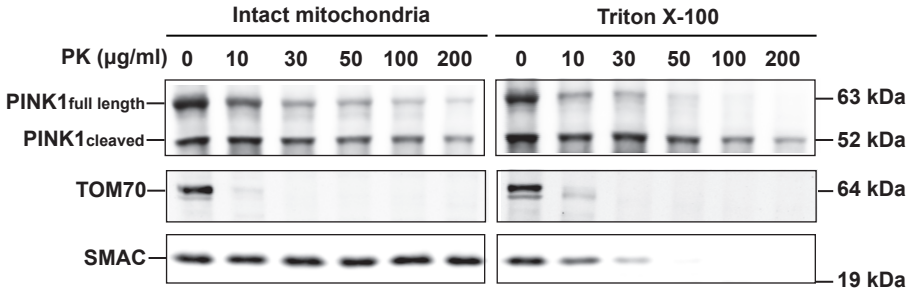
Figure S2. (**A**) Quantification of the ratio of cleaved to full length PINK1 signals from Figure 1A normalized to the ratio of DMSO controls. Two-way ANOVA demonstrates that there is a significant effect of treatment on the ratios ($F_{[3,16]} = 70.10$, $P<0.001$), but that this effect does not differ between cytosolic and mitochondrial faction (interaction: $F_{[3,16]} = 1.59$, $P = 0.23$). Student-Newman-Keuls post-hoc test indicates that the ratios are significantly smaller after valinomycin

treatment than in DMSO control in the mitochondrial but not cytosolic fractions while they are significantly higher after MG132 treatment than in DMSO control in both mitochondrial and cytosolic fractions. The test also shows that the ratios are significantly higher after MG132 treatment than in the combination of valinomycin and MG132 treatments in both mitochondrial and cytosolic fractions. All values are means ± SEM for 3 independent experiments. (**B**) Alkaline extraction of both full-length and cleaved [^{35}S]-labeled PINK1 from isolated mitochondria of HeLa cells. After *in vitro* import of [^{35}S]-labeled PINK1, mitochondria from HeLa cells are subjected to alkaline extraction at the indicated pH values. Samples are then fractionated into particulate and soluble fraction by ultracentrifugation. Upper panel: Representative autoradiogram. Lower panel: Quantification of PINK1 partition. Bars indicate percent of signal intensities for both the soluble and particulate fraction relative to the sum of both fractions for each pH.

Figure S3. Appearance of mitochondrial network as a surrogate of mitophagy. Representative immunocytochemistry images of YFP-Parkin HeLa cells with normal, reduced or no mitochondrial network, as evidenced by TOM-20 imunofluorescence, in response to 16 h of 1 μM valinomacin exposure. *Scale bar* = 20 μm.

