Supplementary Information for

AAA+ ATPase chaperone p97/VCP^{FAF2} governs basal pexophagy

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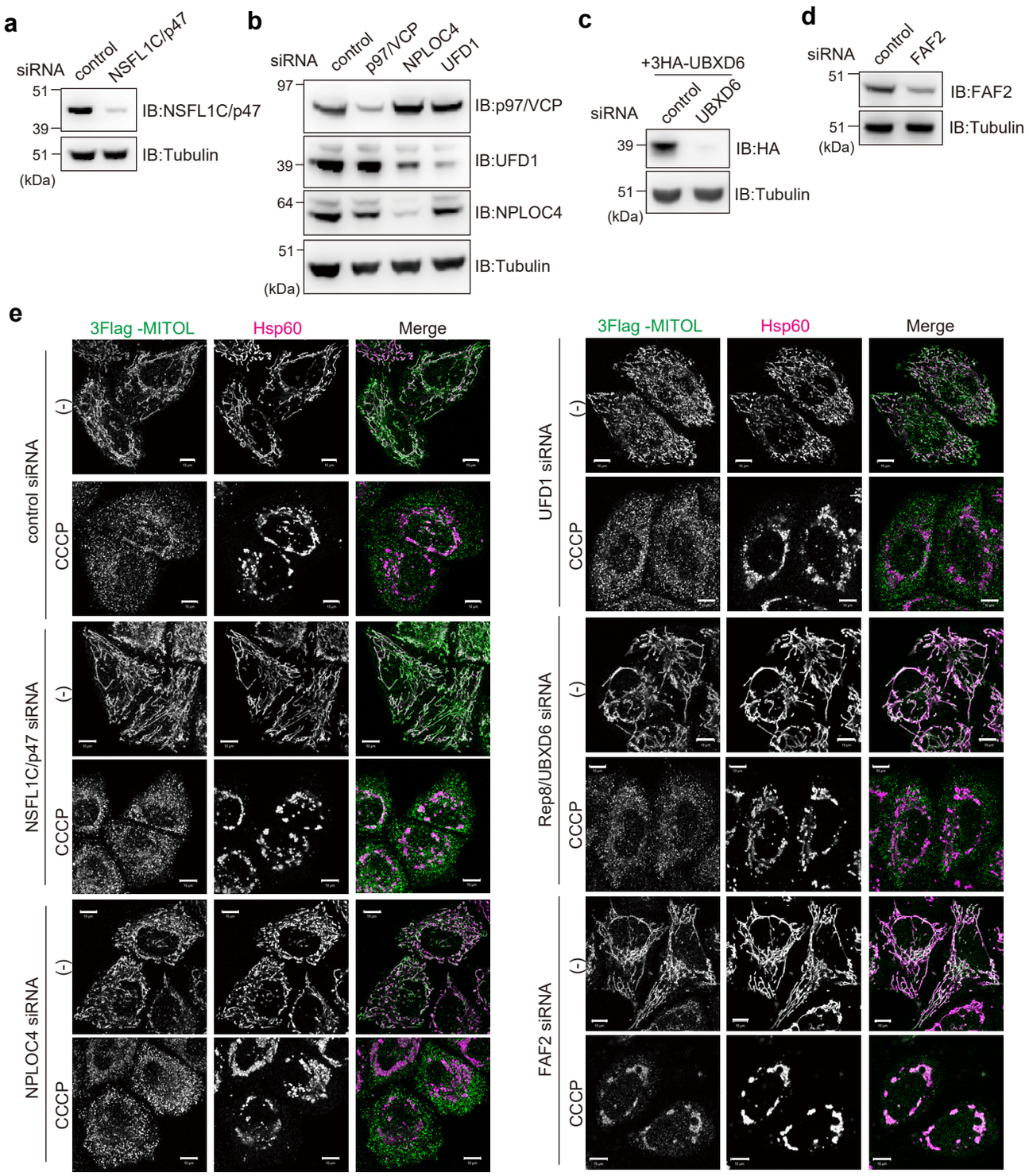
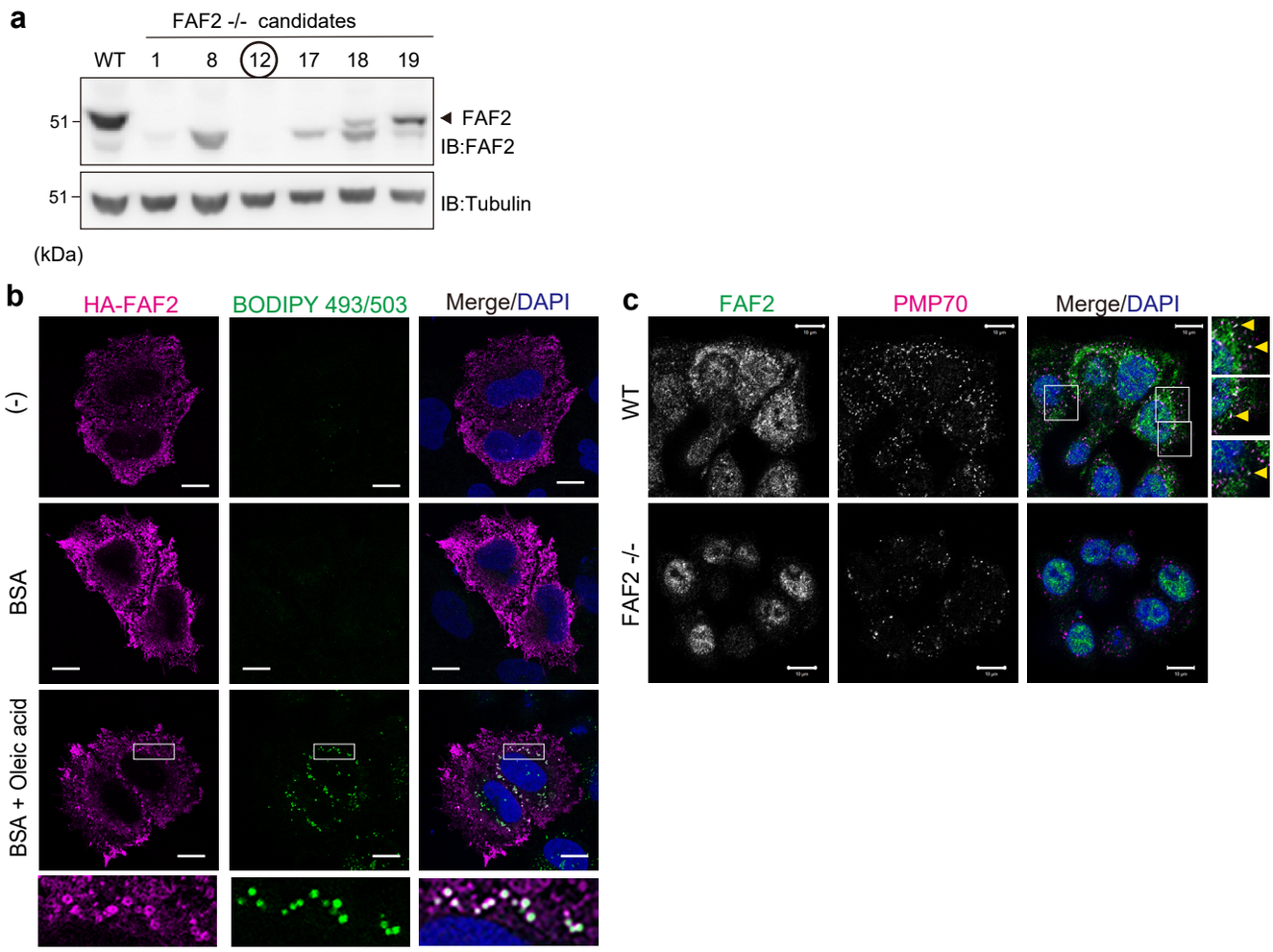
Supplementary Figures 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14



