Supporting Information

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SI Materials and Methods

Reagents and Antibodies. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP; Sigma) was prepared in ethanol and used at a final concentration of 10 μM. Paraquat (Sigma–Aldrich) was prepared in water and used at a final concentration of 10 mM. Genistein (Sigma) was prepared in DMSO and used over a range of concentrations from 10 to 100 μM. Antibodies used for immunoblotting were anti-light chain 3 (anti-LC3; Sigma), anti-PTEN-induced kinase 1 (PINK1) (Novus), anti-Parkin (Abcam), antiactin (Millipore), antitubulin (Sigma), goat anti-mouse IgG horseradish peroxidase (HRP; Abcam) and goat anti-rabbit IgG HRP (Invitrogen). Antibodies used for immunofluorescence were anti-ATP5A (Mitosciences), anti-GFP (Abcam), Alexa Fluor 488 anti-rabbit (Molecular Probes), and Alexa Fluor 594 anti-mouse (Molecular Probes).

Immunofluorescence and Dyes. Cells were cultured in eight-well chamber slides (BD Bioscience) and fixed in ice-cold methanol. Samples were blocked in 1% BSA for 30 min at room temperature before antibody application. The nucleic acid binding dye, Hoechst 33342 (Molecular Probes), was added for 10 min before sample mounting with MOWIOL 4-88 (Calbiochem). Microscopy was performed using a Deltavision RT Deconvolution Widefield microscope or an Olympus FV1000 Fluoview Confocal microscope with a U Plan S Apo N.A. 1.40 100× objective or a Plan Apo N.A. 1.42 60× objective.

High-Content Screening. ***Confirmation screens.*** dsRNA probes corresponding to primary screen hits were arrayed across three 384-well plates and underwent four rounds of confirmation screens, following the same protocol as primary screening.

Secondary screens. Three secondary screens were performed on confirmed primary hits. Paraquat-induced Parkin translocation and mitochondrial perinuclear clustering assays involved seeding 6,000 cells of a Schneider 2 receptor plus (S2R+) cell line stably expressing pMK33-Parkin-GFP into clear-bottomed, dsRNA-containing 384-well plates as in the primary screen. Cells were treated with 10 mM paraquat for 24 h before fixation with ice-cold methanol. Assays were performed in triplicate. Parkin translocation image acquisition and analysis methodologies corresponded to primary screening. Mitochondrial perinuclear clustering assays were acquired using a Plan Fluor N.A. 0.30 10× objective (Nikon), with four images per well. Images were quantified manually using a three-point scoring system, where 1 represents diffuse mitochondria, 2 represents a mixture of diffuse and aggregated mitochondria, and 3 represents aggregated mitochondria. Mitochondrial morphology analysis used nontoxified S2R+ cells, seeded as in the primary screen. After 4 d, the medium was replaced with Schneider's medium (SM) containing MitoTracker Red (Molecular Probes) and Hoechst dye, and incubated for 15 min. That medium was replaced with SM, and samples were imaged live using a CFI S Plan Fluor N.A. 0.60 40× objective (Nikon) at nine images per well. Mitochondrial morphology was assessed manually using a four-point scoring scheme, where 1 indicates fragmented, 2 indicates WT, 3 indicates fused, and 4 indicates clumped. All scoring was done with the experimenter blinded to the conditions.

HeLa screens. We used a custom library of human siRNA probes comprising 84 siGENOME SMARTpool siRNAs (Dharmacon). siRNAs were arrayed over two clear-bottomed 96-well plates (Greiner) at a final concentration of 25 nM. Screening analyzed Parkin translocation and mitophagy in a HeLa cell line stably expressing pLVX-Puro-YFP-Parkin. Dharmafect 1 transfection reagent (Dharmacon) was added to siRNA-containing plates according to the manufacturer's instructions, and cells were plated in antibiotic-free media. Plates were sealed and incubated for 4 d (Parkin translocation) or 3 d (mitophagy) before the addition of CCCP-containing media for 4 h (Parkin translocation) or 24 h (mitophagy). Cells were fixed with ice-cold methanol and processed for imaging. Nine images per well were captured using a Plan Fluor N.A. 0.30 10× objective (Nikon). Images were analyzed manually using a five-point scoring scheme. In each case, a score of 0 indicates dead or low YFP-expressing cells. The degree of Parkin translocation was assessed, where 1 indicates <10% translocation, 2 indicates 10–50% translocation, 3 indicates 50–90% translocation, and 4 indicates >90% translocation. The degree of mitophagy was assessed, where 1 represents a full complement of mitochondria, 2 represents an overall reduced mitochondrial signal, 3 represents ∼10% of cells with no mitochondrial signal, and 4 represents >20% of cells with no mitochondrial signal.

Lysate Preparation and Immunoblotting. Cells were lysed in lysis buffer [50 mM Tris·HCl, 150 mM NaCl, 10% (vol/vol) glycerol, 1% Triton X-100, 10 mM *N*-ethylmaleimide, 2 mM EGTA, 1 mM $MgCl_2$, 50 μM MG-132, and protease inhibitor mixture (Roche)]. Lysates were resolved by SDS/PAGE, transferred to PVDF (Bio-Rad), and incubated with primary antibodies. Immunoreactive bands were detected using enhanced chemiluminescence reagent (Amersham) per the manufacturer's instructions and exposed to light-sensitive photograph film (Amersham). Immunoblots were quantified using the ImageJ (National Institutes of Health) plug-in "Gels" and normalized to loading controls.

Assessing Starvation-Induced Autophagy. HeLa cells were transfected with 25 nM siRNA as indicated and incubated for 4 d. The medium was replaced with fresh medium (U, untreated), HBSS (Invitrogen) (S, starvation), 20 nM bafilomycin A_1-containing media (Sigma) (B, bafilomycin A_1), or 20 nM bafilomycin A_1-containing HBSS (S&B) for 2 h. Protein samples were collected, and endogenous LC3 was analyzed using SDS/PAGE. Immunoblots were quantified using ImageJ. Assays were performed in triplicate, and the mean "LC3-II/total LC3" ratio was calculated per sample.

