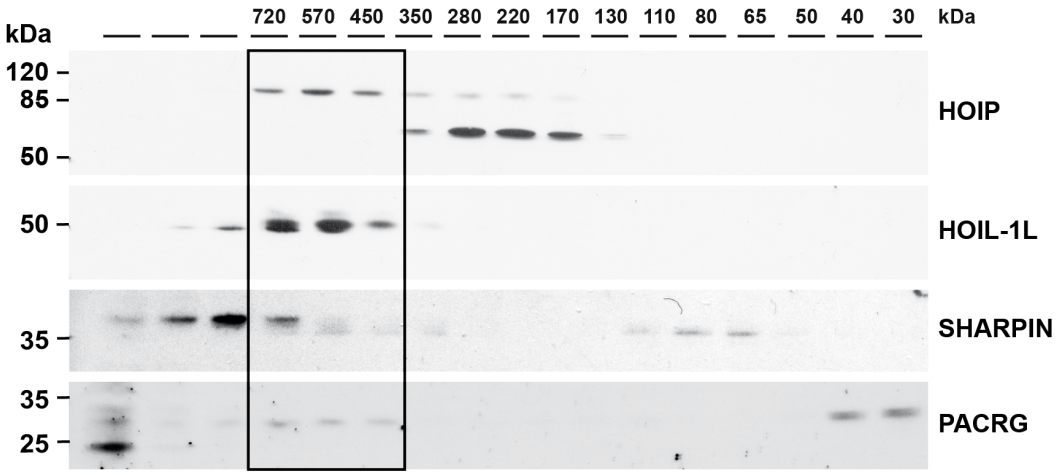


Fig. S1. PACRG co-elutes with endogenous LUBAC components.

HEK293T cells expressing PACRG were lysed, and soluble proteins were separated by size-exclusion chromatography. Fractions were collected and analyzed by Western blotting using antibodies against HOIP, HOIL-1L, SHARPIN, and PACRG. Blot is representative of 3 independent experiments.