Supplementary Figure 1. Knockdown of FAF2 impairs MITOL extraction from damaged mitochondria.

a-d. Immunoblot confirmation of p97/VCP cofactor knockdown. Since an anti-Rep8/UNXD6 antibody was not available, HeLa cells stably expressing 3HA-Rep8/UBXD6 were used to confirm Rep8/UBXD6 knockdown.

e. The extraction of 3Flag-MITOL from damaged mitochondria was inhibited by FAF2 knockdown. HeLa cells stably expressing HA-Parkin and 3Flag-MITOL were transfected with the indicated siRNAs and then treated with or without CCCP for 3 hours. Anti-Flag and anti-Hsp60 antibodies were used for immunostaining. n=2 assays. Scale bars, 10 μm. Source data are provided as a Source Data file.

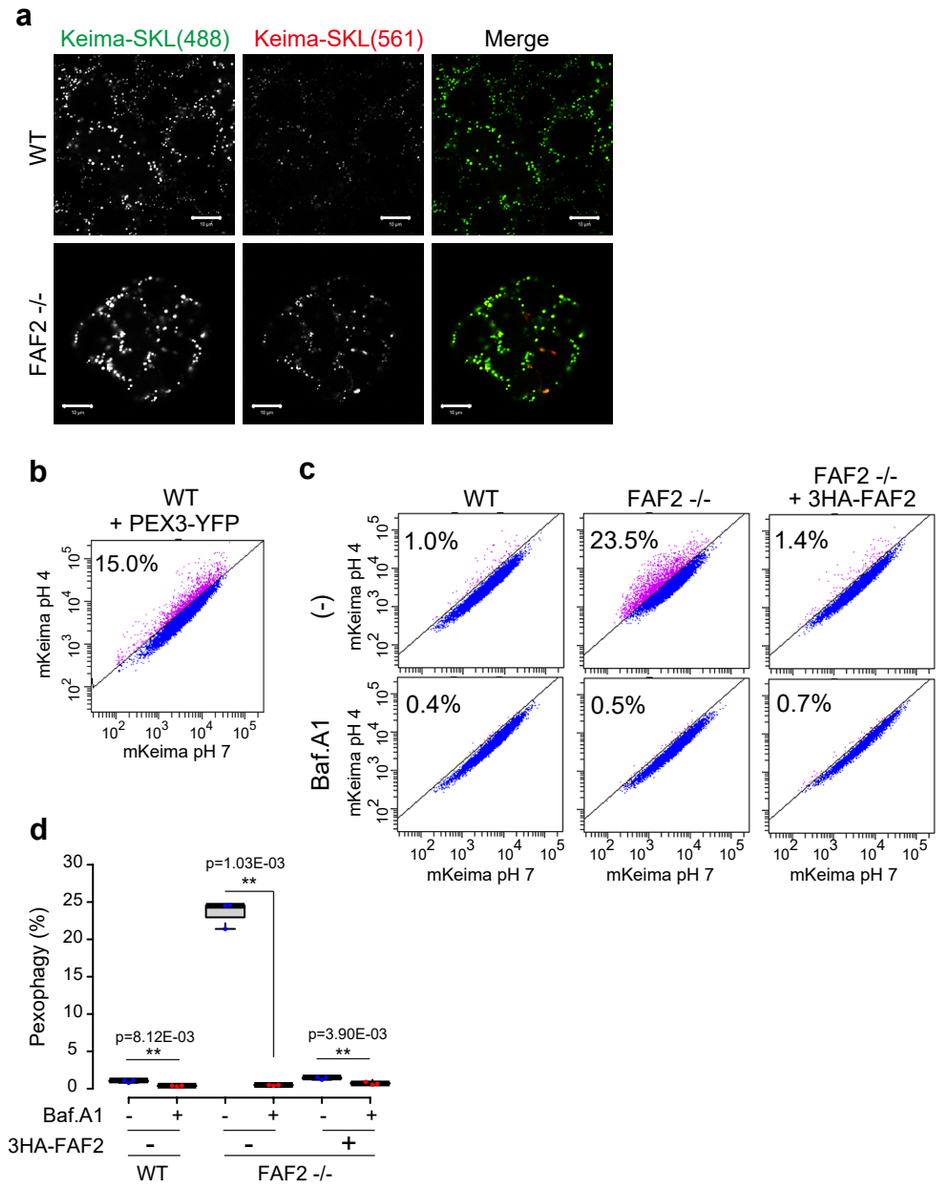


Supplementary Figure 2. Endogenous localization of FAF2

a. Establishment of an FAF2-/- HCT116 cell line. Total cell lysates prepared from FAF2-/- candidates were immunoblotted with the indicated antibodies. FAF2 was depleted in clones #1 and #12. The FAF2-/- HCT116 cell line used in this study was derived from clone #12.

b. HA-FAF2 partially localizes on lipid droplets. HeLa cells transiently expressing HA-FAF2 were treated with BSA alone or BSA with oleic acid for 18 hours and then immunostained with anti-HA antibody and BODIPY 493/503. Higher magnification images of the boxed regions are shown below each panel. n=2 assays. Scale bars, 10 μm.

c. Endogenous FAF2 partially localizes to peroxisomes in WT cells. WT and FAF2 -/- HCT116 cells were immunostained with the indicated antibodies. Higher magnification images of the boxed regions are shown to the right. n=2 assays. Scale bars, 10 μm. Arrowheads indicate representative examples of FAF2-PMP70 co-localization that was only observed in the presence of FAF2. Source data are provided as a Source Data file.