Quantitative Real-Time PCR. RNA was extracted using an RNeasy RNA purification kit (Qiagen), and cDNA was synthesized using a Protoscript II first-strand cDNA synthesis kit (New England BioLabs) according to each manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using a standard protocol, and each sample was normalized to the ribosomal reference gene, *RNA18S5*. A list of primers is available upon request. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ (cycle threshold) method. The primers used for qRT-PCR are as follows:

RNA18S5: CAGCCACCCGAGATTGAGCA, TAGTAGCGACGGGCGGTGTG

PINK1: GCCGGACGCTGTTCCTCGTT, TGGACACCTCTGGGGCCATC

Parkin: CACACTGACAGCAGGAAGGA, AGGGGCCTTTGCAATACA

FBXW7: GGGCACCAGTCGTTAACAAG, GTCACAGCACTCTGATGCTTG

SREBF1: CGTGCTGGGCACCGAGAGC, GGCCGTGTGACTGGCTCACC

Analysis of Mitochondrial Membrane Potential Status. S2R+ cells were cultured in eight-well live imaging trays (Ibidi), followed by the addition of 50 nM MitoTracker Red in SM for 30 min. The medium was replaced and live cells were imaged before (0 min) and 5, 15, or 30 min after the addition of CCCP to culture media.

HeLa cells were cultured in 96-well plates (Corning) in the presence of siRNA as indicated. Samples were incubated with vehicle- or 10 μM CCCP-containing medium for 1 h before the addition of 50 nM tetramethylrhodamine, methyl ester (Molecular Probes) in assay buffer (80 mM NaCl, 75 mM KCl, 25 mM D-glucose, 25 mM Hepes) for 30 min. Cells were washed in vehicle- or 10 μM CCCP-containing assay buffer, and fluorescence was quantified using a Mithras LB940 plate reader. Each well was normalized to the cell number by incubation with CyQuant (Molecular Devices) according to the manufacturer's instructions.

Parkin Translocation Rescue. Initially, deconvolved SMARTpools (Dharmacon) made up of four individual siRNAs against *SREBF1*, *FBXW7*, *GSK3A*, and *parkin* were tested for their ability to block CCCP-induced Parkin translocation in HeLa cells stably expressing YFP-Parkin. The single strongest siRNA for each gene was selected for use in rescue experiments. For this, corresponding cDNA (Source Bioscience) underwent Quikchange mutagenesis (Agilent) according to the manufacturer's instructions, producing three to four synonymous point mutations within the siRNA target region. Mutations were concentrated in the seed region of the siRNA target, producing siRNA-resistant constructs. To demonstrate the rescuing effect, either siRNA alone or siRNA and the corresponding siRNA-resistant construct were transfected into HeLa cells stably expressing YFP-Parkin. After treatment with CCCP (10 μM) for 4 h, the degree of Parkin translocation was assessed by fluorescence microscopy. The assay was performed in triplicate and assessed blindly on a cell-by-cell basis.

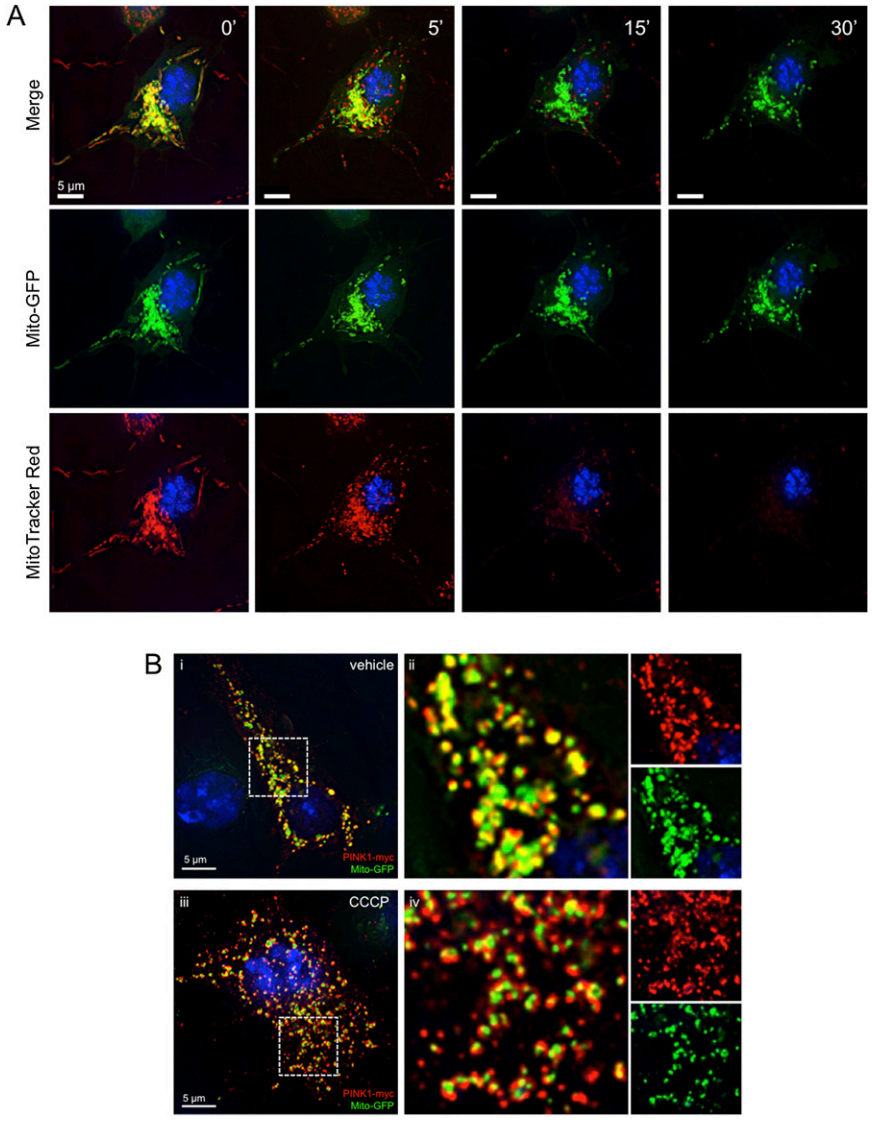

Fig. S1. *Drosophila* S2R+ cells recapitulate mitochondrial membrane potential (ΔΨm) loss and PINK1 stabilization following CCCP toxification. (*A*) Cells transiently expressing Mito-GFP (green), treated with 50 nM MitoTracker Red (red) for 30 min and imaged live over a time course of 10 μM CCCP treatment. (*B*) Cells transiently expressing Mito-GFP (green) and PINK1-myc (red), treated with vehicle (*i* and *ii*) or 10 μM CCCP (*iii* and *iv*) for 4 h. Boxed areas in *i* and *iii* are enlarged in *ii* and *iv*. Images were acquired using a Deltavision RT system with a 100× objective. (Scale bars: 5 μm.)

