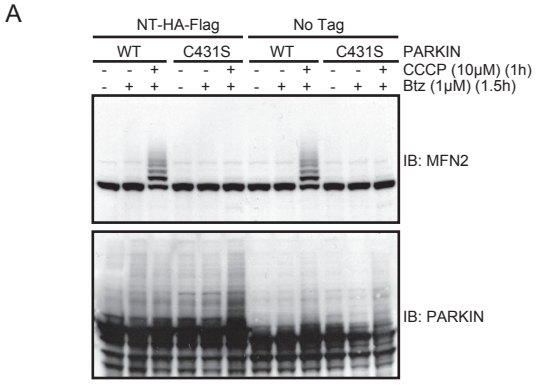
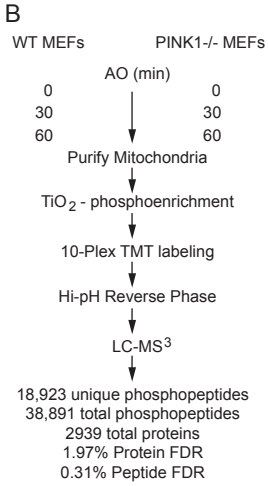
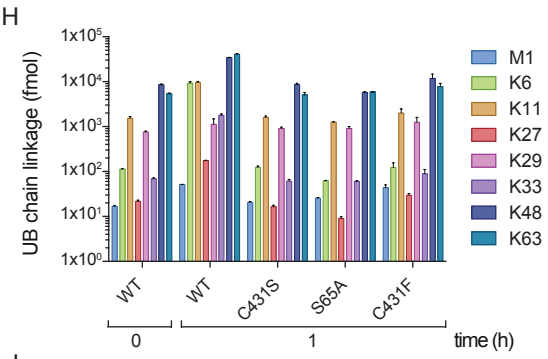
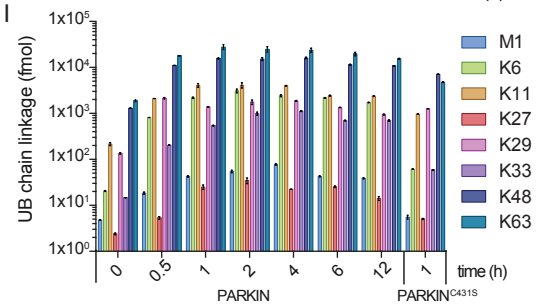
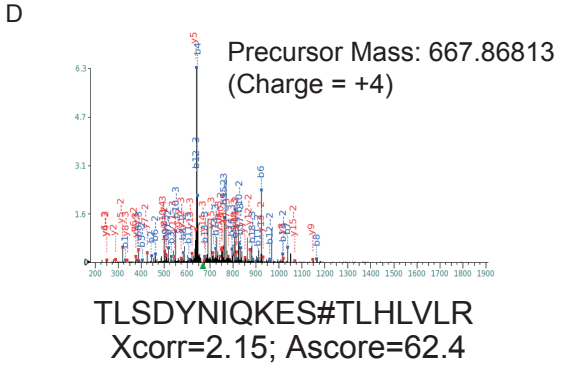
Figure S1

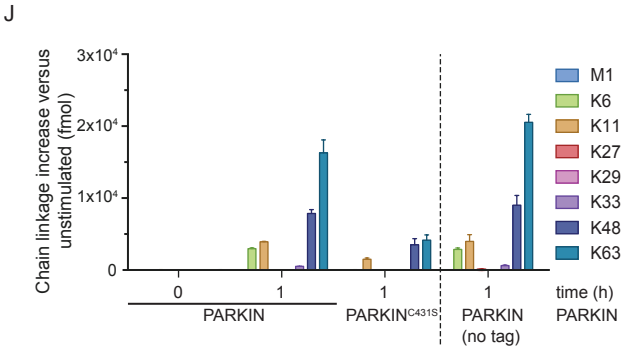












E

+2

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S	3	---	266.171	1113.080	---	16
D	4	---	323.684	**1069.564**	**-497.487**	15
Y	5	**1391.022**	**405.216**	1012.051	---	14
N	6	**750.664**	**462.237**	**930.519**	**89.264**	13
I	7	**396.519**	**518.779**	**873.497**	**69.427**	12
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K	9	**289.292**	**761.437**	**752.926**	**-198.225**	10
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+3

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V	16	**566.070**	**794.422**	129.762	---	3
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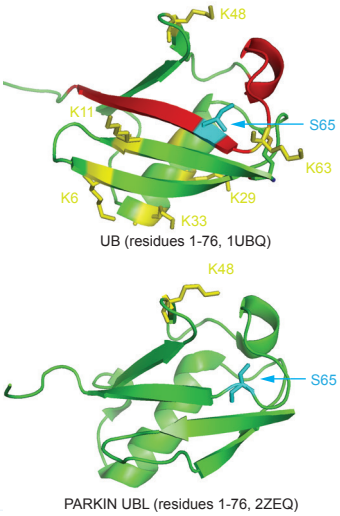
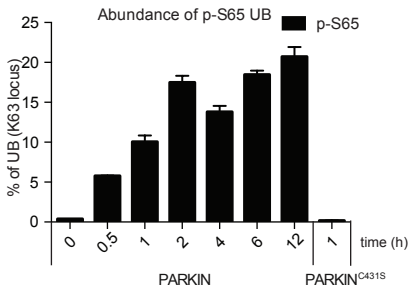
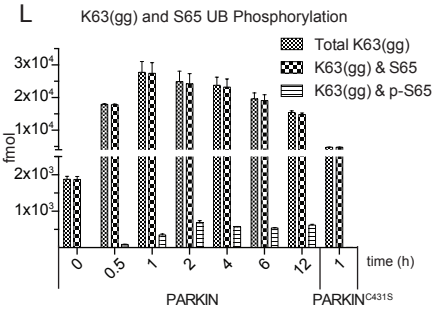
F







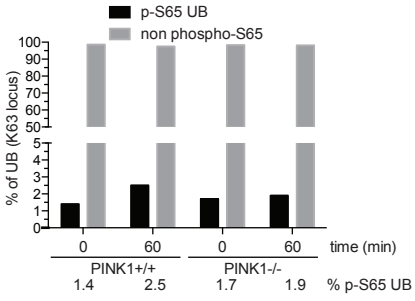
G

UB AQUA peptide	Sequence	Mass (Da) Light	Mass (Da) Heavy
1-6	mQIFVK	780.4204	786.4342
1-6 M1(gg)	**gg**mQIFVK	894.4633	900.4771
1-11 K6(gg)	mQIFVK**gg**LTGK	1394.7591	1401.7763
7-27 K11(gg)	TLTGK**gg**TITLEVEPSDTIENVK	2401.2588	2407.2726
12-27	TITLEVEPSDTIENVK	1786.9200	1792.9338
12-29 K(gg)27	TITLEVEPSDTIENV**Kgg**AK	2100.0950	2106.1025
28-33 K29(gg)	AK**gg**IQDK	815.4501	821.4576
30-42 K33(gg)	IQDK**gg**EGIPPDQQR	1636.8169	1642.8307
30-42	IQDKEGIPPDQQR	1522.7739	1528.7878
34-42	EGIPPDQQR	1038.5094	1044.5232
43-54 K48(gg)	LIFAGK**gg**QLEDGR	1459.7783	1466.7954
43-54	LIFAGK	647.4006	657.4279
49-54	QLEDGR	716.3453	723.3625
55-72 K63(gg)	TLSDYNIQK**gg**ESTLHLVLR	2243.1910	2250.2081
55-63	TLSDYNIQK	1080.5451	1087.5623
64-72	ESTLHLVLR	1066.6135	1073.6306
55-72 K63(gg),pSer65	TLSDYNIQK**gg**E**p**STLHLVLR	2323.1573	2330.1744
55-72 pSer65	TLSDYNIQKE**p**STLHLVLR	2209.1143	2216.1315
64-72 pSer65	E**p**STLHLVLR	1146.5798	1153.5969
PARKIN AQUA peptide			
52-74	NDWTVQNcDLDQQSIVHIVQRPWR	3006.4518	3016.4601
52-74 pSer65	NDWTVQNcDLDQQsIVHIVQRPWR	3086.4181	3096.4264

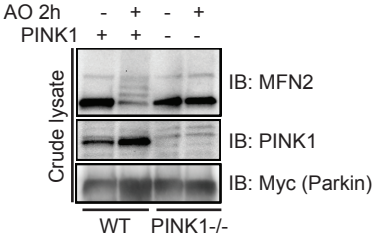
PTM (bold):
m = oxidized
p = phosphorylation
gg = gly gly residue
c = carboxymethylation

Heavy AA (red bold underline)

M



N



Hela FLP-IN TREX line

Figure S1. Application of quantitative proteomics to the PINK1-PARKIN pathway (related to Figure 1).