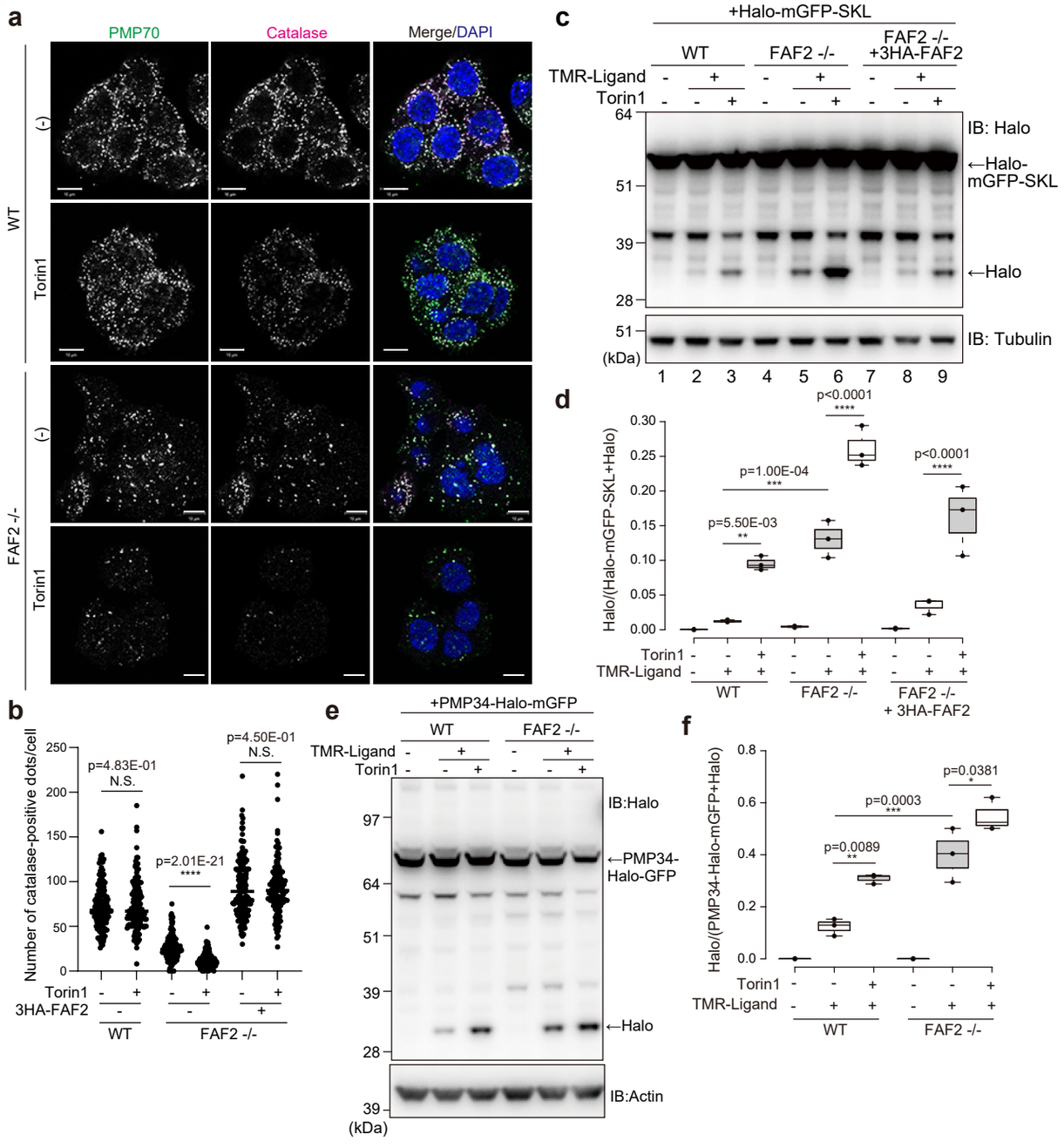
Supplementary Figure 3. Pexophagy is completely blocked by Baf.A1 treatment.

a. mKeima-SKL was stably expressed in WT or FAF2-/- HCT116 cells. FAF2-/- cells that did not have complete peroxisomal loss were used to generate representative images of the cell line. Scale bars, 10 μm.

b. The pexophagy flux in WT HCT116 cells expressing PEX3-YFP was analyzed using mKeima-SKL-based flow cytometry. n=3 assays.

c. Representative FACS data with the percentage of pexophagy-positive cells indicated. Cells were analyzed by FACS after 24 hours bafilomycin. A1 (Baf.A1) treatment. n=3 assays.

d. Quantitative analysis of the pexophagy flux for cells in (c). Dots represent individual data points from three independent experiments. Statistical significance was calculated using a one-tailed Welch' s t-test. ** $p < 0.01$. The center lines correspond to the medians and the box limits indicate the 25th and 75th percentiles. The box-plot whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Source data are provided as a Source Data file.



Supplementary Figure 4. Torin1 treatment reduces peroxisome abundance in FAF2 -/- cells.

a. Peroxisome abundance in FAF2-/- cells was reduced by Torin1 treatment. The cells were treated with Torin1 for 24 hours, and then immunostained with anti-PMP70 and anti-catalase antibodies. Cell nuclei were stained with DAPI. Scale bars, 10 μm.

b. The number of catalase-positive peroxisomes per cell in (a). Dots represent individual data points from three independent experiments. n=150 cells (54, 41, 55 cells/experiments; WT), n=157 cells (66, 42, 49 cells/experiments; WT + Torin1), n=153 cells (57, 43, 53 cells/experiments; FAF2 -/-), n=147 cells (59, 39, 49 cells/experiments; FAF2-/- + Torin1), n=155 cells (45, 49, 61 cells/experiments; FAF2 -/- + 3HA-FAF2), and n=145 cells (56, 36, 53 cells/experiments; FAF2 -/- + 3HA-FAF2 + Torin1). Bars, median. Statistical significance was calculated using a one-tailed Welch's t-test; **** $p < 0.0001$; N.S. - not significant.