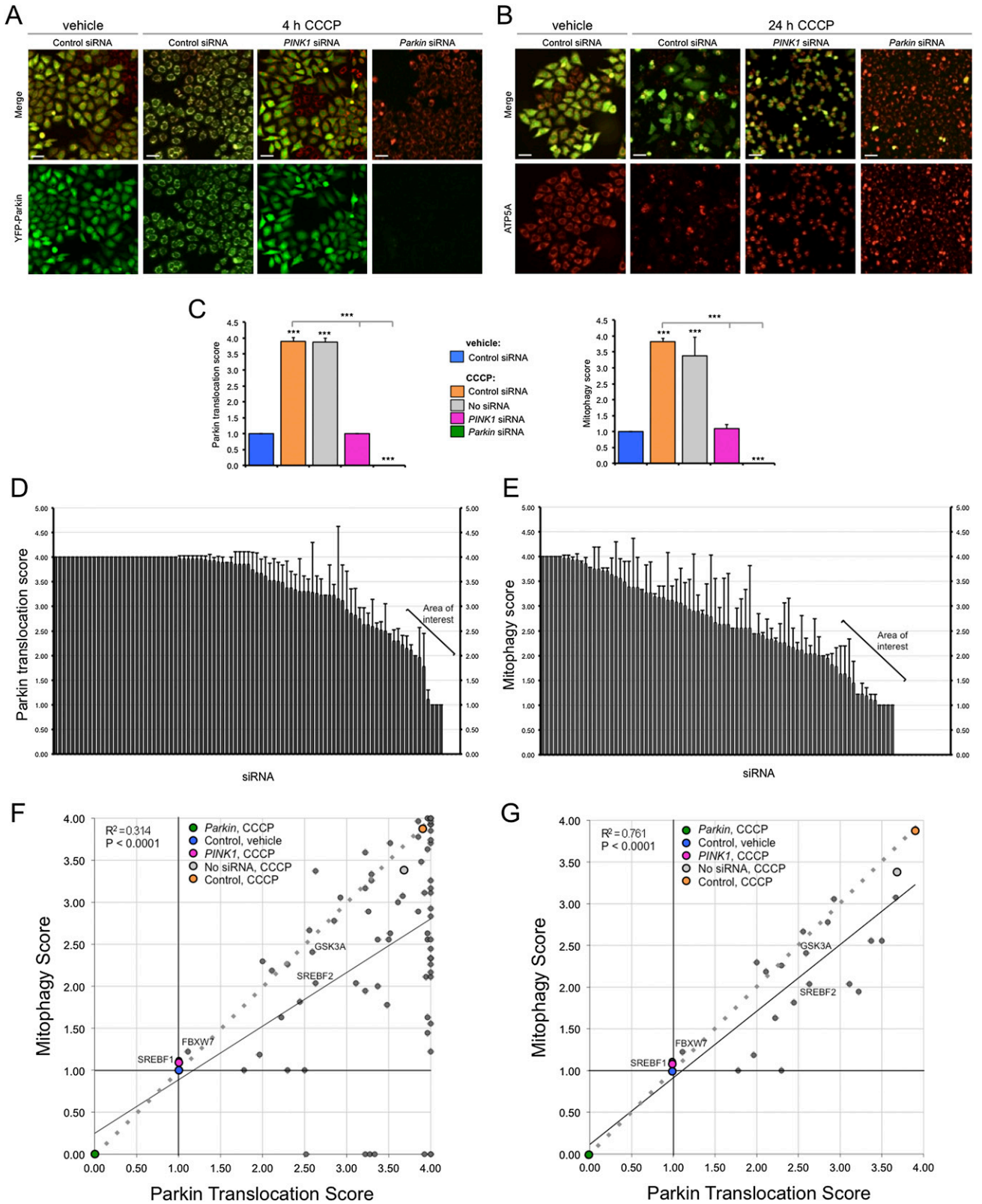

Fig. S2. Rescreening for conserved modulators of Parkin-mediated mitophagy in HeLa cells. Example high-throughput images of HeLa cells stably expressing YFP-Parkin transfected with control, *PINK1*, or *Parkin* siRNAs, following the application of vehicle or CCCP (10 μM) to induce Parkin translocation (*A*) or mitophagy (*B*). (Scale bars: 40 μm.) (*C*) Quantification of Parkin translocation and mitophagy scored in *A* and *B*. Charts represent mean ± SD from triplicate experiments. ***$P < 0.001$ (one-way ANOVA with Bonferroni correction) compared with the vehicle-treated control siRNA unless otherwise indicated. Graphical representation of the Parkin translocation (*D*) and mitophagy score (*E*) of each siRNA. Charts show mean ± SD from triplicate experiments, arranged according to assay score (high to low) and then by gene name (A to Z). siRNAs reducing the degree of Parkin translocation or mitophagy to a level comparable

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to *PINK1* siRNA are highlighted as the "area of interest." (*F* and *G*) Linear regression analysis of Parkin translocation and mitophagy scores. Scatter graph representation of the Parkin translocation score (*x* axis) against the mitophagy score (*y* axis) of all candidate siRNAs tested (*F*) or only the 20 hits (*G*). Colored data points represent screen controls (see key). Solid lines represent the line of best fit and correspond to the linear regression line equation (*y*). Also included is the "coefficient of determination" (R^2) values and significance of the positive data correlation (*P*). Dashed lines represent $R^2 = 1$.