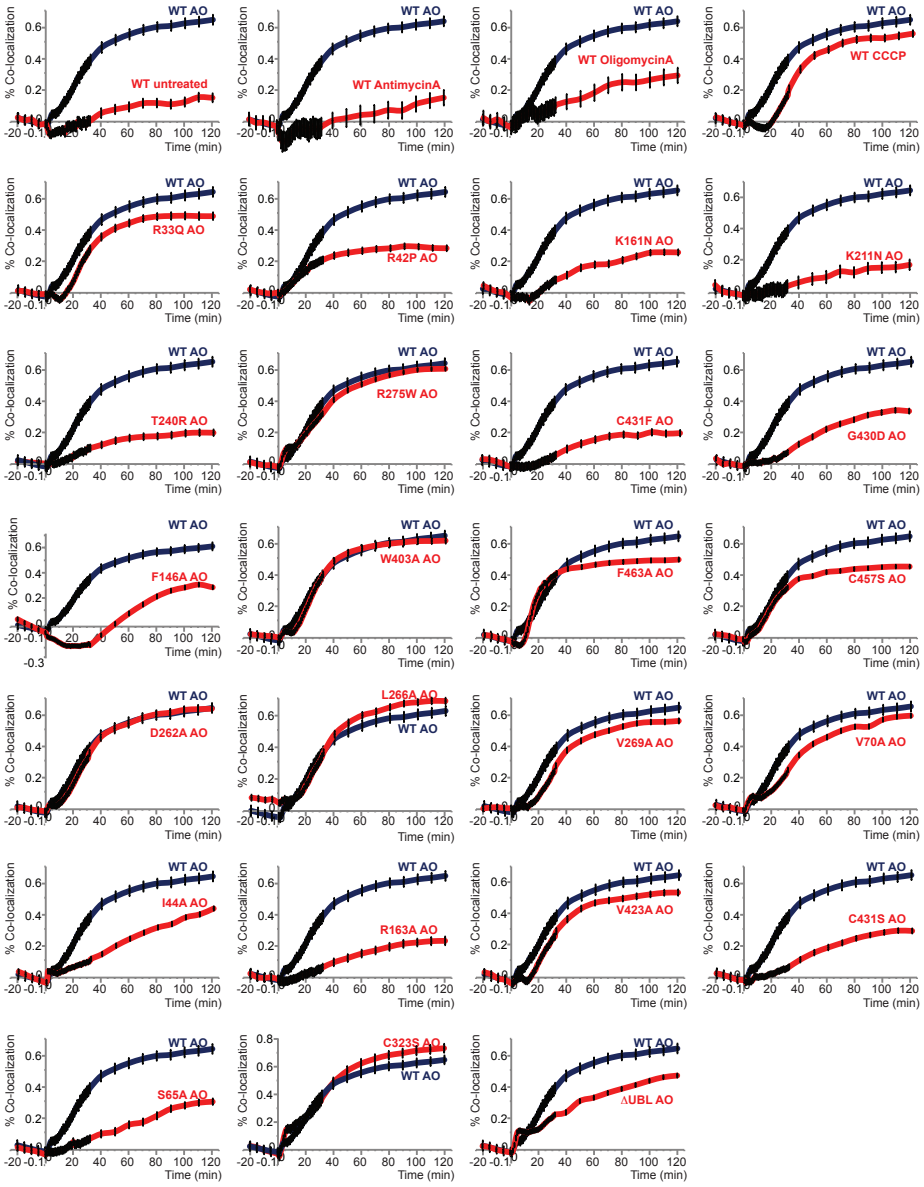
(A) HA-FLAG-PARKIN and untagged PARKIN display indistinguishable activities in MFN2 ubiquitylation in vivo. HeLa cells stably expressing HA-FLAG-PARKIN or untagged PARKIN in either the wild-type or catalytically defective (C431S) form were treated with either CCCP, CCCP plus Bortezomib, or left untreated and after 1 h, cell extracts were immunoblotting with anti-MFN2 or anti-PARKIN.
(B) Scheme depicting approach for identification of cellular substrates of PINK1 in response to mitochondrial depolarization.
(C-F) Characterization of S65 UB phosphorylation. (B) Sequence alignment of the UBL domain of human PARKIN (residues 1-84) and UB. Arrow indicates the position of S65 in both PARKIN and UB. (C) MS2 spectra for the p-S65 tryptic peptide from UB (residues 55-72) showing the precursor mass, Xcorr, and Ascore values. (D) Fragment ions identified in the p-S65 UB^{55-72} peptide. (E) Structures of UB and $PARKIN^{UBL}$. The position of the tryptic peptide identified in UB is shown in red with S65 in cyan. The position of S65 in $PARKIN^{UBL}$ is shown in cyan. The positions of lysine residues are shown in yellow.
(G) AQUA peptides employed in this study.
(H) UB chain linkage abundance in response to mitochondrial depolarization with AO (1h). The number of fmol quantified are shown on a log10 scale, and error bars represent triplicate measurements (+/- SEM).
(I) UB chain linkage abundance in response to mitochondrial depolarization with AO over a time course of 30 min to 12 h. The number of fmol quantified are shown on a log10 scale, and error bars represent triplicate measurements (+/- SEM).
(J) UB chain linkage increase in mitochondria from cells depolarized for 1 h with AO and purified by sucrose gradient centrifugation, normalized against mitochondria in the absence of stimulation. Error bars represent triplicate measurements (+/- SEM).
(K) Time course for p-S65 UB on mitochondria in response to depolarization with AO. Error bars represent triplicate measurements (+/- SEM).
(L) Time course for accumulation of doubly modified (K63gg and p-S65) and singly modified K63gg UB peptides in response to mitochondrial depolarization with AO. Error bars represent triplicate measurements (+/- SEM).
(M,N) Stoichiometry of total UB phosphorylation in HeLa Flp-In T-REx cells with and without PINK1. HeLa Flp-In T-REx cells containing an inducible PARKIN gene were used for gene editing with a zinc finger nuclease targeting PINK1. Clones in which PINK1 was homozygously knocked out were identified by PCR (not shown) and by examining PINK1 dependent mitochondrial ubiquitylation with α-MFN2 (panel N). To examine the stoichiometry of UB phosphorylation (panel M), total cell extracts from cells with our without depolarization were treated with USP2 to collapse all ubiquitin chains to monomers. The sample was then heated to 95^{o}C for 10 min, and UB digested with trypsin prior to AQUA proteomics.

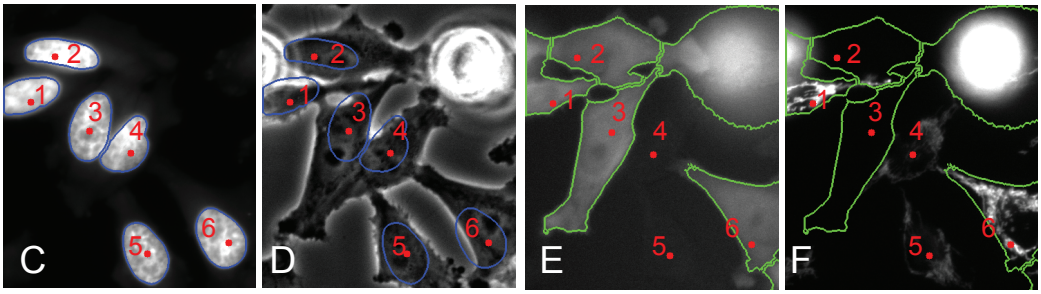
Figure S2

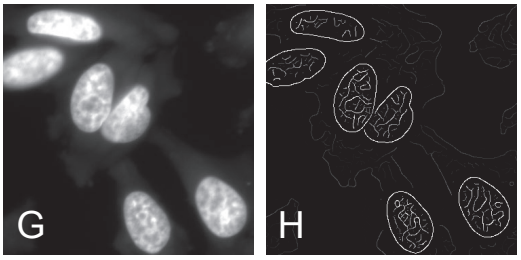
A

Type	Mutant	Description
Controls	WT	Untreated
	WT	Antimycin A
	WT	Oligomycin A
	WT	Antimycin A + Oligomycin A
	WT	CCCP
Patient Mutants	R33Q	UBL domain
	R42P	UBL domain
	K161N	RING0 domain
	K211N	RING0 domain
	T240R	RING1 domain
	R275W	RING1 domain
	G430D	Catalytic domain
	C431F	Catalytic Cysteine
Structure-based Mutants	F146A	Proposed activating mutant
	F463A	Proposed activating mutant
	W403A	Proposed activating mutant
	C457S	Proposed activating mutant
	D262A	UBL-RING1 interface
	L266A	UBL-RING1 interface
	V269A	UBL-RING1 interface
	V70A	UBL-RING1 interface
	I44A	UBL-RING1 interface
	R163A	Proposed phosphate binding site
	V423A	Candidate donor UB binding
	C431S	Catalytic Cysteine
Regulatory Mutants	S65A	PINK1 phophorylation site
	ΔUBL	Regulatory domain
	C323S	Nitrosylated

B







I

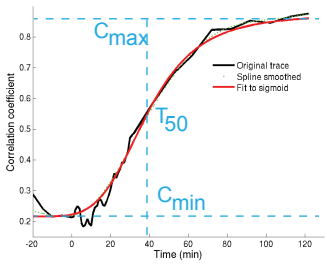


Figure S2. Kinetics of PARKIN and PARKIN mutant localization to mitochondria in response to depolarization (related to Figure 2).

(A) Mutants examined in this study.

(B) Plots for % co-localization for PARKIN and individual PARKIN mutants. Error bars represent measurements at individual time points for >300 cells (+/- SEM).

(C-F) A cropped image illustrating the image segmentation procedure. Red dots are the manually defined seeding points. All images are from the first frame of the movie except panel C, which is from the fixed image acquired after the last frame of the movie. (C) Nuclear segmentation using the DAPI image from the end of the movie. (D) Overlay nuclear mask on the phase contrast channel from the first frame of the movie. (E) Cell segmentation using the GFP-PARKIN channel. (F) Overlay cell mask on the mitochondria channel.

(G,H) Steerable filtering to identify nuclear boundary in the DAPI images. (G) Original DAPi image. (H) Non-maximum suppressed response from the steerable filter.

(I) A typical time trace of correlation coefficient from a cell undergoing Parkin translocation. The black line is original trace, green dotted line is the spline smoothed trace, and the red solid line is the result from a constrained fitting to sigmoid curve. C_{min} and C_{max} are the values of the asymptote on both ends of the fitted curve, while T50 is the time point where the fitted curve reaches 50% of the maximum.