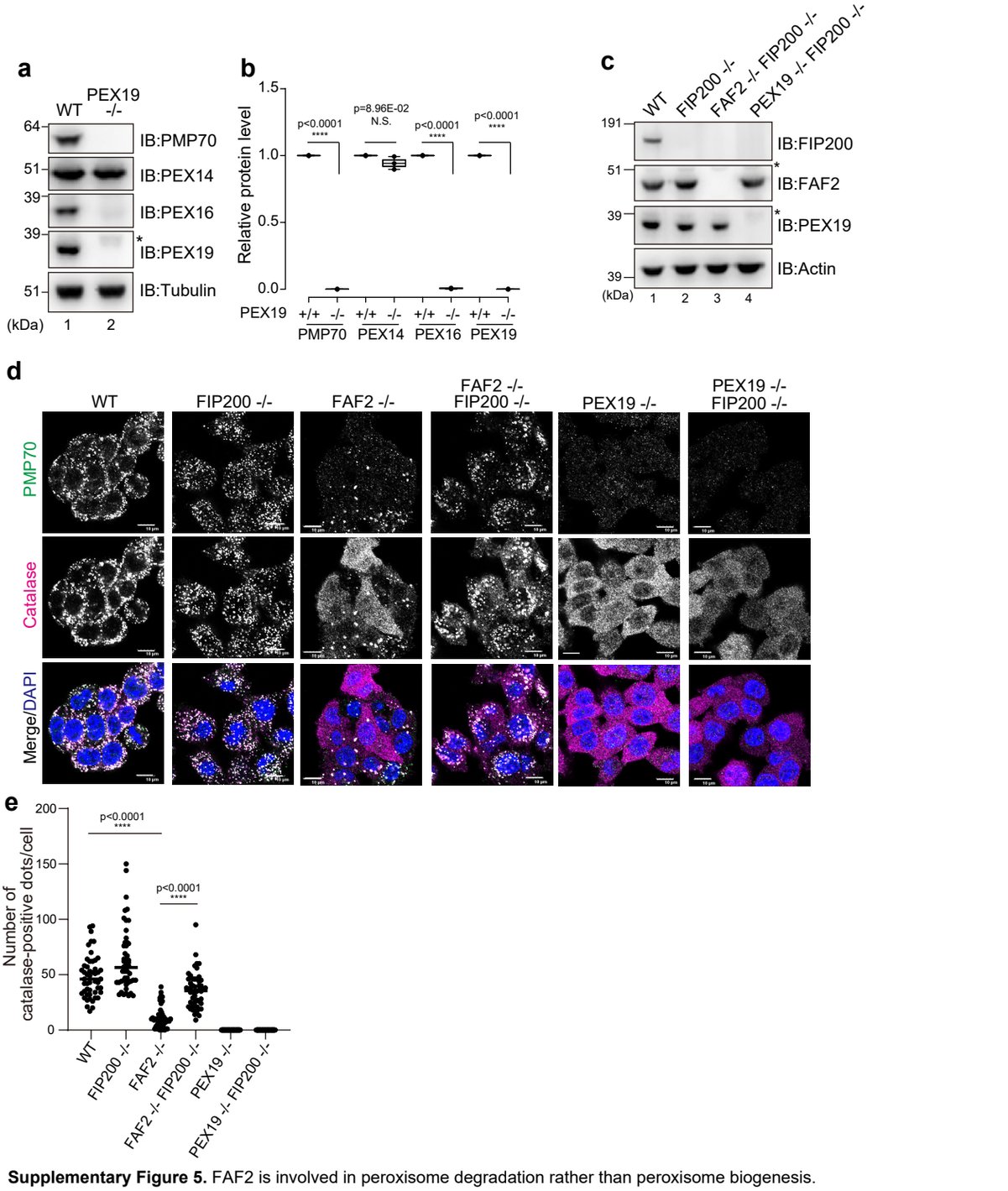
c. The Pexophagy flux was evaluated using a HaloTag assay. The intensity of the Halo fragment was enhanced even in WT cells upon Torin1 treatment. n=3 assays.

d. Quantification of the pexophagy flux for cells in (c). The Halo band intensity was normalized to the sum of the Halo-mGFP-SKL and Halo band intensities. The normalized Halo intensities are shown as as box plots with the dots indicating individual data points from three independent experiments. Statistical significance was calculated using one-way ANOVA; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. The center lines correspond to the medians and the box limits indicate the 25th and 75th percentiles. The box-plot whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

e. Torin1 treatment enhances pexophagy. WT or FAF2 -/- cells stably expressing PMP34-Halo-mGFP were treated with Torin1. Cell lysates were collected and subjected to SDS-PAGE. n=3 assays.

f. Quantitative analysis of the pexophagy flux for cells in (e). Dots represents an individual data points from three independent experiments. Statistical significance was calculated using one-way ANOVA; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.The center lines correspond to the medians and the box limits indicate the 25th and 75th percentiles. The box-plot whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

Source data are provided as a Source Data file.



Supplementary Figure 5. FAF2 is involved in peroxisome degradation rather than peroxisome biogenesis.