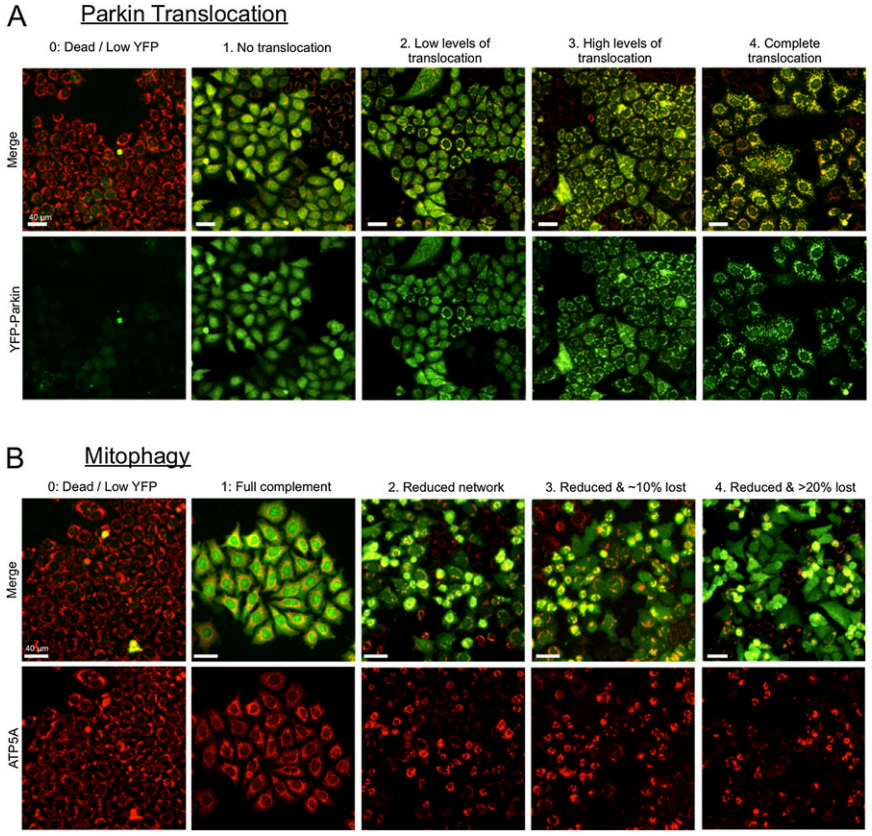

Fig. S3. HeLa cell scoring scheme for Parkin translocation and mitophagy. Example images of the scoring system used to assess Parkin translocation (*A*) and mitophagy (*B*) in a CCCP-treated HeLa cell line stably expressing pLVX-Puro-YFP-Parkin (YFP-Parkin.HeLa) cells. Cells were toxified with 10 μM CCCP for 4 h (*A*) or 24 h (*B*). (*A*) YFP-Parkin translocation (green) was assessed manually according to the following five criteria: 0 = dead cells or low YFP expression, 1 = <10% of cells with Parkin translocation, 2 = 10–50% of cells with Parkin translocation, 3 = 50–90% of cells with Parkin translocation, 4 = >90% of cells with Parkin translocation. (*B*) Mitophagy was assessed manually by loss of the ATP5A marker (red) according to the following five criteria: 0 = dead cells or low YFP expression, 1 = full complement of mitochondria, 2 = cells with a predominantly reduced mitochondrial network, 3 = cells with a reduced mitochondrial network and ~10% of the population with no mitochondria, 4 = cells with a reduced network and >20% of the population with no mitochondria. Images were captured using an ImageXpress Micro wide-field high-content screening microscope with a 10× objective (Molecular Devices). (Scale bars: 40 μm.)