Figure S3

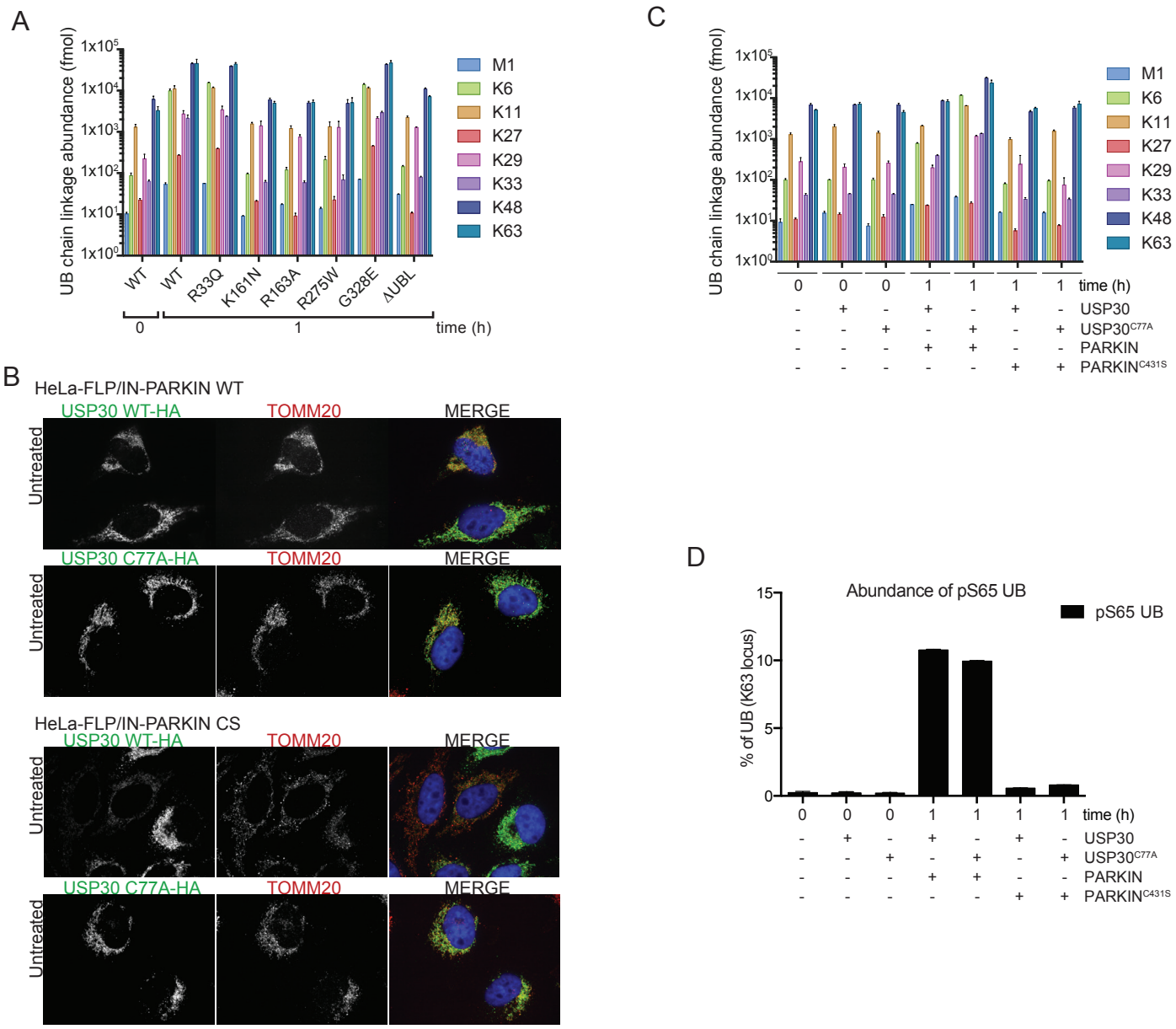

A
UB chain linkage abundance (fmol)
1x10^5
1x10^4
1x10^3
1x10^2
1x10^1
1x10^0
M1
K6
K11
K27
K29
K33
K48
K63
WT
WT
R33Q
K161N
R163A
R275W
G328E
ΔUBL
0
1
time (h)
C
UB chain linkage abundance (fmol)
M1
K6
K11
K27
K29
K33
K48
K63
0 0 0 1 1 1 1 time (h)
- + - + - + - USP30
- - + - + - + USP30C77A
- - - + + - - PARKIN
- - - - - + + PARKINC431S
B
HeLa-FLP/IN-PARKIN WT
USP30 WT-HA
TOMM20
MERGE
Untreated
USP30 C77A-HA
TOMM20
MERGE
Untreated
HeLa-FLP/IN-PARKIN CS
USP30 WT-HA
TOMM20
MERGE
Untreated
USP30 C77A-HA
TOMM20
MERGE
Untreated
D
Abundance of pS65 UB
pS65 UB
% of UB (K63 locus)
15
10
5
0
0 0 0 1 1 1 1 time (h)
- + - + - + - USP30
- - + - + - + USP30C77A
- - - + + - - PARKIN
- - - - - + + PARKINC431S

Figure S3. Mitochondrial UB chain synthesis by patient and structure-based PARKIN mutants and reversal by USP30 (related to Figure 3).

(A) Absolute UB chain linkage abundance in HeLa Flp-In T-REx cells expressing the indicated PARKIN protein with or without depolarization for 1h was determined by UB-AQUA proteomics. Error bars represent triplicate measurements (+/- SEM).

(B) Immunoflourescence of HeLa Flp-In T-REx cells engineered to express inducible untagged PARKIN and either USP30-HA or USP30^{C77A}-HA constitutively. α-HA staining is in green. α-TOMM20 was used as a mitochondrial marker.

(C) Absolute UB chain linkage abundance in HeLa Flp-In T-REx cells engineered to express inducible untagged PARKIN and either USP30-HA or USP30^{C77A}-HA constitutively with or without depolarization for 1h was determined by UB-AQUA proteomics. Error bars represent triplicate measurements (+/- SEM).

(D) Absolute abundance of p-S65 is largely unaltered in UB chains targeted by USP30.

Figure S4

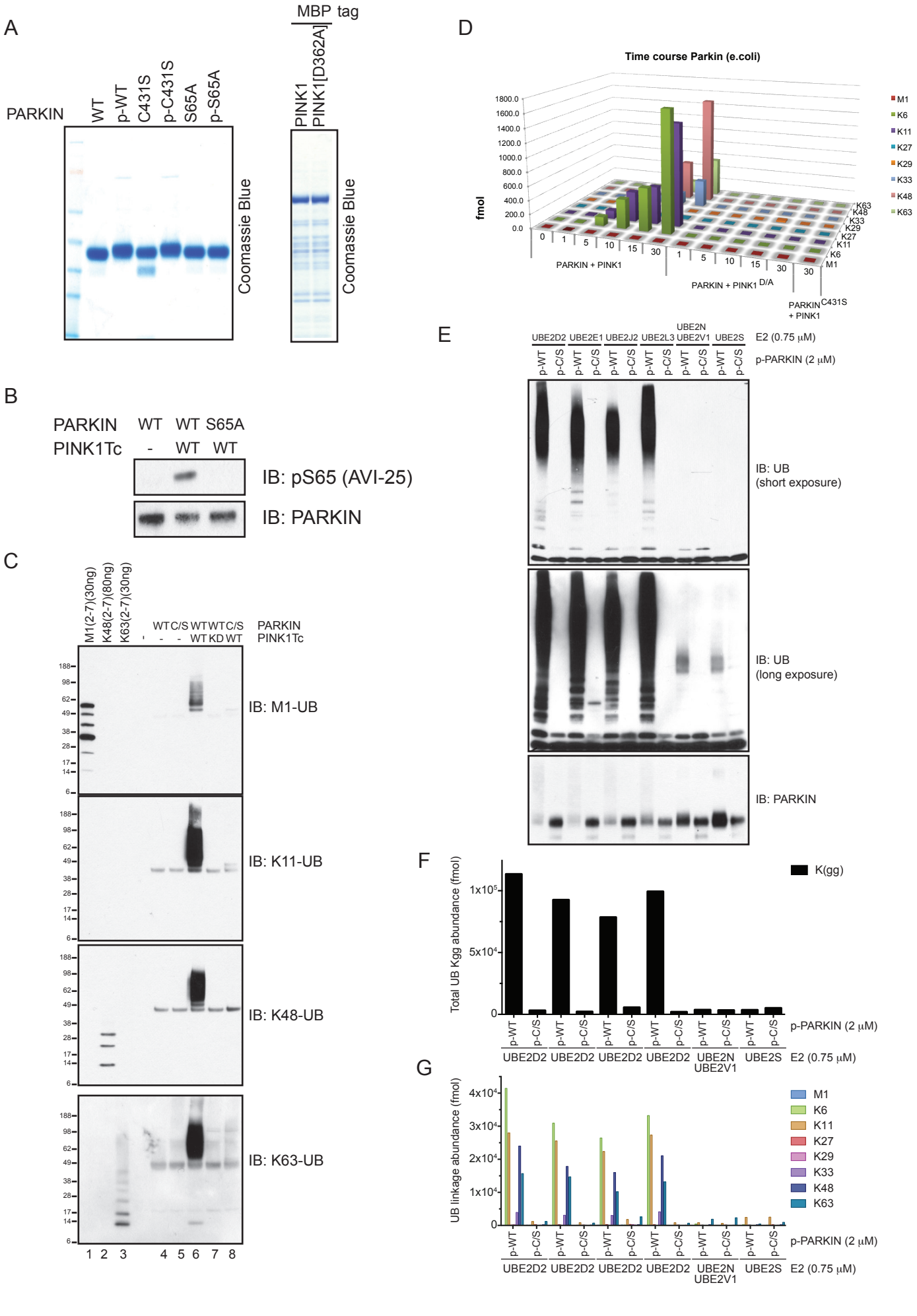

A
MBP tag
PARKIN
WT
p-WT
C431S
p-C431S
S65A
p-S65A
PINK1
PINK1[D362A]
Coomassie Blue
Coomassie Blue
B
PARKIN WT WT S65A
PINK1Tc - WT WT
IB: pS65 (AVI-25)
IB: PARKIN
C
M1(2-7)(30ng)
K48(2-7)(80ng)
K63(2-7)(30ng)
WT C/S WT WT C/S
- - - WT KD WT
PARKIN
PINK1Tc
IB: M1-UB
IB: K11-UB
IB: K48-UB
IB: K63-UB
1 2 3 4 5 6 7 8
D
Time course Parkin (e.coli)
fmol
PARKIN + PINK1
PARKIN + PINK1 D/A
PARKIN + PINK1 C431S
M1
K6
K11
K27
K29
K33
K48
K63
E
UBE2D2 UBE2E1 UBE2J2 UBE2L3 UBE2N UBE2V1 UBE2S
E2 (0.75 μM)
p-PARKIN (2 μM)
IB: UB (short exposure)
IB: UB (long exposure)
IB: PARKIN
F
Total UB Kgg abundance (fmol)
K(gg)
p-PARKIN (2 μM)
UBE2D2 UBE2D2 UBE2D2 UBE2D2 UBE2N UBE2V1 UBE2S
E2 (0.75 μM)
G
UB linkage abundance (fmol)
M1
K6
K11
K27
K29
K33
K48
K63
p-PARKIN (2 μM)
UBE2D2 UBE2D2 UBE2D2 UBE2D2 UBE2N UBE2V1 UBE2S
E2 (0.75 μM)

Figure S4. In vitro analysis of PARKIN activity upon phosphorylation by PINK1Tc (related to Figure 4).