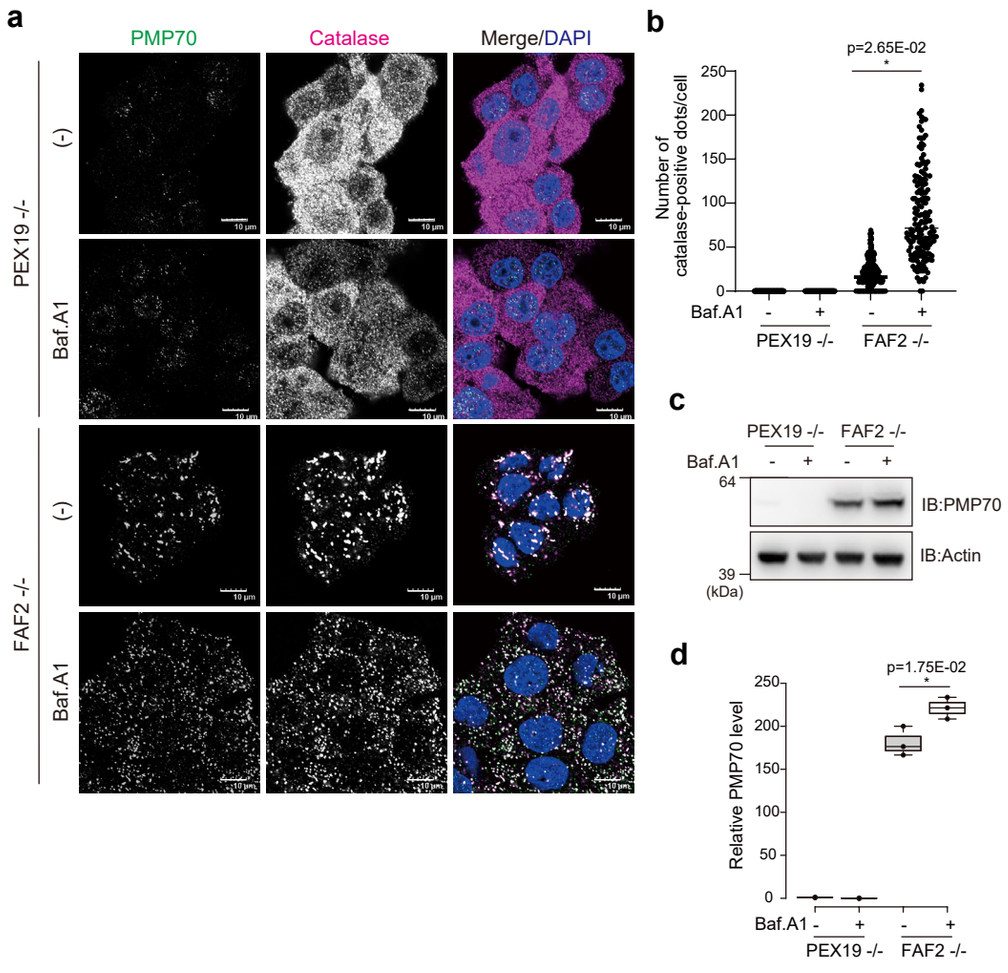
a. PMP70 and PEX16 levels in PEX19-/- cells are reduced. WT and PEX19-/- HCT116 cells were immunoblotted with the indicated antibodies. The asterisk indicates a cross-reactive band.

b. Quantitative analysis of the peroxisomal protein levels for cells in (a). The amount of protein in WT HCT116 cells was set to 1. Dots represent individual data points from three independent experiments. Statistical significance was calculated using a one-tailed Welch's t-test; **** $p < 0.0001$; N.S.- not significant. The center lines correspond to the medians and the box limits indicate the 25th and 75th percentiles. The box-plot whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

c. Immunoblot confirmation of FIP200, PEX19, and FAF2 deletion. The cell lysates were immunoblotted with the indicated antibodies.

d. Peroxisome abundance is increased in FAF2 -/- FIP200 -/- double knockout cells. Each cell line was immunostained using the indicated antibodies. Scale bars, 10 μm. n=3 assays.

e. Quantitative analysis of the number of peroxisomes per cell in (d). The dots indicate individual data points from three independent experiments. Total cell number from three experiments=50 cells. Bars, median. Statistical significance was calculated using one-way ANOVA; **** $p < 0.0001$. Source data are provided as a Source Data file.



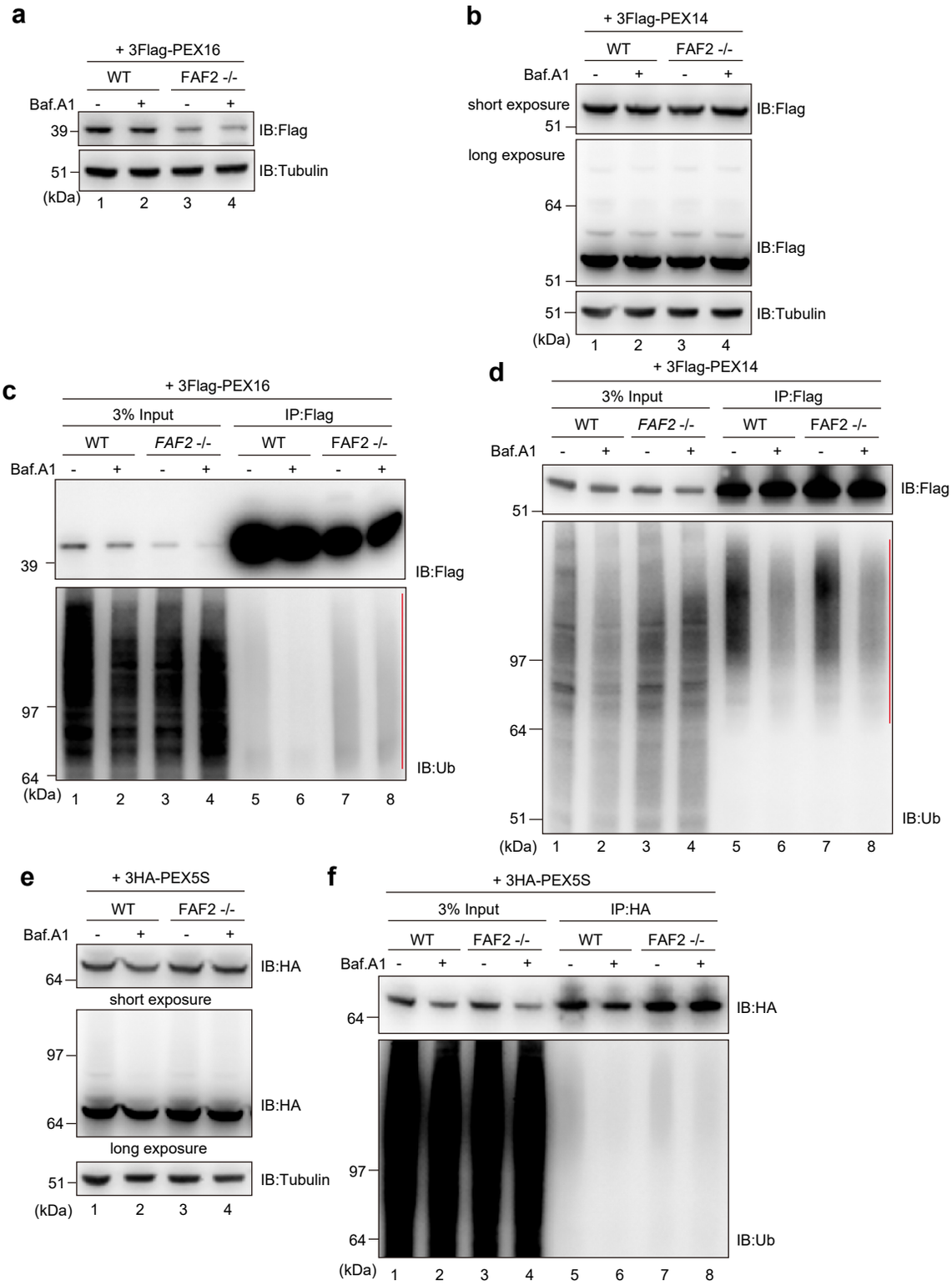
Supplementary Figure 6. Peroxisome number increases in Baf.A1-treated FAF2 -/- cells but not in PEX19 -/- cells.