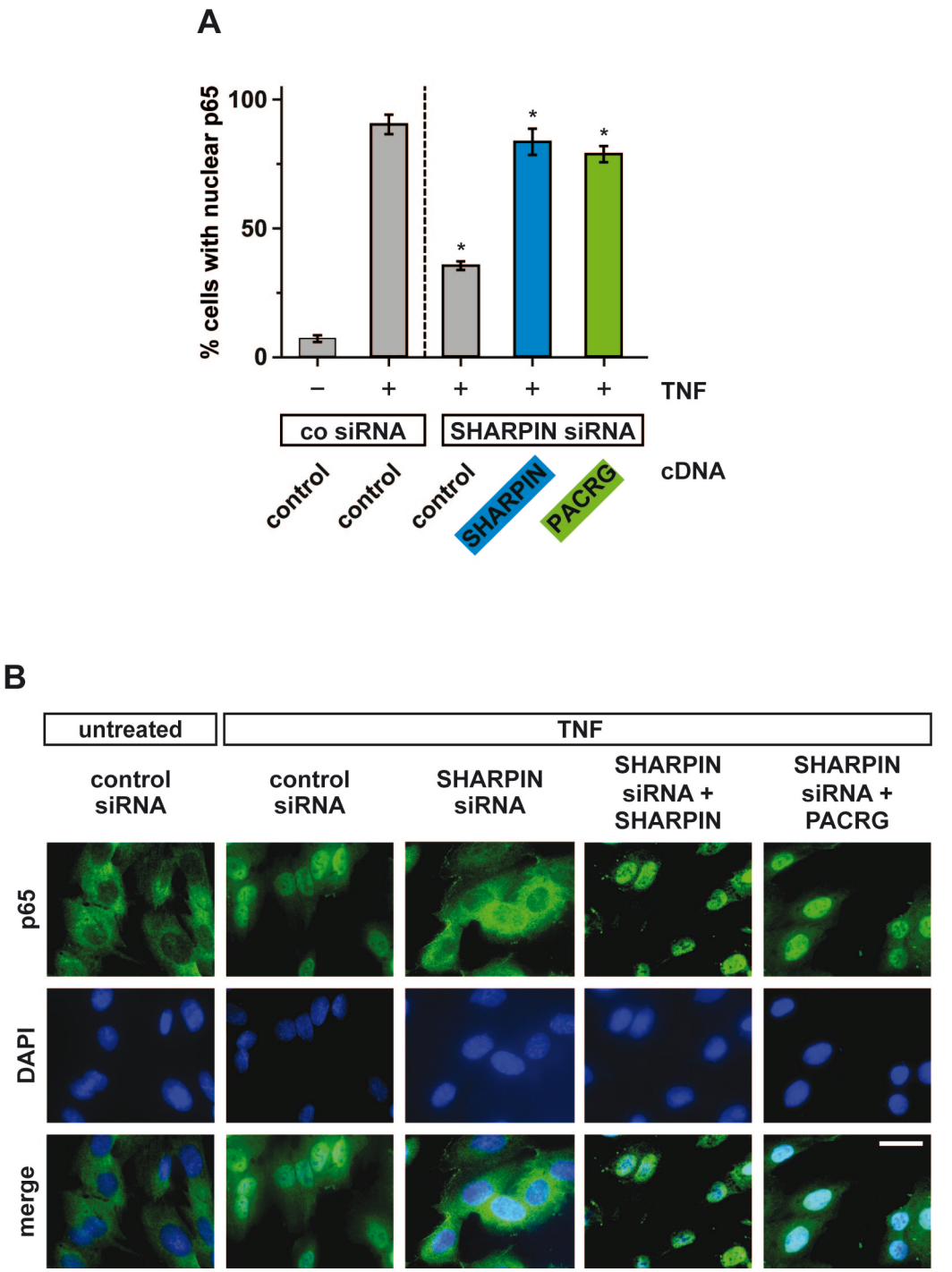


Fig. S2. PACRG restores defective p65 translocation in SHARPIN-deficient cells.

(A) SH-SY5Y cells were transfected with control or SHARPIN-specific siRNA with or without plasmids coding for SHARPIN or PACRG as indicated. One day after transfection, cells were stimulated with TNF, and nuclear translocation of p65 was determined by indirect immunofluorescence using an antibody against p65. Nuclei were stained with DAPI. Data represent the mean ± SEM of 4 independent experiments. For statistical analysis one-tailed

Mann-Whitney U-test was performed. At least 100 cells were analyzed per condition. *$p \leq 0.05$. **(B)** Representative examples of the indirect immunofluorescence experiment quantified in (A). Scale bar, 100 µm.