(A) Purified PARKIN proteins with or without phosphorylation by PINK1. Proteins were detected by Coomassie staining in SDS-PAGE gels. PARKIN proteins were purified away from PINK1 was described in Supplemental Experimental Procedures (Method 1).

(B) Characterization of the AVI-25 α-pS65 PARKIN antibody selected by phage display (see Supplemental Experimental Procedures). WT or S65A PARKIN was purified from insect cells and either left untreated or treated with PINK1Tc produced in E. coli. Proteins were then separated by SDS-PAGE and immunoblotted with either α-PARKIN antibodies or with AVI-25, a single chain antibody selected for binding to a p-S65 PARKIN peptide as described in Supplemental Experimental Procedures.

(C) Immunoblotting of in vitro PARKIN UB chain assembly reactions using α-M1, α-K11, α-K48 and α-K63 UB chain specific antibodies.

(D) UB-AQUA analysis of chain assembly reactions produced using PARKIN purified from E. coli and activated by phosphorylation with wild-type or kinase inactive (D/A) PINK1Tc. PARKIN^{C431S} prepared similarly was used as a negative control.

(E) Immunoblotting analysis of in vitro UB chain assembly reactions (30 min) using phosphorylated (99%) PARKIN wild-type (p-WT) or inactive mutant PARKIN^{C431S} (p-C/S) in combination with various E2 conjugating enzymes.

(F, G) Samples from panels E were subjected to UB-AQUA proteomics.

Figure S5

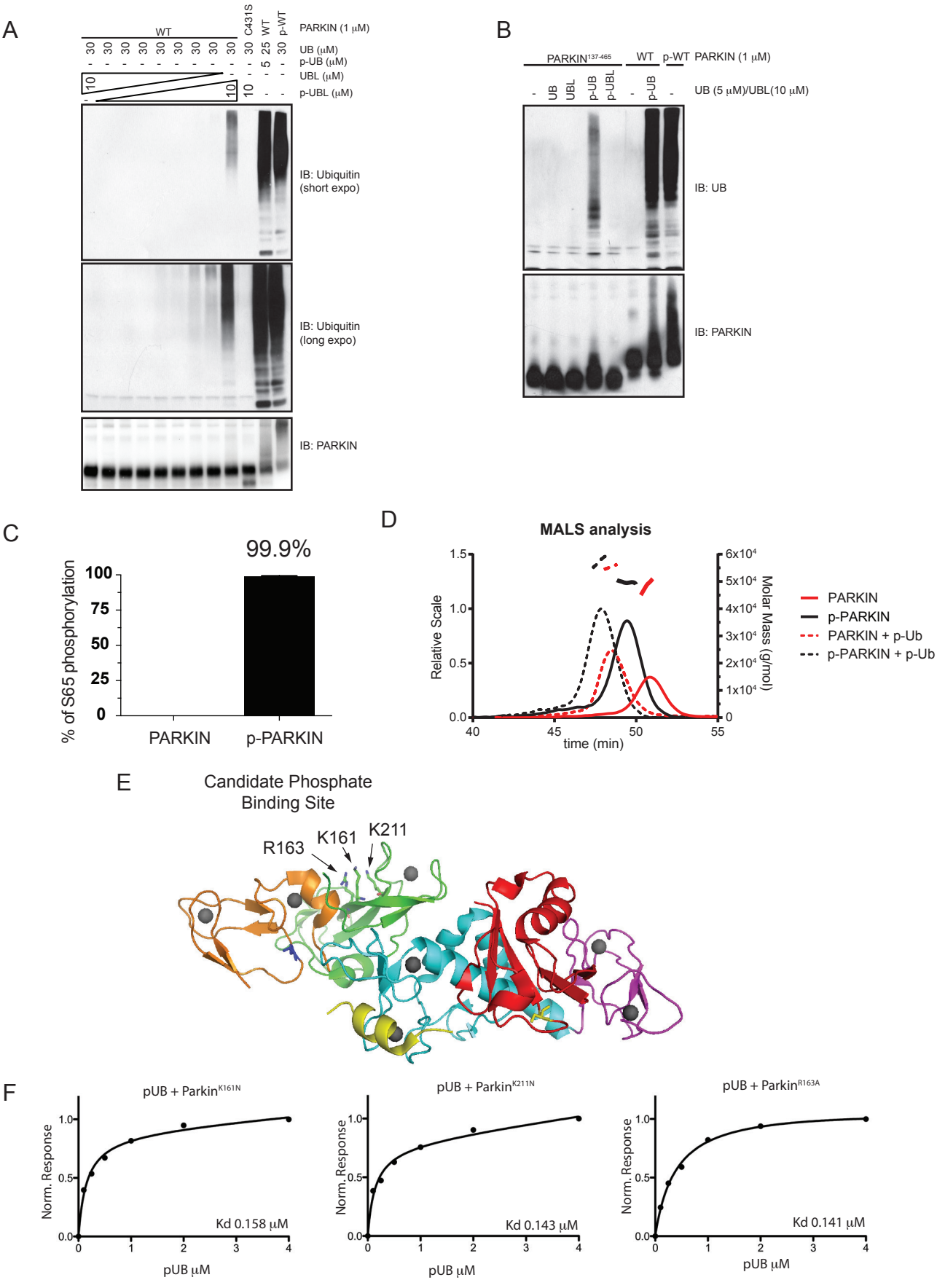

A
WT
C431S
WT
p-WT
PARKIN (1 μM)
UB (μM)
p-UB (μM)
UBL (μM)
p-UBL (μM)
IB: Ubiquitin (short expo)
IB: Ubiquitin (long expo)
IB: PARKIN
B
PARKIN137-465
WT
p-WT
PARKIN (1 μM)
UB (5 μM)/UBL(10 μM)
UB
UBL
p-UB
p-UBL
IB: UB
IB: PARKIN
C
99.9%
% of S65 phosphorylation
PARKIN
p-PARKIN
D
MALS analysis
Relative Scale
Molar Mass (g/mol)
time (min)
PARKIN
p-PARKIN
PARKIN + p-Ub
p-PARKIN + p-Ub
E
Candidate Phosphate Binding Site
R163
K161
K211
F
pUB + ParkinK161N
pUB + ParkinK211N
pUB + ParkinR163A
Norm. Response
pUB μM
Kd 0.158 μM
Kd 0.143 μM
Kd 0.141 μM

Figure S5. Biophysical analysis of the interaction of PARKIN with p-UB (related to Figure 5).

(A) Activation of UB chain synthesis by full length PARKIN (30 min reaction) upon addition of stoichiometrically PINK1-phosphorylated UBL domain (p-UBL) from rat PARKIN. The concentrations of p-UBL used in panel were 0, 0.1, 0.25, 0.5, 1, 2, 3.5, 5, 10 μM. The total UBL concentration was 10 μM and total UB concentration 30 μM.

(B) Activation of UB chain synthesis by $PARKIN^{\Delta UBL}$ (residues 137-465) (30 min reaction) upon addition of stoichiometrically PINK1-phosphorylated UBL domain (p-UBL) from rat PARKIN or UB (p-UB). The total UB concentration was 30 μM.

(C) AQUA proteomics was used to determine the stoichiometry of PARKIN phosphorylation by PINK1 (Method 2, see Supplemental Experimental Procedures).

(D) Time traces for SEC-MALS analysis of PARKIN proteins with and without phosphorylation by PINK1Tc and in the presence of absence of p-UB (premixed at a 1:5 PARKIN:p-UB molar ratio). The p-PARKIN and p-UB used for these experiments were 99% phosphorylated based on AQUA proteomics.

(E) Structure of auto-inhibited PARKIN (Trempe et al., 2013) showing the positions of R163, K211, and K161 suggested to be involved in phosphate binding (Wauer and Komander, 2013). Color code: UBL domain, red; RING0, green; RING1, cyan; IBR, magenta; REP, yellow; RING2, orange.

(F) Binding of p-UB (99% phosphorylated) to PARKIN mutants was measured using Bio-layer interferometry.