a. FAF2 -/- and PEX19 -/- cells were incubated with or without Baf.A1 for 24 hours. Cells were immunostained with the indicated antibodies. Scale bars, 10 μm.

b. Quantitative analysis of the number of peroxisomes per cell in (a). The dots indicate individual data points from three independent experiments. n=314 cells (130, 113, 71 cells/experiments; PEX19 -/- cells), n=228 cells (95, 53, 80 cells/ experiments; PEX19 -/- cells + Baf.A1), n=213 cells (50, 65, 98 cells/experiments; FAF2 -/-), and n=168 cells (50, 58, 60 cells/experiments; FAF2 -/- + Baf.A1). Bars, median. Statistical significance was calculated using a one-tailed Welch' s t-test; * $p < 0.05$.

c. PMP70 levels in FAF2 -/- cells increase following Baf.A1 treatment. Cell lysates were subjected to SDS-PAGE and immunoblotted with the indicated antibodies. n=3 assays.

d. Quantitative analysis of PMP70 levels for cells in (c). PMP70 levels were normalized to WT cells, which were set to 1. The relative levels are shown as box plots with the dots indicating individual data points from three independent experiments. The center lines correspond to the medians and the box limits indicate the 25th and 75th percentiles as determined using R. The box plot whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Statistical significance was calculated using a one-tailed Welch' s t-test; * $p < 0.05$. Source data are provided as a Source Data file.



Supplementary Figure 7. Ubiquitylated PEX14 and PEX5S do not accumulate in FAF2-/- cells.

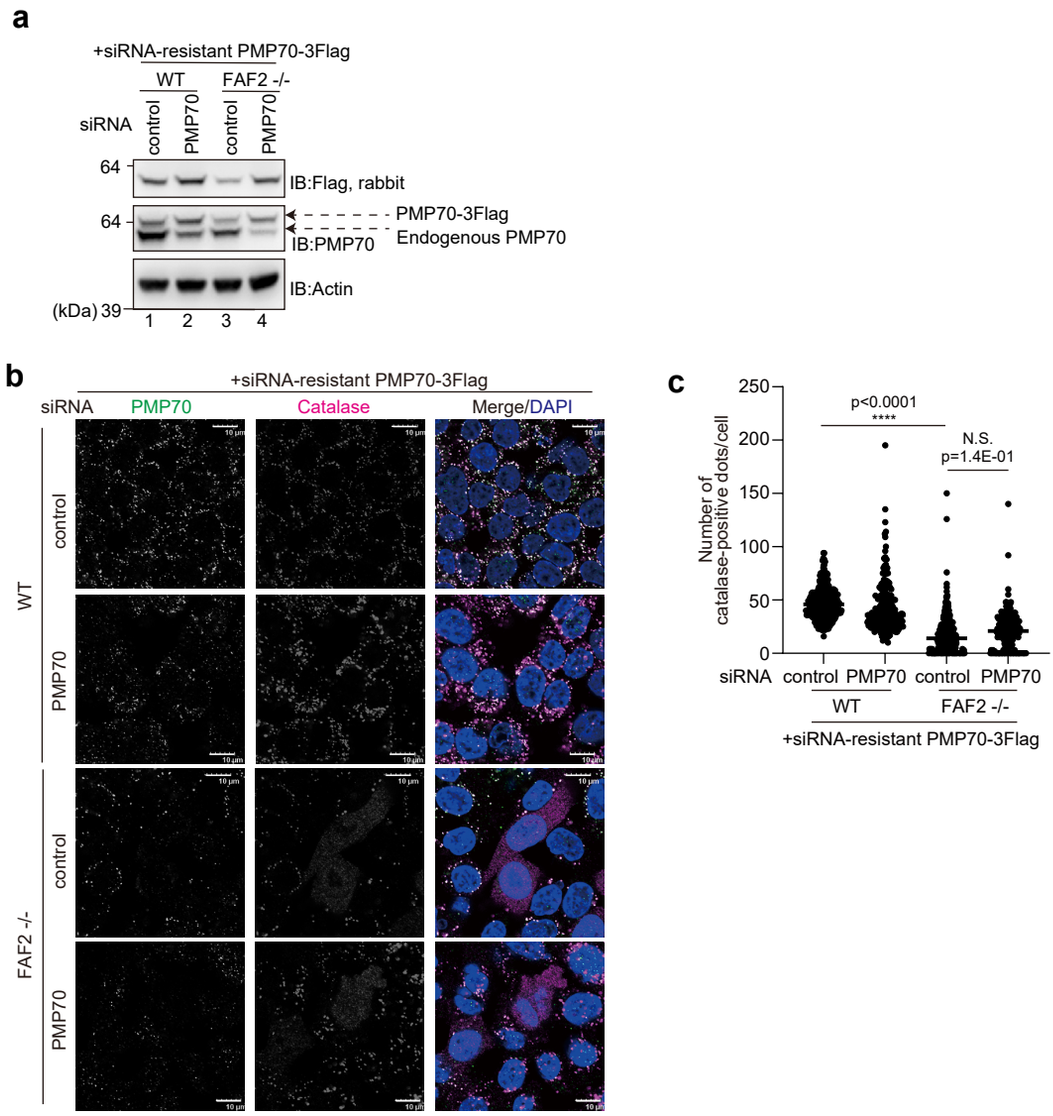
a. WT or FAF2-/- cells stably expressing 3Flag-PEX16 were treated with or without Baf.A1 for 24 hours and then total cell lysates were immunoblotted with the indicated antibodies. n=2 assays.