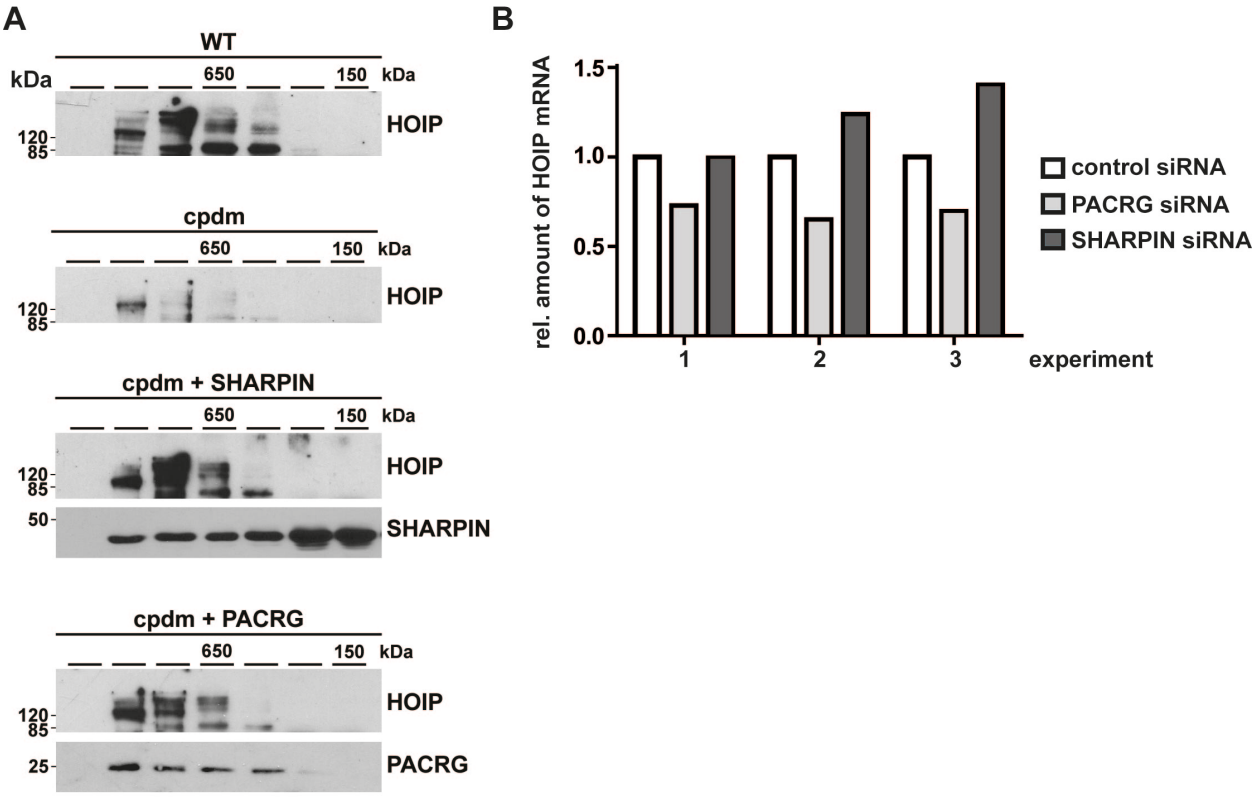
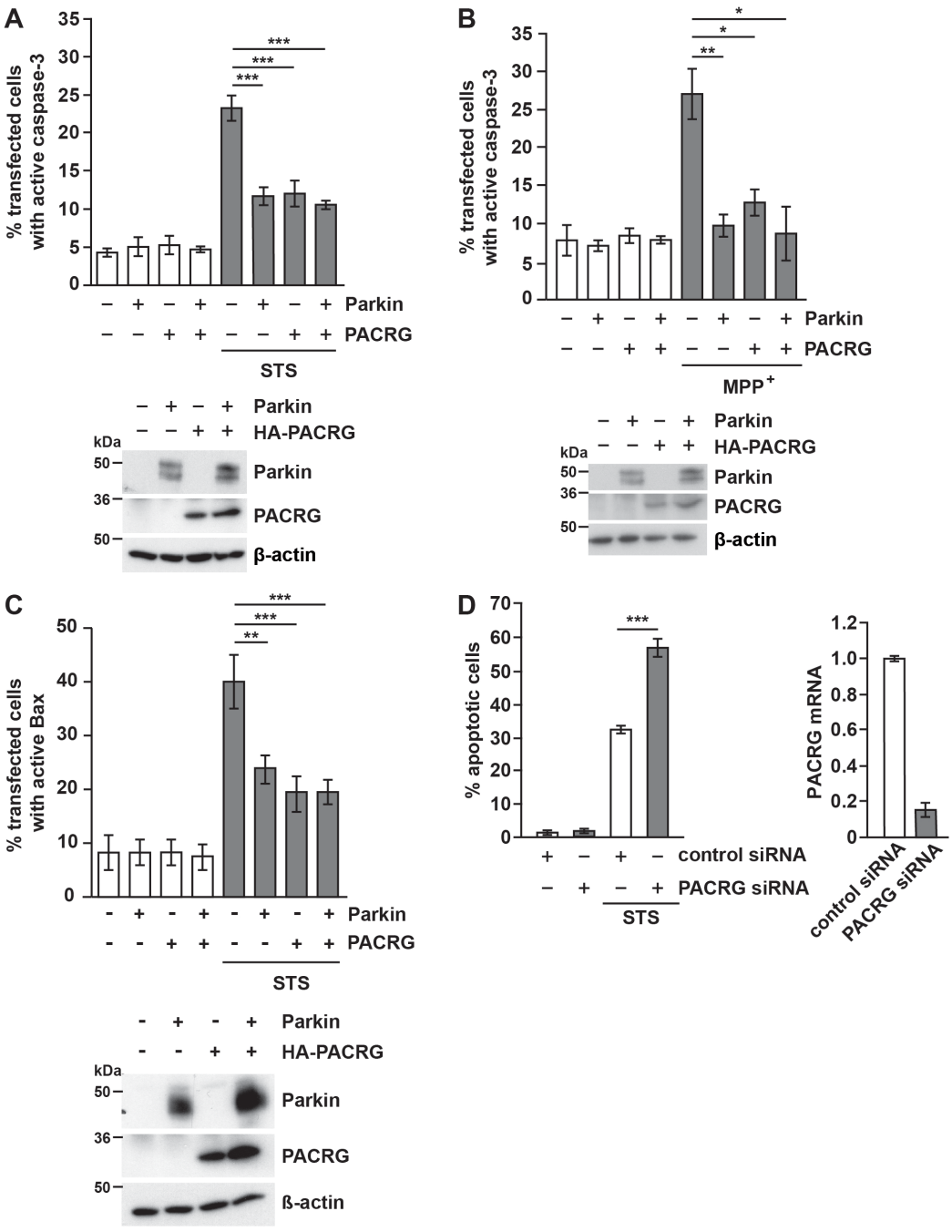