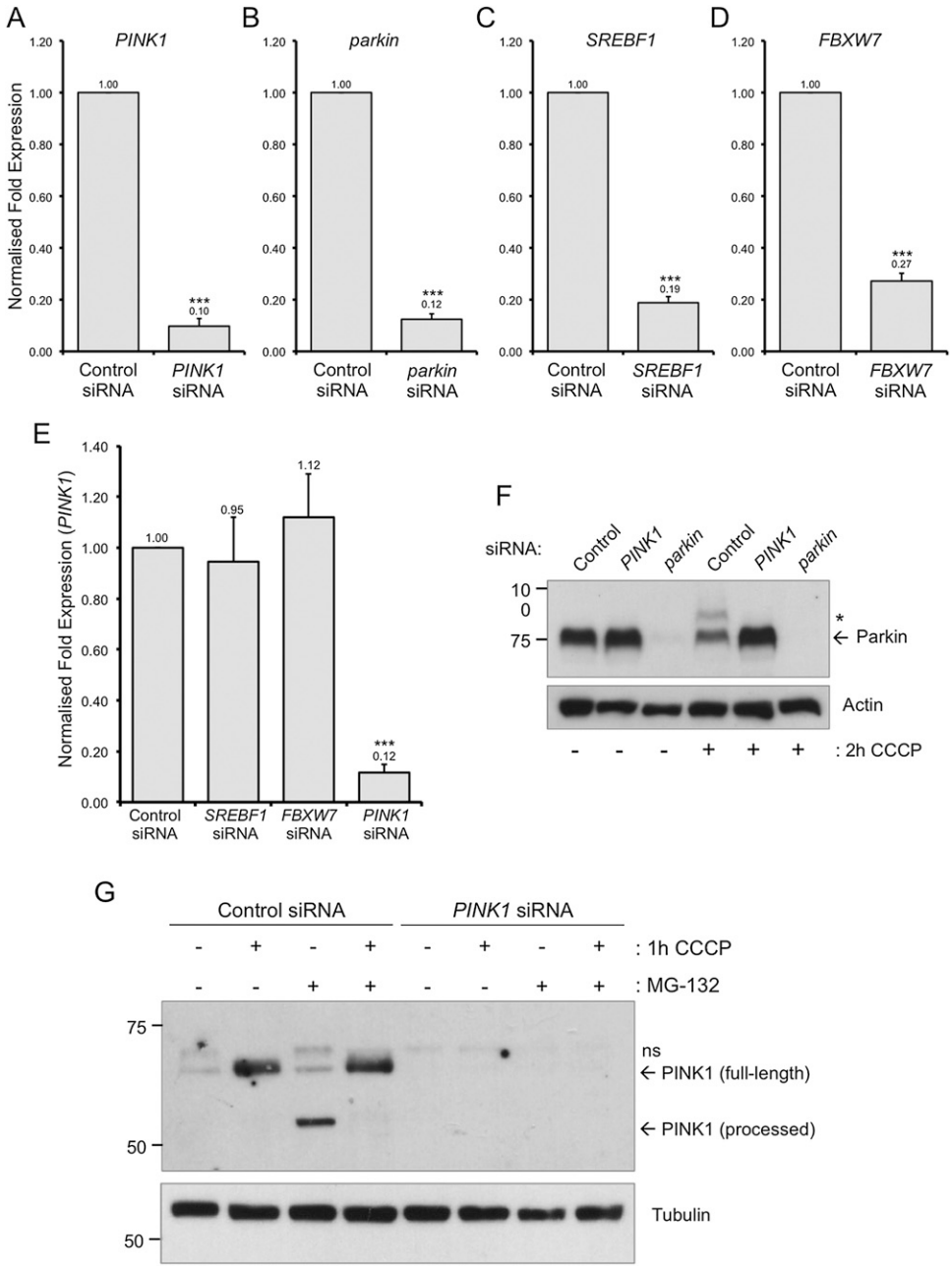

Fig. S4. Expression analysis following siRNA treatment. HeLa cells expressing YFP-Parkin were exposed to 25 nM control, *PINK1*, *parkin*, *SREBF1*, or *FBXW7* siRNA, as indicated, for 4 d. Expression of *PINK1* (*A* and *E*), *parkin* (*B*), *SREBF1* (*C*), and *FBXW7* (*D*) was analyzed using qRT-PCR against the housekeeping gene *RNA18S5*. Graphs show the mean ± SD of triplicate experiments. ***$P < 0.001$ (Student's *t* test, compared with control siRNA-treated samples). (*F* and *G*) Immunoblots to test the efficacy of knockdown and specificity of antibodies. HeLa cells were treated with siRNAs and CCCP as indicated and immunblotted for Parkin (*F*) or endogenous PINK1 (*G*). The asterisk indicates a ubiquitinated Parkin species upon depolarization. Proteasome inhibitor MG-132 (50 μM for 1 h) allows detection of endogenous processed PINK1. Actin or tubulin served as a loading control. ns, nonspecific band in an anti-PINK1 immunoblot.

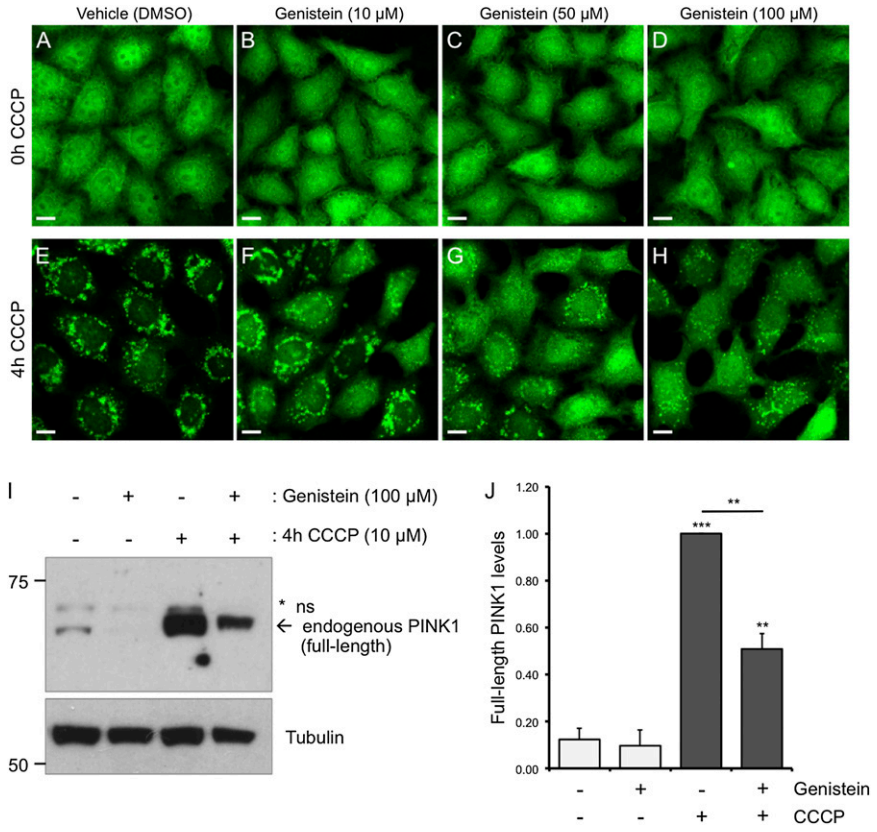

Fig. S5. Chemical inhibition of the sterol regulatory binding protein (SREBP) pathway by genistein treatment. HeLa cells expressing YFP-Parkin were treated with either vehicle (*A* and *E*) or genistein (*B–D* and *F–H*) over a range of concentrations [10 μM (*B* and *F*), 50 μM (*C* and *G*), and 100 μM (*D* and *H*)] for 24 h before the application of vehicle (*A* and *E*) or 10 μM CCCP (*B–D* and *F–H*) for 4 h. YFP-Parkin translocation was assessed qualitatively. (Scale bars: 10 μm.) (*I*) HeLa cells were treated with either vehicle or 100 μM genistein for 24 h before the application of vehicle or 10 μM CCCP for 4 h. Alterations in endogenous full-length PINK1 levels were assessed by immunoblotting. Tubulin was used as a loading control. (*J*) Quantification of endogenous full-length PINK1 levels. The chart represents mean ± SEM of triplicate assays. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with vehicle-treated samples unless otherwise indicated (one-way ANOVA with Bonferroni correction).