b, e. PEX14 and PEX5S do not accumulate in FAF2-/- cells following Baf.A1 treatment. WT or FAF2-/- HCT116 cells stably expressing 3Flag-PEX14 or 3HA-PEX5S were treated with Baf.A1 for 24 hours and then total cell lysates were immunoblotted with the indicated antibodies. n=2 assays.

c. PEX16 is ubiquitylated in FAF2-/- cells. WT or FAF2-/- cells stably expressing 3Flag-PEX16 were treated with or without Baf.A1 for 24 hours. 3Flag-PEX16 was immunoprecipitated with anti-Flag beads. The samples (IP products and 3% of input) were immunoblotted with the indicated antibodies. The red vertical line denotes ubiquitylation. n=2 assays.

d, f. The cells in (b) and (e) were immunoprecipitated with anti-Flag beads or anti-HA beads. The samples (IP products and 3% of input) were immunoblotted with the indicated antibodies. The red vertical line denotes ubiquitylation. n=2 assays.

Source data are provided as a Source Data file.

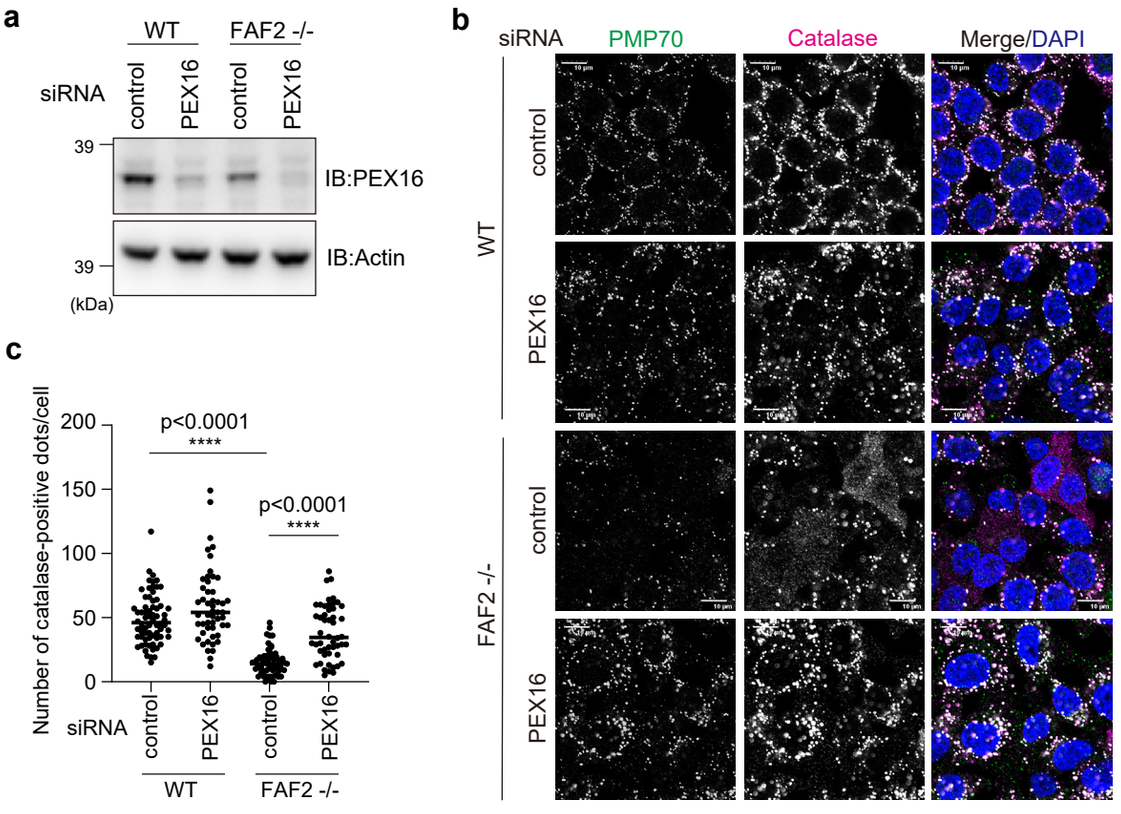


Supplementary Figure 8. Peroxisome abundance does not change in siPMP70-treated FAF2 -/- cells expressing siRNA-resistant PMP70-3Flag.

a. The levels of siRNA resistant PMP70-3Flag were not reduced by PMP70 knockdown, indicating that siRNA-resistant PMP70-3Flag is not a target of siPMP70. After siRNA transfection, cell lysates were collected and immunoblotted.

b. Peroxisome abundance is not impacted by PMP70 knockdown in FAF2 -/- cells expressing siRNA-resistant PMP70-3Flag. WT or FAF2 -/- cells were treated with each siRNAs and were immunostained with the indicated antibodies. Scale bars, 10 μm.

c. Quantitative analysis of the per cell number of peroxisomes for cells in (b). Dos indicate individual data points from three independent experiments. n=293 cells (128, 75, 90 cells/experiments; WT + sicontrol), n=205 cells (56, 67, 82 cells/ experiments; WT + siPMP70), n=343 cells (84, 81, 78 cells/experiments; FAF2 -/- + sicontrol), and n=202 cells (83, 57, 62 cells/experiments; FAF2 -/- + siPMP70). Bars, median. Statistical significance was calculated using one-way Anova; **** $p < 0.0001$; N.S. - not significant. Source data are provided as a Source Data file.



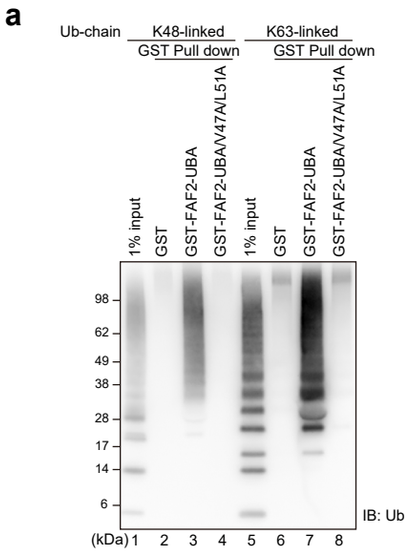
Supplementary Figure 9. PEX16 knockdown increases peroxisome abundance in FAF2 -/- cells.