Fig. S3. PACRG stabilizes LUBAC.

(A) Wild-type (WT) MEFs, cpdm MEFs and cpdm MEFs expressing either SHARPIN or PACRG were lysed, and soluble proteins were separated by size-exclusion chromatography. Fractions were collected and analyzed by Western blotting using antibodies against HOIP, SHARPIN or PACRG. Blot is representative of 3 independent experiments. **(B)** Quantification of HOIP mRNA by real-time RT-PCR in MEFs silenced for PACRG or SHARPIN expression (corresponding to Fig. 5E).


A
% transfected cells with active caspase-3
35
30
25
20
15
10
5
0
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***
***
– + – + – + – + Parkin
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STS
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Parkin
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% transfected cells with active caspase-3
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Parkin
PACRG
ß-actin
D
% apoptotic cells
70
60
50
40
30
20
10
0
***
+ – + – control siRNA
– + – + PACRG siRNA
STS
PACRG mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0
control siRNA
PACRG siRNA

Fig. S4. PACRG protects against STS- and MPP^+-induced apoptotic cell death.

(A, B) SH-SY5Y cells transiently expressing EYFP (control), HA-PACRG, Parkin, or HA-PACRG plus Parkin were treated with STS or MPP^+. Apoptotic cell death was visualized by indirect immunofluorescence using an antibody against active caspase-3 and quantified by counting transfected cells positive for active caspase-3. Lysates were immunoblotted using antibodies specific for Parkin and HA. β-actin is a loading control. (**C)** SH-SY5Y cells transiently expressing EYFP, HA-PACRG, Parkin, or HA-PACRG plus Parkin were treated with STS. Apoptotic cell death was visualized by indirect immunofluorescence using an antibody against active Bax and quantified by counting transfected cells positive for active Bax. Lysates were immunoblotted using antibodies specific for Parkin, HA, and β-actin. **(D)** SY5Y cells transfected with control or PACRG siRNA were treated with STS. Apoptotic cell death was visualized by indirect immunofluorescence using an antibody against active caspase-3 and quantified by counting transfected cells positive for active caspase-3. PACRG knockdown efficiencies were determined by real-time RT-PCR. For all panels, data represent the mean ± SEM of at least 3 independent experiments. At least 900 transfected SH-SY5Y cells were assessed per condition. Data represent the mean ± SEM. Statistical analysis was carried out using the unpaired Student's t-test. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