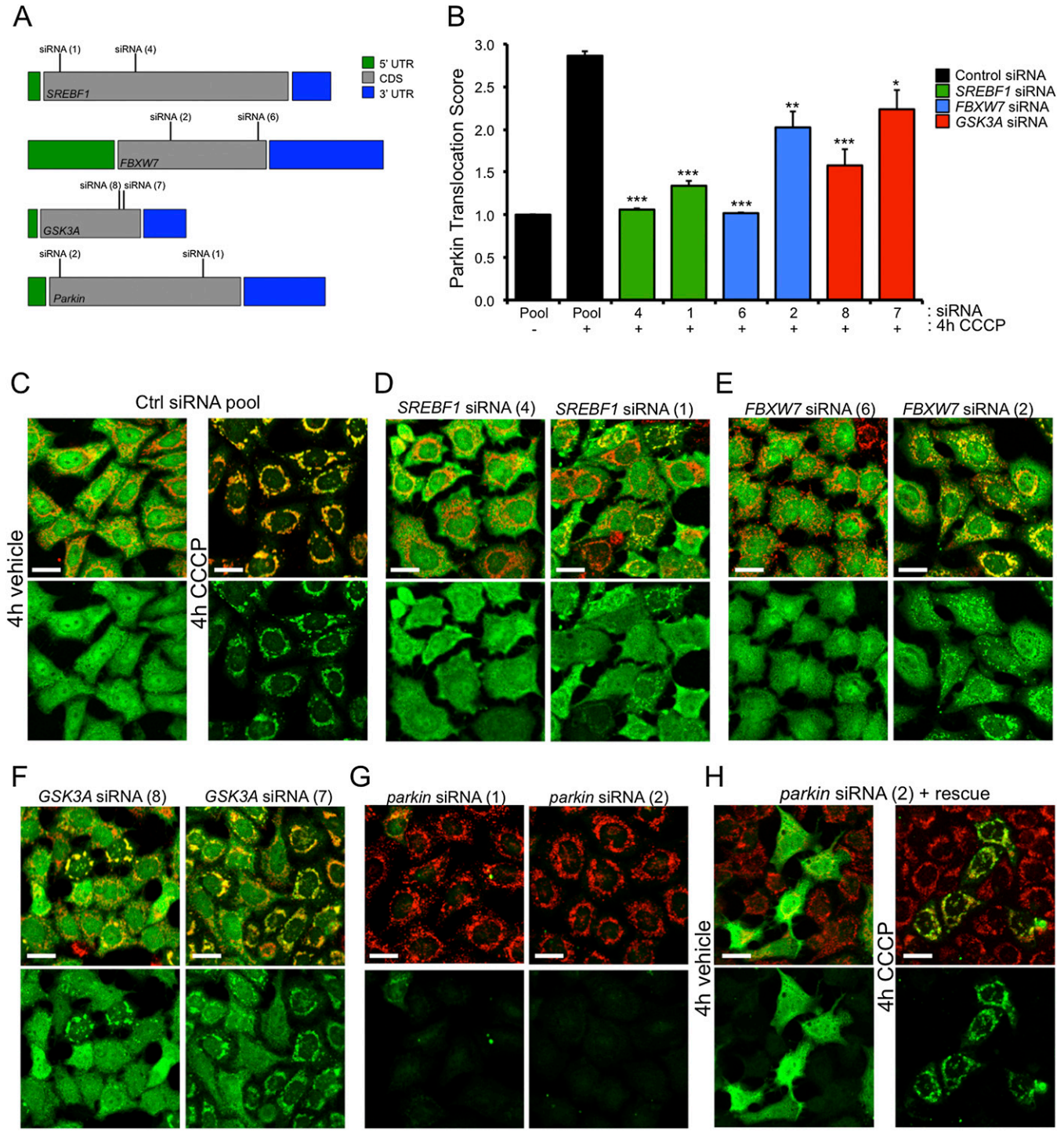

Fig. S6. Deconvolution of siRNA SMARTpools. (*A*) Schematic of individual siRNA target regions within corresponding mRNAs. (*B–G*) HeLa cells expressing YFP-Parkin (green) were transfected with either pooled (*C*) or individual (*D–G*) siRNAs and treated with either vehicle (*C*) or CCCP (10 μM) (*C–G*) for 4 h as indicated. (*B*) YFP-Parkin translocation to mitochondria (anti-ATP5A; red) was analyzed, producing a Parkin translocation score for each condition. The chart represents mean ± SEM of triplicate assays. ***$P < 0.0001$; **$P < 0.01$; *$P < 0.05$ (one-way ANOVA with Bonferroni correction, compared with "Control, 4-h CCCP" samples). (*G*) HeLa cells expressing YFP-Parkin (green) and transfected with two individual siRNAs targeting *Parkin* lead to a marked decrease of YFP-Parkin signal. (*H*) Cotransfection of an individual *parkin* siRNA and a corresponding "siRNA-resistant" YFP-Parkin construct leads to the rescue of YFP-Parkin expression, and its translocation to mitochondria (red) following treatment with CCCP (10 μM) for 4 h. (Scale bars: 20 μm.)