Figure S6

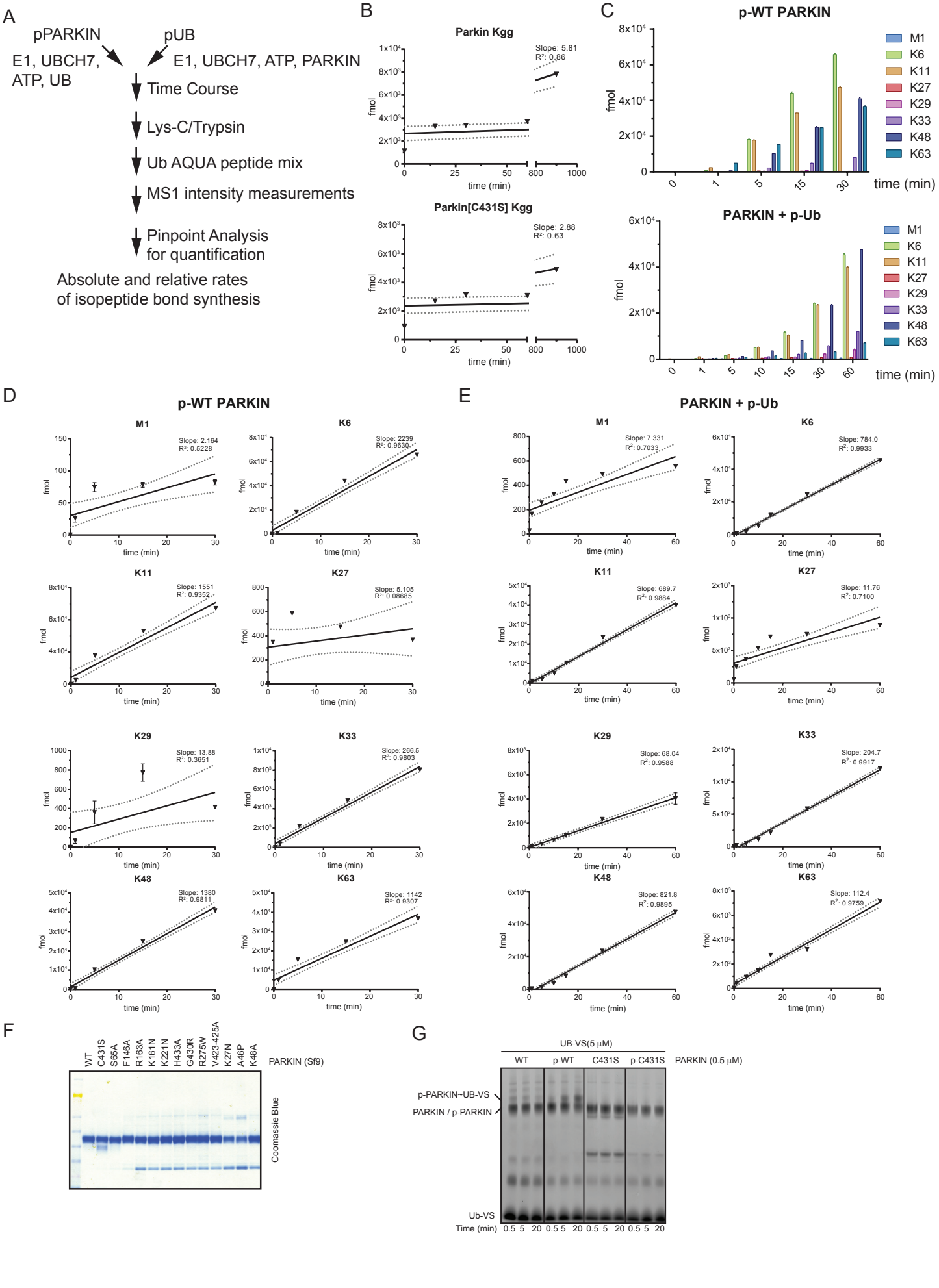

A
pPARKIN
pUB
E1, UBCH7, ATP, UB
E1, UBCH7, ATP, PARKIN
Time Course
Lys-C/Trypsin
Ub AQUA peptide mix
MS1 intensity measurements
Pinpoint Analysis for quantification
Absolute and relative rates of isopeptide bond synthesis
B
Parkin Kgg
Slope: 5.81
R²: 0.86
Parkin[C431S] Kgg
Slope: 2.88
R²: 0.63
fmol
time (min)
C
p-WT PARKIN
PARKIN + p-Ub
M1
K6
K11
K27
K29
K33
K48
K63
fmol
time (min)
D
p-WT PARKIN
M1
Slope: 2.164
R²: 0.5228
K6
Slope: 2239
R²: 0.9630
K11
Slope: 1551
R²: 0.9352
K27
Slope: 5.105
R²: 0.08685
K29
Slope: 13.88
R²: 0.3651
K33
Slope: 266.5
R²: 0.9803
K48
Slope: 1380
R²: 0.9811
K63
Slope: 1142
R²: 0.9307
E
PARKIN + p-Ub
M1
Slope: 7.331
R²: 0.7033
K6
Slope: 784.0
R²: 0.9933
K11
Slope: 689.7
R²: 0.9884
K27
Slope: 11.76
R²: 0.7100
K29
Slope: 68.04
R²: 0.9588
K33
Slope: 204.7
R²: 0.9917
K48
Slope: 821.8
R²: 0.9895
K63
Slope: 112.4
R²: 0.9759
F
WT
C431S
S65A
F146A
R163A
K161N
K221N
H433A
G430R
R275W
V423-425A
K27N
A46P
K48A
PARKIN (Sf9)
Coomassie Blue
G
UB-VS(5 μM)
WT
p-WT
C431S
p-C431S
PARKIN (0.5 μM)
p-PARKIN~UB-VS
PARKIN / p-PARKIN
Ub-VS
Time (min) 0.5 5 20 0.5 5 20 0.5 5 20 0.5 5 20

Figure S6. Analysis of PARKIN UB chain synthesis kinetics using UB-AQUA proteomics (related to Figure 6).

(A) Schematic representation of the approach used to measure PARKIN rate constants for chain assembly.

(B) Kinetics of formation of UB conjugates for unphosphorylated WT or C431S PARKIN. Each point represents the average of triplicate measurements.

(C) Histogram showing the number of fmol of each chain type formed by p-WT PARKIN or unphosphorylated PARKIN plus p-UB.

(D,E) Velocity plots for each UB chain type formed by p-WT PARKIN (D) or unphosphorylated PARKIN plus p-UB (E). Dotted lines represent the 95% confidence intervals for the calculated slope (rate of UB conjugation) and R^2.

(F) Coomassie Blue analysis of various PARKIN proteins used in this study after separation by SDS-PAGE.

(G) WT or C431S PARKIN with or without phosphorylation (99%) was incubated with UB-VS for the indicated times and reaction products examined by SDS-PAGE and stained with SYPRO-Ruby.

SUPPLEMENTAL TABLE LEGENDS

Supplemental Table 1 (related to Figure 1).

This table contains the identity and spectral counts of di-GLY modified mitochondrial proteins identify by LC-MS/MS of ubiquitylated proteins purified from depolarized mitochondria over a time course. This Table also includes the identification of pS65 in UB.

SUPPLEMENTAL MOVIE LEGENDS

Movie S1 (related to Figure2).

This movie is a live imaging experiment of WT GFP-PARKIN recruitment to mitochondria in response to depolarization. GFP-PARKIN, Green; TOMM20, Red.

Movie S2 (related to Figure 2).

This movie is a live imaging experiment of GFP-PARKINC431S recruitment to mitochondria in response to depolarization. GFP-PARKIN, Green; TOMM20, Red.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibodies and other reagents

The following antibodies were used in this study: MFN2 ([NIAR164], Abcam), CISD1 (16006-1-AP, proteintech), PCNA (sc-56, Santa-Cruz), TOMM20 (sc-11415, Santa-Cruz), PARKIN (sc-32282, Santa-Cruz, [EPR5024(N)], Abcam), UB (Dako), M1-UB (generously provided by Vishva Dixit, Genentech), K11-UB (Matsumoto et al., 2010, generously provided by Vishva Dixit, Genentech), K48-UB (clone *Apu2*, Millipore,[EP8589] Abcam), K63-UB (clone *Apu3*, Millipore,[EPR8590-448] Abcam), Anti-HA (11867423001, Roche), Anti-Flag M2 (F1804, Sigma) and anti-His (631212; Clonetech) and Neutravidin-HRP (Pierce) was used to detect pS65-PARKIN antibody. Recombinant HA-UB-VS, E1, UBE2D2, UBE2E1, UBE2J2, UBE2L3, UBE2N/UBE2V1, UBE2S, OTULIN, OTUB1, AMSH, and Cezanne deubiquitylating enzymes, as well as K48 and K63 UB chains were generously provided by Francesco Melandri (Boston Biochem, Cambridge MA). Ubiquitin (U6253**),** Antimycin A (A8674**)** and Oligomycin A (75351) were from Sigma. Phos-Tag gels were from Wako chemicals.

Cell culture, immunoblotting, and mitochondrial protein isolation

HeLa Flp-In T-REx cells (generously provided by Brian Raught, Ontario Cancer Institute) were grown in DMEM with 10% fetal calf serum, 15µg/ml Blasticidin and 100µg/ml Zeocin. The indicated PARKIN proteins and mutants (ΔUBL correspond to PARKIN$^{81-465}$) were transferred from pDNR221-based plasmids into either pcDNA5-FRT/TO-FLAG-HA-Gateway-FRT-Hygromycin based vectors (or the corresponding GFP-tagging vector) using λ recombinase in vitro and these plasmids transfected into HeLa Flp-In T-REx cells followed by selection with Hygromycin (200ug/ml) instead of 100µg/ml Zeocin. To induce protein expression, cells were treated with 0.5 µM doxycycline (DOX) for 16 h to

induce low levels of PARKIN proteins. Cells were either left untreated or depolarized with a mixture of Antimycin A (10 μM) and Oligomycin A (5 μM) for the indicated time period. Where noted CCCP (10 μM) was also used for depolarization. At the indicated times, cells were washed twice with ice cold PBS and lysed in lysis buffer (50 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium 2-glycerol 1-phosphate, 1mM sodium orthovanadate, 0.27M sucrose, 1% (v/v) NP-40, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM PMSF), to produce whole cell extracts.