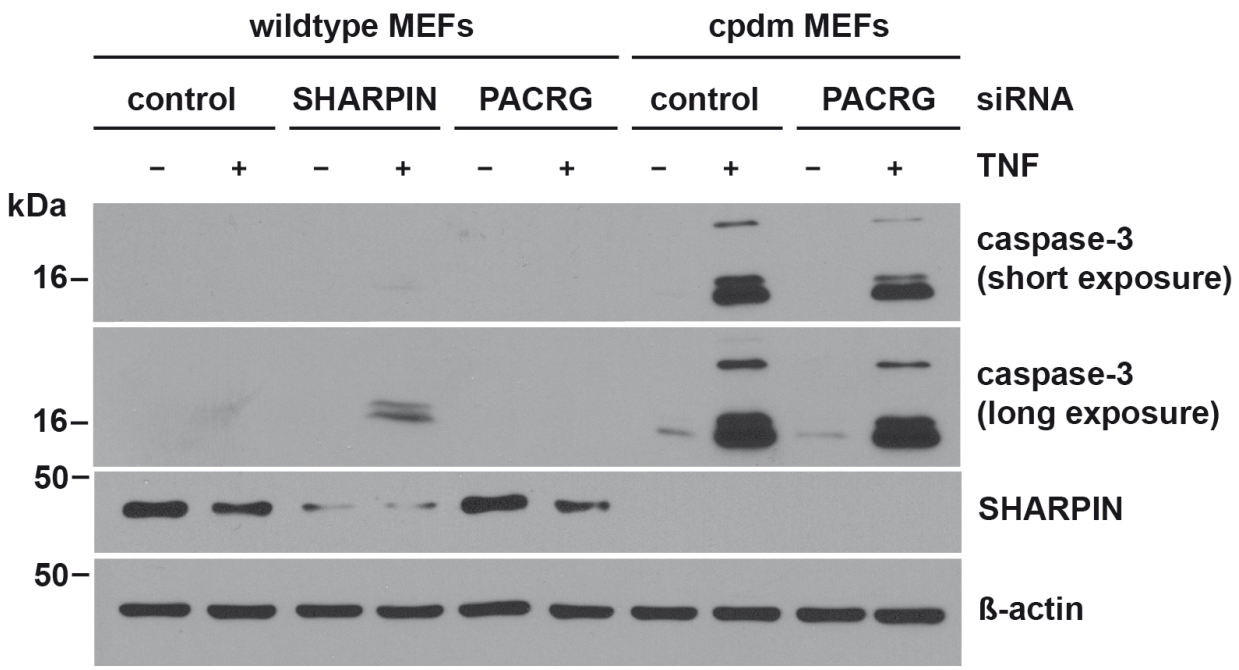

Fig. S5. TNF treatment of PACRG-silenced MEFs does not activate caspase-3.

Wild-type MEFs and cpdm MEFs were transfected with the indicated siRNAs then treated with TNF. Cell lysates were analyzed by Western blotting using an antibody against active caspase-3. Blot is representative of 3 independent experiments.

Table S1. Antibodies

Antibody Target	Clone Name	Supplier	Dilution Western Blot	Dilution Imuno-cytochemistry
PACRG	C-8	Santa Cruz	1:1000	
Parkin	PRK8	Santa Cruz	1:1000	1:500
Hsp60	N-20	Santa Cruz	1:1000	1:500
p65	A	Santa Cruz	1:1000	1:500
M1 ubiquitin	1E3	Millipore	1:1000	1:200
M1 ubiquitin	1F11/3F5/Y10 2L	Genentech	1:10000	1:4000
HOIL-1L	AB38540	Abcam	1:1000	
Cleaved Caspase-3	5A1E	Cell Signalling	1:1000	1:400
SHARPIN	4444	Cell Signalling	1:1000	
SHARPIN	14626-1-AP	Proteintech	1:2000	
IκBα	9242	Cell Signalling	1:1000	
HA tag	16B12	Covance	1:1000	1:1000
β-actin	AC-74	Sigma Aldrich	1:5000	1:1000
HOIP	SAB2102031	Sigma Aldrich	1:1000	
HOIP	A303-560A	Bethyl Laboratories	1:1000-1:2000	1:500
NEMO	HPA000426	Sigma Aldrich	1:1000	
BAX	6A7	eBioscience	1:500	1:500
anti-rabbit IgG (H+L)	Alexa488	Thermo Scientific		1:1000
anti-mouse IgG (H+L)	Alexa488	Thermo Scientific		1:1000
anti-rabbit IgG (H+L)	Alexa555	Thermo Scientific		1:1000
anti-goat IgG (H+L)	Alexa555	Thermo Scientific		1:1000
anti-mouse IgG (H+L)	HRP-tagged	Promega	1:10000	
anti-rabbit IgG (H+L)	HRP-tagged	Promega	1:10000	
anti-goat IgG (H+L)	HRP-tagged	Promega	1:10000	
anti-HA agarose (mouse)	A2095	Sigma Aldrich		
anti-cMyc agarose (mouse or rabbit)	#20168	Thermo Scientific		
anti-V5 agarose (mouse)	A7345	Sigma Aldrich		