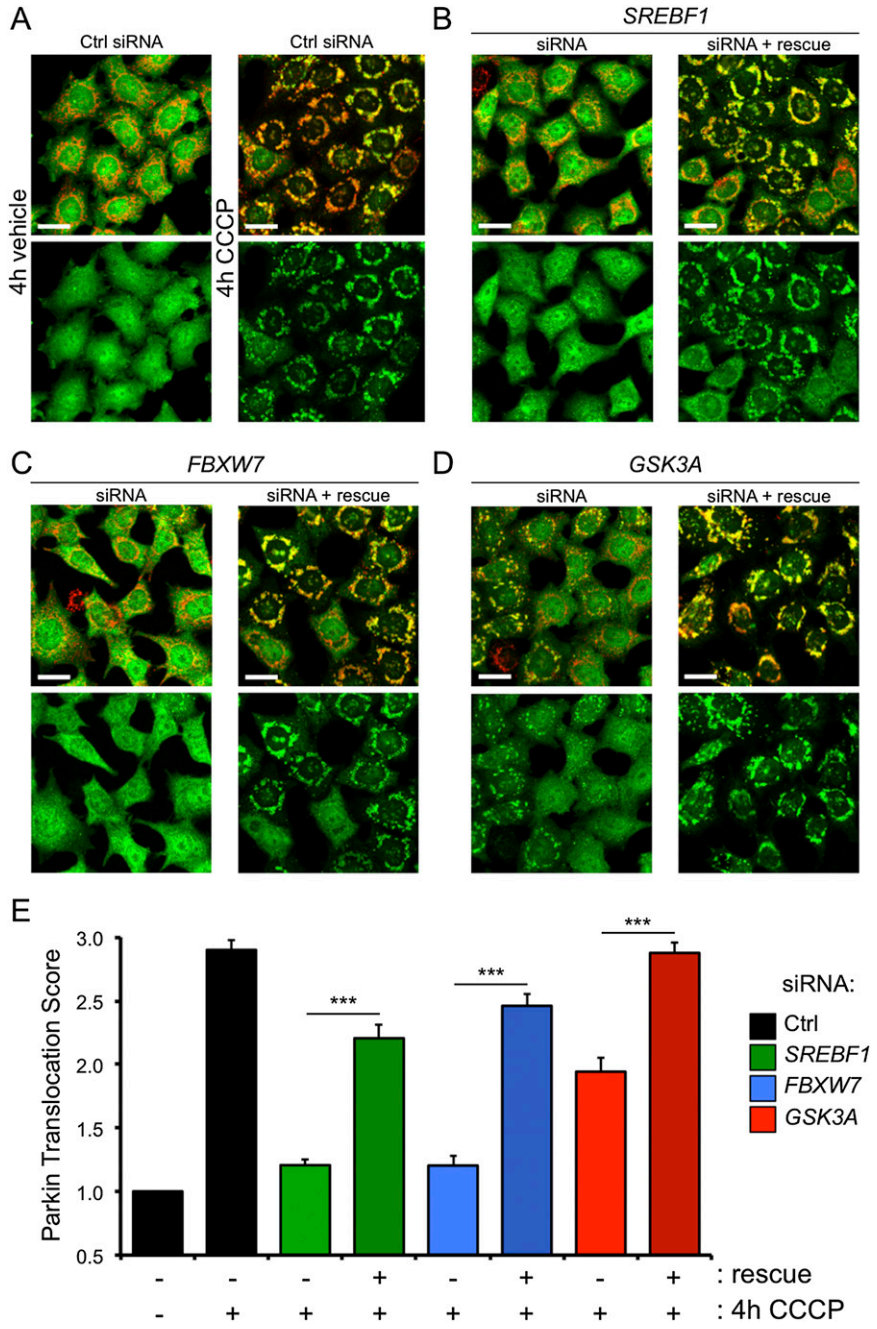

Fig. S7. Rescue of Parkin translocation with siRNA-resistant constructs. HeLa cells expressing YFP-Parkin (green) were transfected with either pooled (*A*) or individual (*B–D*) siRNAs alone or with corresponding siRNA-resistant "rescue" constructs (*B–D*) and treated with either vehicle (*A*) or CCCP (10 μM) (*A–D*) for 4 h as indicated. (*E*) YFP-Parkin translocation to mitochondria (anti-ATP5A; red) was assessed, producing a Parkin translocation score for each condition. The chart represents mean ± SD of triplicate assays. ***$P < 0.001$ (Student's *t* test between equivalent siRNA-treated samples). (Scale bars: 20 μm.)

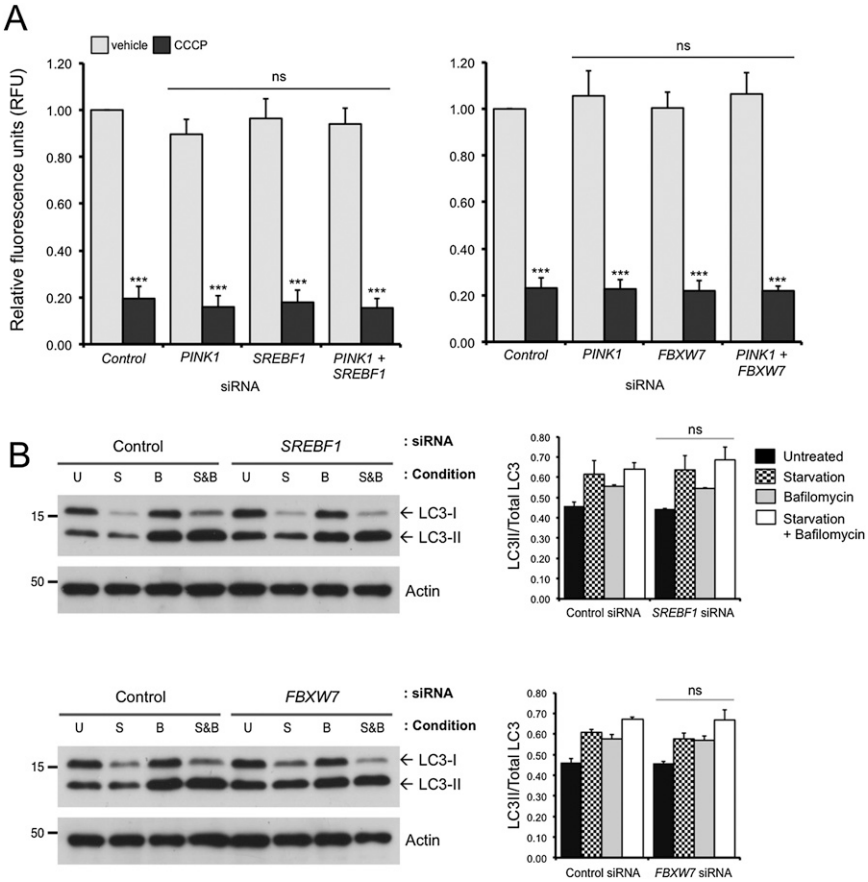

Fig. S8. Mitochondrial membrane potential and starvation-induced autophagy are not affected by *SREBF1* and *FBXW7* knockdown. (*A*) HeLa cells were treated with control, *PINK1*, *SREBF1*, or *FBXW7* siRNAs or combinations for 2 d, before the addition of either vehicle or 10 μM CCCP for 1 h as indicated. The mitochondrial membrane potential ($\Delta\Psi$m) was assessed by application of the potentiometric dye, tetramethylrhodamine, methyl ester. Charts represent mean ± SEM of triplicate assays. ***$P < 0.001$ (Student's *t* test between vehicle and CCCP-treated pairs). No significance was found across vehicle-treated and CCCP-treated groups, compared with control siRNA samples. ns, nonsignificant (one-way ANOVA with Bonferroni correction). (*B*) HeLa cells were transfected with control, *SREBF1*, or *FBXW7* siRNAs and subjected to 2 h of control (U, untreated), starvation (S), 20 nM bafilomycin (B), or starvation with 20 nM bafilomycin (S&B) treatment. LC3 lipidation (shift from LC3-I to LC3-II) was assessed by immunoblotting. Actin was used as a loading control. Quantification of LC3-II formation was assessed as LC3-II/total LC3 levels, using ImageJ. Charts represent mean ± SD of triplicate assays. ns, nonsignificant (Student's *t* test with respective control siRNA sample).