Crude mitochondria was purified after 2 two wash in ice cold PBS by scrapping cells in PBS containing 200 mM chloroacetamide (3 ml per 15 cm dish). Cells were then collected and centrifuged at 450xg for 5 minutes at 4C. Cell pellet was resuspended in 5 ml (per 15 cm dish) mitochondrial isolation buffer (MIB) (50 mM Tris HCl, pH 7.5, 70 mM Sucrose, 210 mM Sorbitol, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium 2-glycerophosphate, 0.2 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine, 1 μg/ml Leupeptin & Aprotinin) plus 100 mM chloroacetamide and centrifuged at 1400xg for 5 minutes at 4^{o}C. The cell pellet was re-suspended in 5 ml MIB buffer plus 100 mM chloroacetamide and sonicated twice for 20 seconds at lowest settings. Sample were spun 10 min at 1400xg to remove unbroken cells/debris and supernatant was collected, this correspond to the “total protein” fraction, and transferred into round-bottom tube prior to centrifugation for 10 min at 15000xg. Supernatant which correspond to cytosolic fraction and crude ER fraction was removed and the pellet corresponding to the crude mitochondria fraction was resupsended in 10 ml of MIB buffer plus 100 mM chloroacetamide, prior to centrifugation for 10 min at 15000xg. The mitochondrial pellet was wash two more time and pellet was then lysed in lysis buffer plus 100 mM chloroacetamide. In some experiments, we additionally purified

mitochondria using a sucrose gradient (Bozidis et al., 2007).

Whole cell extracts or mitochondrial extracts were sonicated and clarified by centrifugation (16000xg for 10 min at 4C) and protein concentrations determined by the Bradford assay. Cell extracts (25 µg) or mitochondrial extracts were separated by SDS/PAGE, transferred to PVDF membranes, and proteins detected by immunoblotting.

Generation of USP30 cell lines were performed essentially as described previously, but with small modifications (Sowa et al., 2009), Briefly, HeLa Flp-In T-REx cells were transduced with a lentiviral vector expressing HA–Flag–USP30 or the catalytically inactive USP30^{C77A} mutant, and stable cell lines selected in puromycin. Cells stably expressing USP30 were then used as parental cell line to make untagged PARKIN stable cell line using the Flp-In T-REx system describe earlier.

Mitochondrial poly-ubiquitin capture and proteomics

Mitochondrially-derived ubiquitylated proteins were purified using Halo-4xUBA^{UBQLN1}. Briefly, whole cell extracts (1 mg) or mitochondrial extracts (1.5 mg) that were lysed in Lysis buffer containing 100 mM chloroacetamide were incubated at 4^{o}C for 3 hours with 30-50 µl of Halo-4xUBA^{UBQLN1} beads (pack volume). Following 4 washes with lysis buffer containing 1M NaCl and one final wash in 10 mM Tris pH 8.0, proteins were released from Halo-4xUBA^{UBQLN1} using sample buffer prior to analysis by SDS-PAGE and immunoblotting or by 6M Guanidine HCL when analyzed by mass spectrometry. For immunoblotting, samples were separated on 4-12% Bis-Tris gradient gels. For mass spectrometry, samples were subjected to reduction (10 mM TCEP) and alkylation (20 mM chloroacetamide) followed by TCA precipitation. Samples were digested overnight at 37^{o}C with Lys-C and trypsin (in 100 mM TEAB, 0.1% Rapigest, 10% ACN). Digests were acidified with equal volume of 5% formic acid (FA) to a pH ~ 2, dry down,

resuspended in 5% FA, and subjected to C18 StageTip desalting. Samples were analyzed by LC-MS/MS on an Orbitrap-Elite mass spectrometer to identify ubiquitylation sites in mitochondrial proteins (Table S1) as described previously (Sarraf et al., 2013) or used for AQUA as described below.

UB-AQUA proteomics

UB-AQUA was performed largely as described previously but with several modifications (Phu et al., 2011). A collection of 19 heavy-labeled reference peptides (Figure S1G) each containing a single 13C/15N-labeled amino acid was produced at Cell Signaling Technologies and quantified by amino acid analysis. Ub-AQUA peptides from working stocks (in 5% FA) were diluted into the digested sample (in 5% FA) to be analyzed to an optimal final concentration predetermined for individual peptide such that each peptide's intensity would be ranging between e^6-e^8. Samples and AQUA peptides were oxidized with 0.1% Hydrogen Peroxide for 30 min and subjected to C18 StageTip desalting and remove the hydrogen peroxide and resuspended in 5% FA. Both in vitro and in vivo experiments were performed in triplicate and analyzed sequentially by LC-MS on an Exactive-Orbitrap mass spectrometer equipped with a Thermo Fisher nanospray source, a PAL HTC autosampler for sample handling, and an Accela HPLC pump for liquid chromatography (LC) separations. Nanoliter flow rates across the column were achieved using a flow-split method. Peptides were separated on a 125 µm × 18 cm hand-pulled fused silica microcapillary column with a needle tip diameter of <10 µm and was packed with 3-µm 200 Å Maccel C18 AQ beads. The column was equilibrated with buffer A (3% ACN + 0.125% FA). Peptides were loaded onto the column at 100% buffer A. Separation and elution from the column was achieved using a 32-min 0%−42% gradient of buffer B (100% ACN + 0.125% FA). The total LC-method with loading, separation, and column equilibration was 50 min. The Exactive-Orbitrap MS was operated using the following

parameters: AGC, 3×10^6; resolution, 5×10^4; m/z range, 350−1000; maximum ion time, 500 ms; and lock masses, 371.1012 and 445.1200 m/z. LC-MS data analysis was performed using Pinpoint software (Thermo Fisher Scientific) with manual validation of peptide masses (Figure S1G). MS1 intensities from Pinpoint were exported as a .CSV file for further analysis in Excel and GraphPad Prism. Total Ub was determined as the average of the total Ub calculated for each individual locus (Phu et al., 2011). For UB chain synthesis kinetics, data were further analyzed in GraphPad Prism and linear regression analysis was performed to fit slopes with quantitative MS data. R^2 is the coefficient of determination and the 95% confidence intervals are indicated in dotted lines.

S65-PARKIN AQUA proteomics

Heavy-labeled reference peptides for S65-PARKIN (Figure S1G) were from JPT Peptides Technologies (SpikeTides™_TQL Proteotypic Peptides). S65-PARKIN AQUA peptides (non-phospho and phospho heavy peptide) were reduced (10 mM TCEP) and alkylated (20 mM chloroacetamide), dried down and resuspended in 5% FA. The desired amount of AQUA peptide was mixed with previously TCA precipitated sample prior to Lys-C and Trypsin enzymatic digestion (in 100 mM TEAB, 0.1% Rapigest, 10% ACN). Samples were then handled as previously described in the UB-AQUA samples section. MS1 signal for both cleaved and miss-cleaved form of S65-peptide (NDWTVQNCDLDQQSIVHIVQR, NDWTVQNCDLDQQSIVHIVQRPWR) were measured and monitored in Pinpoint software.

Immunoprecipitation of PARKIN and p-S65-PARKIN quantification

Hela Flp-In T-REx cells at 80% confluence expressing Flag-HA-PARKIN or catalytically inactive $PARKIN^{C431S}$ mutant, were treated with doxycycline (0.5 μg.ml) for 16 hours and

then treated with Antimycin A (10 μM) and Oligomycin A (5 μM) for the time indicated. Cells from 2x15 cm dishes were washed twice with ice cold PBS and lysed in 3 ml of lysis buffer supplemented with protease and phosphatase inhibitors. Whole cell extracts were sonicated, clarified by centrifugation (16000xg for 10 min at 4°C), filtered through 0.45μ filters (Sartorius) and protein concentrations determined by the Bradford assay. Parkin from whole cell extracts (14 mg) was immunoprecipitated with 75 μl Anti-FLAG M2 Magnetic Beads (Sigma) for 4 hours at 4°C. Beads were washed 3 times with lysis buffer containing 500 mM NaCl and twice with 50 mM Tris pH 8.0, 150 mM NaCl. Elution with 3xFlag peptide (250 μg/ml) diluted in 50 mM Tris pH 8.0, 150 mM NaCl, was performed twice (375 μl for 10 min), eluates were combined and reductively carboxymethylated, and precipitated with 20% trichloro-acetic acid (TCA). TCA-precipitated proteins were then subject to the AQUA proteomic workflow.

Video microscopy analysis of GFP-PARKIN translocation

The methods for generation of cells expressing inducible GFP-PARKIN and mutant proteins, depolarization and imaging are provided in the main text. In order to determine translocation of GFP-PARKIN to mitochondria (marked by RFP expression), the following method was developed an implemented.

Nuclear segmentation: Manual seeding of nuclear locations enabled the algorithm to detect partial nuclei on the edges of a given field, (cell # 1 in Figure S2D-F). The contribution of data extracted from partial cells around the image border was not critical to the statistics of the final results. However, including the nuclear mask for those partial cells ensured that the algorithm did not associate mitochondria regions from the partial cells with their neighboring cells (mitochondria from cell # 1 will be associated with cell # 2 in Figure S2D-F). If not taken into account, cell-to-cell variability in absolute signal level and translocation kinetics in conjuction with this potential mis-assignment could

significantly affect the accuracy of individual cells' correlation measurements. Therefore, along with manual seeding points, nuclear masks were generated using an edge-based approach for all cells in a field of view. First steerable filter (Ridler et al., 1978) was applied to the DAPI images to generate non-maximum suppressed (NMS) response images as depicted in Figure S2G,H. A series of threshold values was determined by calculating the intensity values in the NMS images stepping down from the 99^{th} to 96^{th} percentile. For each threshold value, the algorithm thresholds the NMS images and detects enclosed rings that represent nuclear boundaries by performing a morphological operation on the thresholded NMS images. Enclosed rings that satisfy the size and roundness criteria and also enclose one seeding point are kept. By looping through all NMS image thresholds from high to low, the algorithm identified on average more than 90% of seeded nuclei. For the remaining nuclei for which the algorithm failed to detect a matching object, a sphere with average nuclear radius was placed on top of each seeding point. Normally those seeding points represented nuclei with either very low DAPI signal or sitting on the image border.