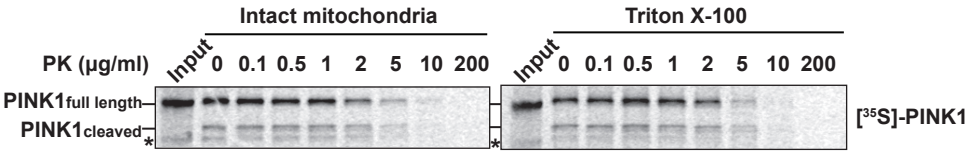
A

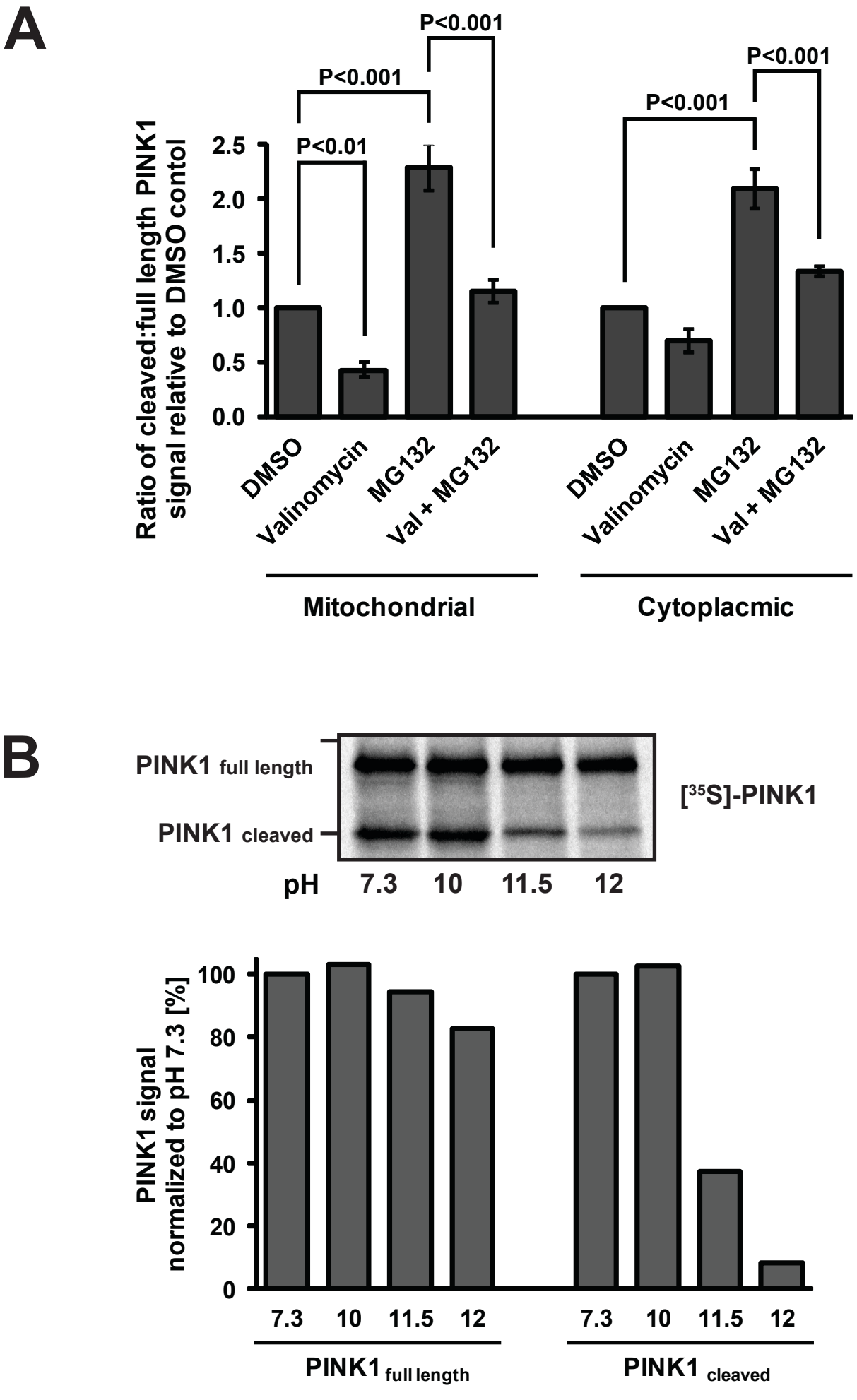
Intact mitochondria from HeLa cells over-expressing PINK1

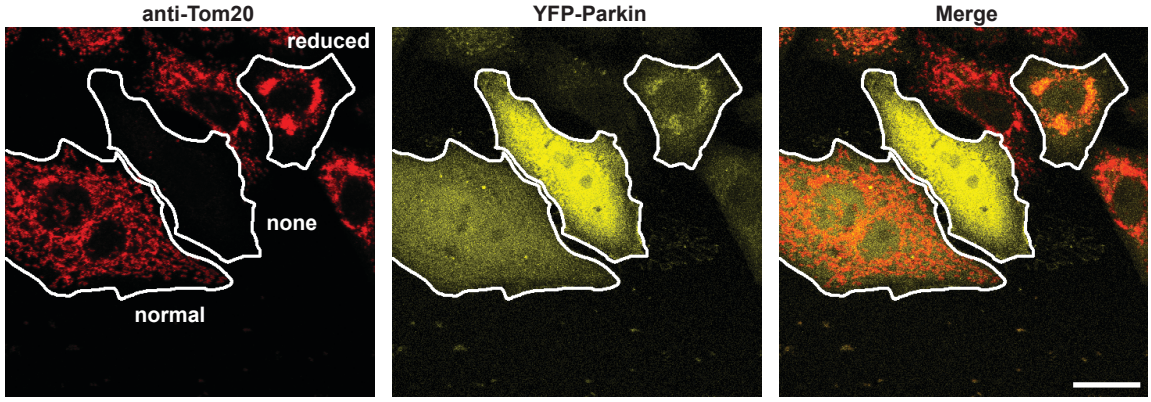


B

Radiolabled PINK1 inported into intact mitochondria from HeLa cells




A
P<0.001
P<0.001
P<0.001
P<0.001
P<0.01
Ratio of cleaved:full length PINK1 signal relative to DMSO contol
2.5
2.0
1.5
1.0
0.5
0.0
DMSO
Valinomycin
MG132
Val + MG132
DMSO
Valinomycin
MG132
Val + MG132
Mitochondrial
Cytoplacmic
B
PINK1 full length
PINK1 cleaved
[35S]-PINK1
pH 7.3 10 11.5 12
PINK1 signal normalized to pH 7.3 [%]
100
80
60
40
20
0
7.3 10 11.5 12
7.3 10 11.5 12
PINK1 full length
PINK1 cleaved


anti-Tom20
YFP-Parkin
Merge
reduced
none
normal