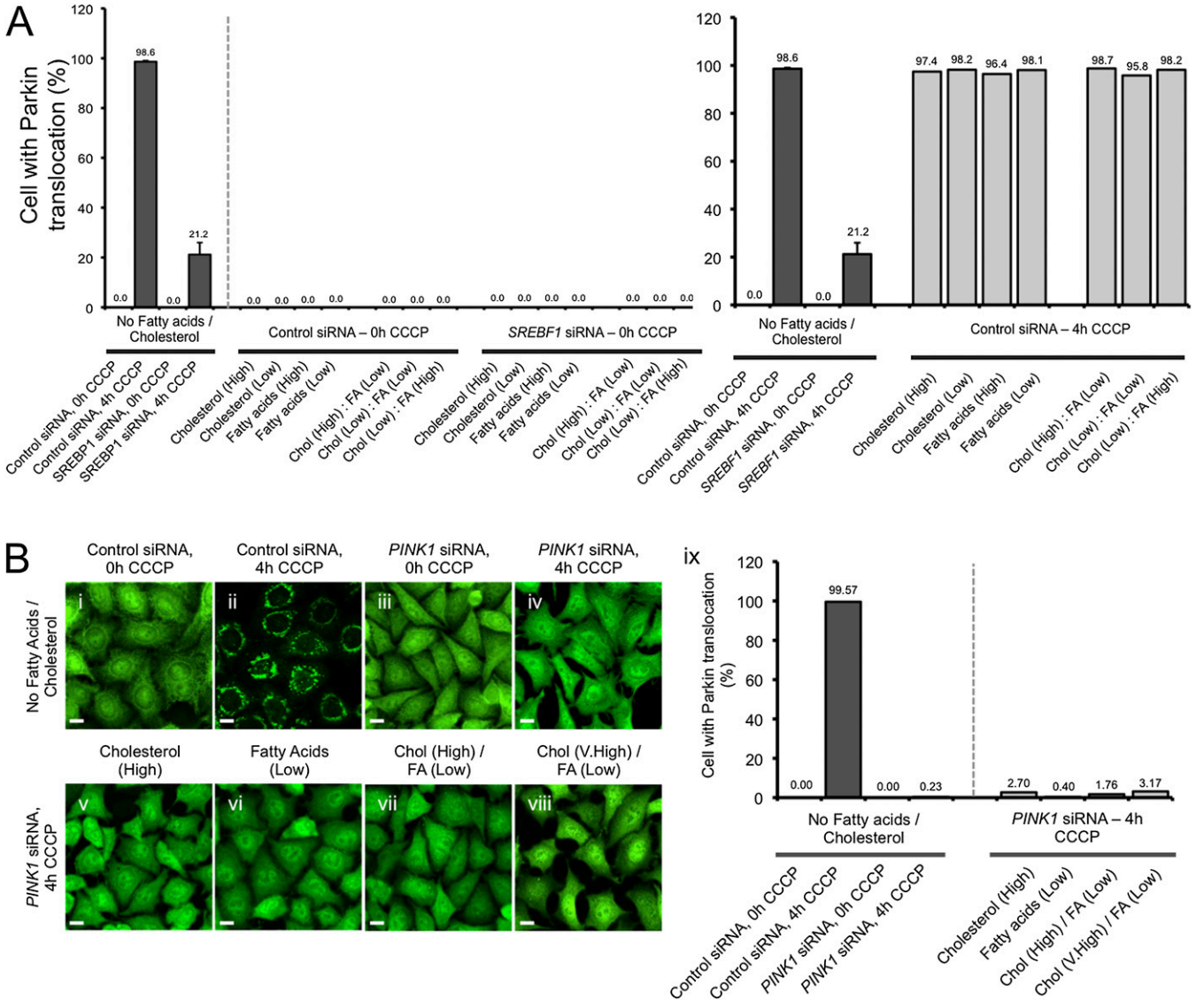

Fig. S9. Parkin translocation with fatty acids and cholesterol; analysis of controls. (*A*) HeLa cells expressing YFP-Parkin were treated with control or *SREBF1* siRNA and exposed to cholesterol, fatty acids, or a combination of the two at varying concentrations. Cells were treated with 10 μM CCCP or vehicle for 4 h as indicted, and the degree of Parkin translocation was quantified manually as the percentage of cells with Parkin translocation. (*B*) HeLa cells expressing YFP-Parkin were treated with control or *PINK1* siRNA for 4 d before the addition of vehicle (*i*–*iv*), cholesterol (*v*), fatty acids (*vi*), or a combination (*vii* and *viii*) for 24 h. Samples were then treated with either vehicle (*i* and *iii*) or 10 μM CCCP (*ii* and *iv*–*viii*) for 4 h. YFP-Parkin translocation (green) was assessed quantitatively as the percentage of cells with Parkin translocation. (*ix*) Quantification of Parkin translocation as in *i*–*viii*. Charts represent the mean of five fields of view per condition, from a single assay. Chol, cholesterol; FA, fatty acids; High, high concentration; Low, low concentration; V.High, very high concentration. (Scale bars: 10 μm.)

Table S1. ***Drosophila*** **genes that inhibited Parkin-GFP translocation in the primary screen, showing their effects in secondary screen assays**

No.	Name	P	M	C	Human homolog(s)
1	ago				FBXW7
2	atms				PAF1
3	CG2469				CTR9
4	Ctr1A				SLC31A1
5	Lilli				AFF4, AFF1, AFF3, AFF2
6	MED14				MED14
7	MED19				MED19
8	MED24				MED24
9	MED7				MED7
10	Pcaf				KAT2A, KAT2B
11	pUf68				PUF60
12	Rpb10				POLR2L
13	Rpb5				POLR2E
14	Rpb8				POLR2H
15	sgg*				GSK3B, GSK3A
16	tlk*				TLK2, TLK1
17	U2af50				U2AF2
18	ab				ZBTB22, ZNF295, ZBTB37, ZBTB17, ZBTB39
19	BAP55				ACTL6A, ACTL6B
20	Cdk9				CDK9
21	CG12050				WDR75
22	CG14023				NCOA6
23	CG14239				—
24	CG17209				POLR3A
25	CG34449				ZDHHC8, ZDHHC5
26	CG6220				CDC73
27	CG9883				—
28	CkIIα				CSNK2A1, CSNK2A2
29	cn				KMO
30	Gclm				GCLM
31	l(2)01810				SLC17A3, SLC17A9
32	Pros25				PSMA2
33	RpL28				RPL28
34	scny				USP36, USP42
35	U2af38				U2AF1, U2AF1L4
36	CG2685				WBP11
37	CG5591				MLL3
38	CG7886				CEP78
39	Cp1				CTSL2, CTSL1, CTSS, CTSK
40	Dis3				DIS3
41	l(1)G0007				DHX38
42	ptip				PAXIP1
43	Rrp6				EXOSC10
44	Srp54				SREK1, SRSF11
45	TfIIFα				GTF2F1
46	Atu				LEO1
47	CG33051				POLR3G, POLR3GL

Table S1. Cont.

No.	Name	P	M	C	Human homolog(s)
48	HLH106				SREBF1, SREBF2
49	Maltase A5				SLC3A1
50	pic				DDB1
51	Prp31				PRPF31
52	CG15459				USMG5
53	faf				USP9X, USP9Y
54	fz3				FZD5
55	GATAe				GATA4
56	MED28				MED28
57	pont				RUVBL1
58	rept				RUVBL2
59	sec10				EXOC5
60	Tango6				TMCO7

C, mitochondrial perinuclear clustering; M, mitochondrial morphology; P, paraquat-induced Parkin translocation. Green indicates a gene scored as a hit, and red indicates no effect (not a hit). The closest human homologs of the *Drosophila* genes are shown.
*Genes with multiple amplicons recovered in the primary screen.

Other Supporting Information Files

Dataset S1 (XLS)