Correction for mis-alignment between channels: Slight mis-alignment of the GFP-PARKIN and mitochondria channels caused by chromatic aberration was detected upon image overlay. To ensure that this would not undermine the correlation analysis, this was corrected at each time point by using the mitochondria channel as the reference image upon which the GFP-PARKIN image was shifted to determine the maximum overlay through image correlation.

GFP-PARKIN channel segmentation: To obtain the mask for the regions of each cell from which the correlation between GFP-PARKIN and RFP-mitochondria channels was computed, a threshold was determined using the Ridler-Calvard method (Ridler et al., 1978) on a Gaussian-smoothed GFP-PARKIN image. The thresholded GFP-PARKIN image was then watershed segmented based on the nuclear mask from the fixed DAPI

image. Though the resultant masks do not closely follow the actual cell boundaries, they enclose the neighborhood of all mitochondria in cells with detectable GFP-PARKIN expression. Cells expressing no or very low-level GFP-PARKIN are not detected by the algorithm and therefore not scored.

Time trace smoothing and fitting: Before fitting the time traces, each line was smoothed using the MATLAB build-in spline fitting function. The smoothed time traces were then fed to a fitting function available through MATLAB central (http://www.mathworks.com/matlabcentral/fileexchange/4363-doseresponse) that is designed to fit dose response data to sigmoid curves. This function fits four parameters that are the minimum and maximum plateau values, the EC50 values (T_{50} in our case) and the Hill coefficient. In order to ensure the fitting converges for all traces, we decided to constrain the minimum and maximum plateau values to be the minimum and maximum values within the spline smoothed data, and only fit the remaining two parameters. A typical time trace, along with its spline smoothed curve and sigmoid function fitted curve are shown in Figure S2I.

Protein Expression, Purification, and Phosphorylation

Human full-length Parkin DNA was codon-optimized for E.coli expression (Invitrogen) and subcloned into pOPIN-K vector for bacterial expression (Berrow et al., 2007) or pFL plasmid (multibac system) for expression in insect cells (Bieniossek et al., 2008). PCR mutagenesis was used to generate all constructs containing point mutation. GST-PreScission-PARKIN and mutants were expressed in BL21-pLys (DE3) E. coli cells using conditions previously described (Kondapalli et al., 2012; Wauer et al., 2013). Baculovirus encoding for 6his-GST-PreScission-PARKIN wild-type and mutants were generated following the multibac system protocol and protein were expressed in Sf9 or High Five cells. Human PARKIN and mutants were expressed in insect cells at 27

degrees for 48-72 hours post infection. Proteins were purified by GST-Sepharose 4B (GE healthcare) and eluted with 20 mM reduced glutathione in 20mM Tris/HCl, 150 mM NaCl, 2 mM DTT, pH 7.5. When necessary, eluted proteins were cleaved with 3c protease overnight at 4^{o}C and/or applied onto Superose 6 10/300 GL (GE healthcare) in 25 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.5 mM TCEP. The peak containing cleaved PARKIN were collected and concentrated. Rattus norvegicus full-length Parkin DNA was codon-optimized for E.coli expression (Invitrogen) and the UBL domain (1-85) subcloned into pOPIN-K vector for bacterial expression. Rat UBL domain was expressed and purified from e.coli as described above for human PARKIN.

Human Ubiquitin DNA was subcloned into a modified pDEST-60 vector encoding for Ubiquitin-6His-tag. Ubiquitin-6His-tag wild-type and mutants were produced in Rossetta (DE3) E. coli cells and purified by Ni-NTA followed by removal of the c-terminal 6His-tag by incubation with GST-USP2cd (1.5 μM). Ubiquitin was filtered through a 30kDa Vivaspin filter to remove GST-USP2cd, concentrated in a 3 kDa molecular-mass cut-off filter device, and loaded on a gel-filtration Superose 6 10/300 GL (GE healthcare). Plasmid encoding for GST-tagged Rat USP2[271-618] (USP2cd) was a gift from Philip Cohen (University of Dundee).

Tribolium castaneum PINK1 (PINK1Tc) [a gift from M. Muqit, MRCPPU, University of Dundee] was expressed as a 6HIS-MBP fusion in BL21 (DE3) Rosetta cells (Kondapalli et al., 2012). Cells were lysed in 1xPBS, 15 mM imidazole, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol and 1 mM Benzamidine. 6HIS-MBP-PINK1 was affinity purified on NiNTA resin and eluted with 1xPBS, 250mM imidazole, 0.1mM EGTA, 0.25% (v/v) Triton X-100, 0.1% (v/v) 2-marcaptoethanol, 1mM Benzamidine and 0.1mM PMSF. Fractions were dialyzed into 50 mM Tris-HCl (pH 7.6),

150 mM NaCl, 0.1 mM EGTA, 0.25% (v/v) Triton X-100, 0.1% (v/v) 2-marcaptoethanol and 10% glycerol.

Two methods were used for phosphorylation by PINK1Tc and subsequent purification. In Method 1, GST-PARKIN was eluted from GSH-Sepharose, incubated with PINK1Tc at a 6:1 ratio (6 h) prior to re-capture of GST-PARKIN on GSH-beads. After washing, GST-PARKIN was proteolyzed with PreScission protease to remove GST and PARKIN purified by gel filtration. In Method 2, fractions containing GST-PARKIN were purified by gel filtration chromatography in 25 mM Tris-HCl (pH 7.6), 200 mM NaCl, 1 mM DTT. PARKIN to be phosphorylated was proteolyzed to remove GST, diluted and applied to a Q-sepharose column and eluted with a linear salt gradient. Fractions were concentrated (Amicon Ultra) and further purified by gel filtration chromatography. The cleaved PARKIN (30 μM) was incubated with 6 μM PINK1Tc for 3 h at 30°C in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM EGTA and 2 mM DTT. Phosphorylated PARKIN (p-WT PARKIN or $PARKIN^{S65A}$ or $PARKIN^{C431S}$ treated in the same manner) was purified by Q-sepharose in 25 mM Tris-HCl (pH 8.0), 1 mM DTT using a linear salt gradient. The stoichiometry of PARKIN and UB phosphorylation was determined by AQUA proteomics using peptides that map to the pS65 locus of each of the respective proteins. UB (Boston Biochem) or the indicated UB mutant were phosphorylated at 30°C in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM EGTA and 2 mM DTT. 20 μM UB was incubated with 4 μM PINK1Tc for 24 h. Phosphorylated UB (p-UB) was purified by gel filtration chromatography in 25 mM Tris-HCl (pH 7.6), 200 mM NaCl, 1 mM DTT, buffer exchanged into pure water using a PD10 desalting column (GE Healthcare), applied to a Q-sepharose column and eluted with 50 mM Tris-HCl (pH 7.6). The extent of UB phosphorylation at S65 was determined by UB-AQUA, electrophoresis

on PhosTag gels, and intact mass analysis. Intact mass measurements were performed using nano-electrospray ionization and time-of-flight mass spectrometry (Waters LCT Premier XE MS) by the St. Jude Proteomics Facility.

After purification on GSH-Sepharose, the UBL domain from rat PARKIN was cleaved, cleaned up by ion exchange and then sizing. The UBL domain from rat PARKIN was phosphorylated under the same conditions as for p-UB. Reaction was ran over

sizing to clean it up (final buffer 25mM Tris 7.6/200mM NaCl). The extent of rat UBL domain phosphorylation (p-UBL) (>98%) was determined by electrophoresis on native gel as well as PhosTag gels.

Halo-tag-4xUBQLN1^{UBA} resin was prepared as in (Emmerich et al., 2013 and the plasmid was a generous of P. Cohen, MRCPPU, University of Dundee) with the following modifications: a 6his-tag was added N-terminal to the Halo-tag and upon expression in Rossetta (DE3) E. coli cells, was purified by Ni-NTA and stored at 80°C. Covalent coupling of Halo-4xUBA^{UBQLN1} to the resin was done by mixing 5 mg of purified Halo-4xUBA^{UBQLN1} to 1 ml of HaloLink resin.

All purified proteins used in experiments were run on SDS-PAGE, followed by staining with Colloidal Coomassie Blue for quality control.

In vitro ubiquitination analysis and UB AQUA proteomics

Unless specified in the figure legends, all ubiquitylation experiments were performed as follows. E1 (UBE1 0.1 μM), E2 conjugating enzyme (UBE2L3 1 μM), E3 ligase (PARKIN 1 or 2 μM), total UB (30 μM) were incubated for the indicated time at 30°C in a 25 μl reaction mixture containing 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM TCEP. The reaction was started by the addition of 2 mM ATP and terminated by the addition of SDS sample buffer containing 10 mM EDTA when analyzed on SDS-PAGE or by the addition

of 10 mM EDTA and TCA (20% final) precipitated when the reaction was further analyzed by mass spectrometry (see UB AQUA section). When PARKIN had to be activated by PINK1Tc prior to ubiquitylation assay, 2 μM PARKIN was incubated with 0.1 μM MBP-PINK1Tc for 60 min at 30°C in a 20 μl reaction mixture containing 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM TCEP, 2 mM ATP). After 60 min, ubiquitylation components (E1, E2, Ub) diluted in 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM TCEP, were added to the samples and incubated for a further 45 min at 30°C. Reaction were stopped by the addition of SDS sample buffer containing 10 mM EDTA when analyzed on SDS-PAGE or by the addition of 10 mM EDTA and TCA (20% final) precipitated when the reaction was further analyzed by mass spectrometry (see UB AQUA section). For Figure S4 E-G, the minimal amount of E2 necessary for the assay was first determined in order to avoid any artifactual PARKIN-independent chain formation that occur with higher than necessary concentration of E2s and can complicate interpretation of results.

In vitro Deubiquitination assay

Unless specified in the figure legends, all deubiquitination experiments were performed as follows: DUBs were diluted in DUB buffer (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM DTT), incubated at 25°C for 10 min for activation. Kinase and ubiquitylation assays were setup as described above and reactions were stopped by the addition of 10 mM EDTA. Aliquots (15 μl) of ubiquitylation assay were mixed with indicated amount of DUBs to a final volume of 30 μl with reaction mixture containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM DTT. The reaction was then incubated for the time indicated at 30ºC with gentle agitation and terminated by the addition of SDS sample buffer. Free M1 or K48-linked recombinant poly-UB chains (2-7) were used as positive controls for

OTULIN and USP5 activity. Ubiquitin chain cleavage was detected by immunoblotting using a polyclonal anti-UB antibody.

Binding and Affinity Measurements

Isothermal calorimetry measurements were made on an ITC200 (MicroCal). PARKIN, p-WT PARKIN and p-UB were buffer exchanged into 25 mM Hepes (pH 7.0), 200 mM NaCl, 1 mM 2-mercaptoethanol using Nap5 desalting columns (GE Healthcare). Proteins were concentrated to 15 μM (PARKIN and p-PARKIN) and 150 mM (p-UB). p-UB was titrated against either PARKIN or p-PARKIN in the sample cell at a constant temperature of 18^{o}C. Sixteen titration measurements were made and the data were analyzed using Origin software provided by MicroCal. Bio-layer interferometry (BLI) measurements were made on an Octet RED96 (ForteBio). GST-PARKIN was immobilized on a GST-capture biosensor equilibrated in 20 mM Tris-HCl (pH 7.6), 200 mM NaCl, 0.1 mg/mL BSA and 0.01% Tween. Equilibrated biosensors were exposed to 0.1-4.0 μM pUB for 40 sec followed by a 60 sec dissociation period in buffer. Affinity measurements were performed using data analysis software provided by ForteBio and fit in Prism5 (Graphpad Software). The mass of PARKIN and the mass/stoichiometry of PARKIN/p-UB complexes was measured by size exclusion chromatography (Superose 6, 10/300 GL) mounted on a high-pressure liquid chromatography system (1260 Infinity, Agilent) coupled to in-line Multi-Angle Light Scattering (SECMALS) (Wyatt DAWN HELEOS II) with in-line refractive index detection (Wyatt Optilab T-rEX). The wavelength used was 658 nm and the calibration constant was 2.32950e-4 1/(V cm). The column was equilibrated with 25 mM Tris pH 8.0, 200 mM NaCl, 2 mM DTT and experiments were conducted at 25 °C. The injected sample volume was 50 μl and experiments were conducted at a flow rate of 0.4 ml/min. Eluted protein was detected via light scattering and refractive index and data were recorded and analysed with Wyatt Astra software

(version 6). We acknowledge the generous assistance of Steve Harrison and Yoana Dimitrova (Harvard Medical School for assistance in SEC-MALS analyses. To examine reaction of PARKIN with UB-VS, the indicated proteins were incubated in reaction buffer (25mM Tris pH 7.6, 50mM NaCl and 5mM DTT) and samples removed over time prior to analysis by SDS-PAGE.

Generation of PINK1 Knockout Hela Flp-In T-REx cell line

To generate PINK1 knockout (KO) cells, a PINK1 KO TALEN pair (Human-H73593_TALEN_L1 and R1, target site: TGGGCCGCAGGACCGGGCGC GGAGCCTCGCAGGGTCGGGCTCGGGCTCCCTA) was purchased from Toolgen.com. HeLa Flp-In T-REx/PARKIN cells were then transfected with TALEN_L1, TALEN_R1 and flag-GFP constructs at the ratio of 2:2:1 by using lipofectamine 2000. After 48h, cells with the top 1% GFP signal intensity were single-cell sorted in 96-well plate using a BD FACSAria at the DFCI flow cytometry facility. To screen PINK1 KO clones, PCR-based genotyping was performed as follow. Primers encompassing TALEN target site (forward: GGCAGCGGCGGCTGCGGGGG, reverse: CCGCCGGCTCTCCGCCTGTTTTTCC) were used to amplify the target region from the genomic DNA isolated from each clone. Upon amplification, PCR products were subjected to sequencing at the DF/HCC DNA resource core to confirm the PINK1 KO clone. To further verify its deletion, western blotting against PINK1 was performed as well.

Whole cell lysate pS65-UB measurement

Parental Hela Flp-In T-REx/PARKIN cells (PINK1+/+) and PINK1 -/- HeLa Flp-In T-REx/PARKIN cells at 80% confluence, were treated with DMSO or with Antimycin A (10 μM) and Oligomycin A (5 μM) for an hour. Cells from 2x15 cm dishes were washed twice with ice cold PBS and harvested in 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$,

10 mM glycerol 2-phosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.5 mM EGTA, 1 mM sodium orthovanadate, 5 mM TCEP, 1 mM PMSF, 1 µg/mL aprotinin, 1 µg/mL leupeptin and phosphatase inhibitor cocktail. Extracts were briefly sonicated and recombinant USP2cd was added to a final concentration of 0.5 µM. Samples were incubated at 30°C with shaking at 1100 r.p.m. for two hours. After centrifugation at 20000 x g for 15 minutes, the supernatants were heated at 95^{o}C for 20 minutes to denature and precipitate the majority of cellular proteins. Protein concentration of the heat-resistant supernatants containing ubiquitin was determined by bicinchoninic acid assay. Equal amount of samples were subject to TCA precipitation and digested overnight at 37°C with Lys-C and trypsin (in 100mM TEAB, 0.1% Rapigest, 10% ACN). Measurement of pS65-UB was done as described in the UB-AQUA section.

In vitro PARKIN binding to 6his-UB chains experiments

K48- or K63-linked 6his-UB$^{(2-7)}$ chains were incubated with MBP-PINK1Tc or catalytically inactive MBP-PINKTc[D359A] at 30°C in a 100 µl reaction mixture containing 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM TCEP, 1 mM ATP for 60 min with shaking at 1100 r.p.m. When needed, recombinant wild-type PARKIN or indicated mutants were phosphorylated in a parallel kinase assay reaction with MBP-PINK1Tc, similarly to 6his-UB$^{(2-7)}$ chains. After 60 min, MBP-PINK1Tc was removed by affinity purification using amylose resin. Phosphorylated or unphosphorylated 6his-UB$^{(2-7)}$ chains (3 µg) were mixed with wild-type PARKIN (1 µg) or indicated mutants in 300 µl reaction mixture containing 25 mM Hepes (pH 7.5), 1 mM EGTA, 2 mM $MgCl_2$, 0.5% (vol/vol) Triton X-100 (binding buffer) plus 150 mM NaCl and incubated for 30 min at 25°C. After 30 min 6his-UB$^{(2-7)}$ chains were captured by adding nickel-charged affinity resin (10 µl pack beads) and incubated for a further 15 min, after which beads were washed three times with 1 mL of buffer plus 250 mM NaCl and once with buffer without NaCl and

Triton X-100. Bound proteins were released by denaturation with 1% LDS, the beads were removed by centrifugation for 1 min at 2,000 × g, and the supernatants were heated for 5 min at 75 °C and subjected to SDS/PAGE.

Evolution of phospho-specific antibodies for S65 in PARKIN

The phospho-specific S65 antibodies were isolated from a recently developed phospho-specific antibody library (Koerber et al., 2013) using standard phage display protocols. Selections were performed using a biotinylated phosphopeptide antigen [Biotin-linker-DLDQQpSIVHIVQR-amide] captured with streptavidin-coated magnetic beads (Promega). Three rounds of selection were performed with decreasing amounts of peptide antigen (500, 250, and 100 nM) and individual phage clones from the third round of selection were analyzed by ELISA. Briefly, 96-well Maxisorp plates were coated with 10 μg/mL NeutrAvidin overnight at 4^{o}C and subsequently blocked with 0.5% BSA for two hours at 20^{o}C. Plates were then incubated with either 250 nM phosphorylated peptide, 250 nM nonphosphorylated peptide, or buffer only. Serial dilutions of phage were captured on the peptide-coated wells for fifteen minutes and bound phage were then detected using a horseradish peroxidase (HRP)-conjugated anti-M13 monoclonal (GE Healthcare). Selected phospho-specific scFv clones were subsequently cloned into a bacterial expression vector with an Avi-tag that enables in vivo biotinylation. ScFvs were then expressed in the C43 (DE3) bacterial strain and purified via Protein A chromatography.

Phosphoproteomic analysis of PINK1 KO MEFs

WT and PINK1-/- MEFs were grown in DMEM with 10% bovine serum and treated with Antimycin A (10 μM) and Oligomycin A (5 μM) for the indicated time period, prior to the

purification of mitochondria as described (Bozidis et al., 2007). Mitochondria were lysed and digested with LysC and trypsin, prior to selection of phosphopeptides as described (Kettenbach and Gerber, 2011). Peptides derived from the indicated times after depolarization were subjected to 10-plex TMT analysis in triplicate and analyzed on an LTQ Orbitrap Velos or Elite (Thermo Scientific San José, CA) mass spectrometer using MS3-based reporter ion quantification (Ting et al., 2011; McAlister et al., 2012). For quantification, a 0.03 *m/z* window centered on the theoretical *m/z* value of each ten reporter ions and the closest signal intensity from the theoretical *m/z* value was recorded. Reporter ion intensities were further de-normalized based on their ion accumulation time for each MS^3 spectrum and adjusted based on the overlap of isotopic envelopes of all reporter ions (manufacturer specifications). Total signal to noise values for all peptides were summed for each TMT channel, and all values were adjusted to account for variance in sample handling. For each peptide, a total minimum signal to noise value of 100 was required (Ting et al., 2011; McAlister et al., 2012).

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