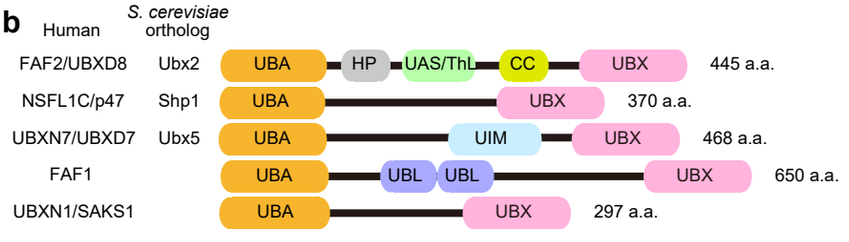
a. WT and FAF2 -/- HCT116 cells were treated with control or PEX16 siRNAs and then immunoblotted with the indicated antibodies.

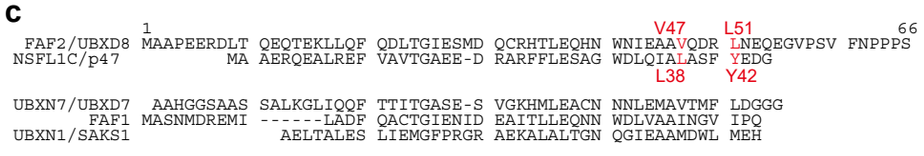
b. Peroxisome abundance increases following PEX16 knockdown in FAF2 -/- cells. WT or FAF2 -/- cells were treated with control or PEX16 siRNAs and then immunostained with the indicated antibodies. Scale bars, 10 μm.

c. Quantitative analysis of the per cell number of peroxisomes for cells in (b). Dots indicate individual data points from two independent experiments. n=68 cells (37, 31 cells/experiments; WT + sicontrol), n=51 cells (21, 30 cells/experiments; WT + siPEX16), n=61 cells (31, 30 cells/experiments; FAF2 -/- + sicontrol), and n=50 cells (24, 26 cells/experiments; FAF2 -/- + siPEX16). Bars, median. Statistical significance was calculated using one-way ANOVA; **** $p < 0.0001$.

Source data are provided as a Source Data file.





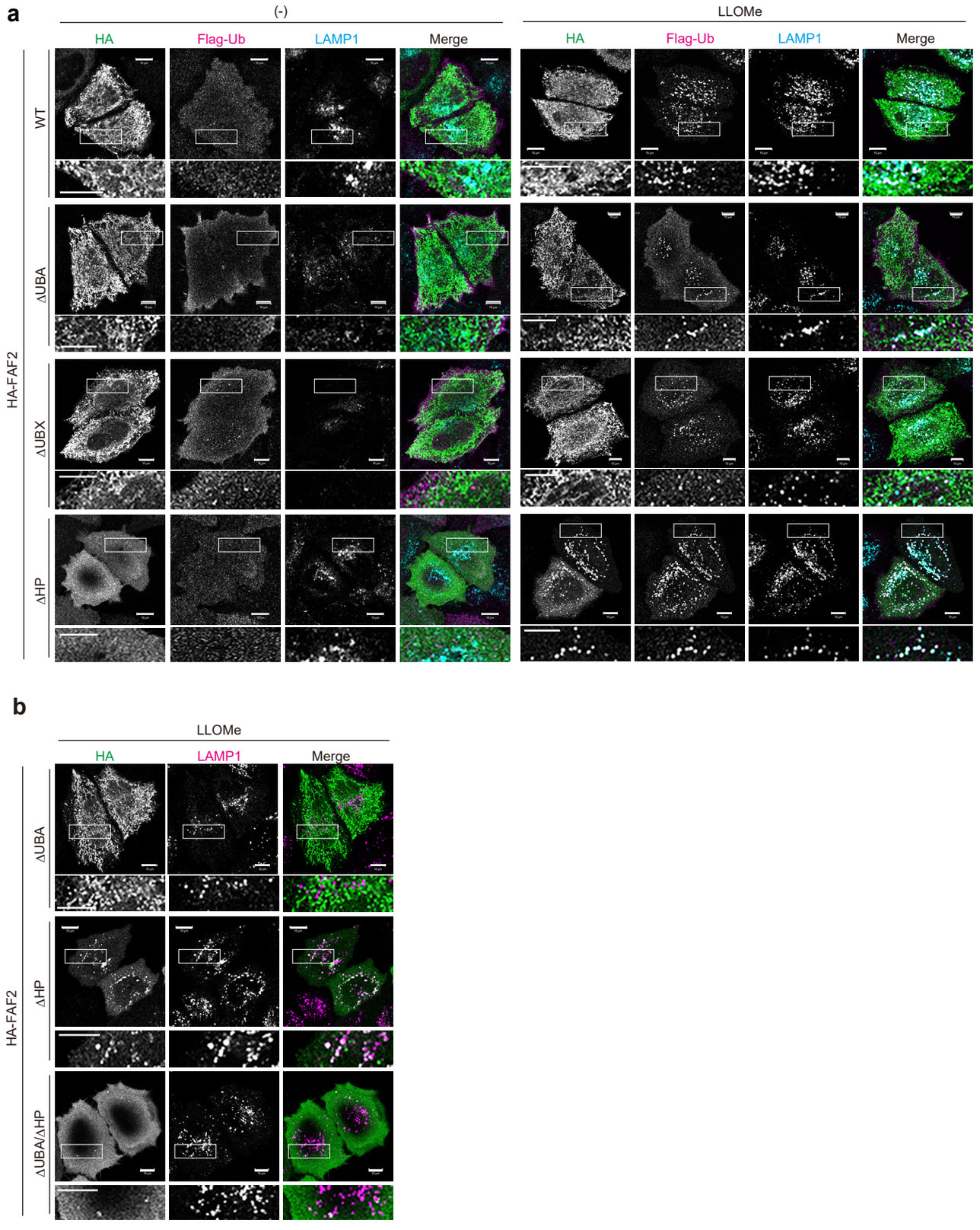


Supplementary Figure 10. The FAF2 UBA domain interacts with ubiquitin.

a. In vitro pull-down assay for GST-FAF2 UBA and ubiquitin chains. GST-FAF2 UBA or GST-FAF2 UBA V47A/L51A were incubated with K48- or K63-linked ubiquitin chains. GST proteins were pulled down using glutathione sepharose resin. The samples (pull-down products and 1% input of K48-linked and K63-linked Ub-chains) were immunoblotted with an anti-Ub antibody.

b. Domain structure of select proteins with UBA-UBX containing cofactors domains.

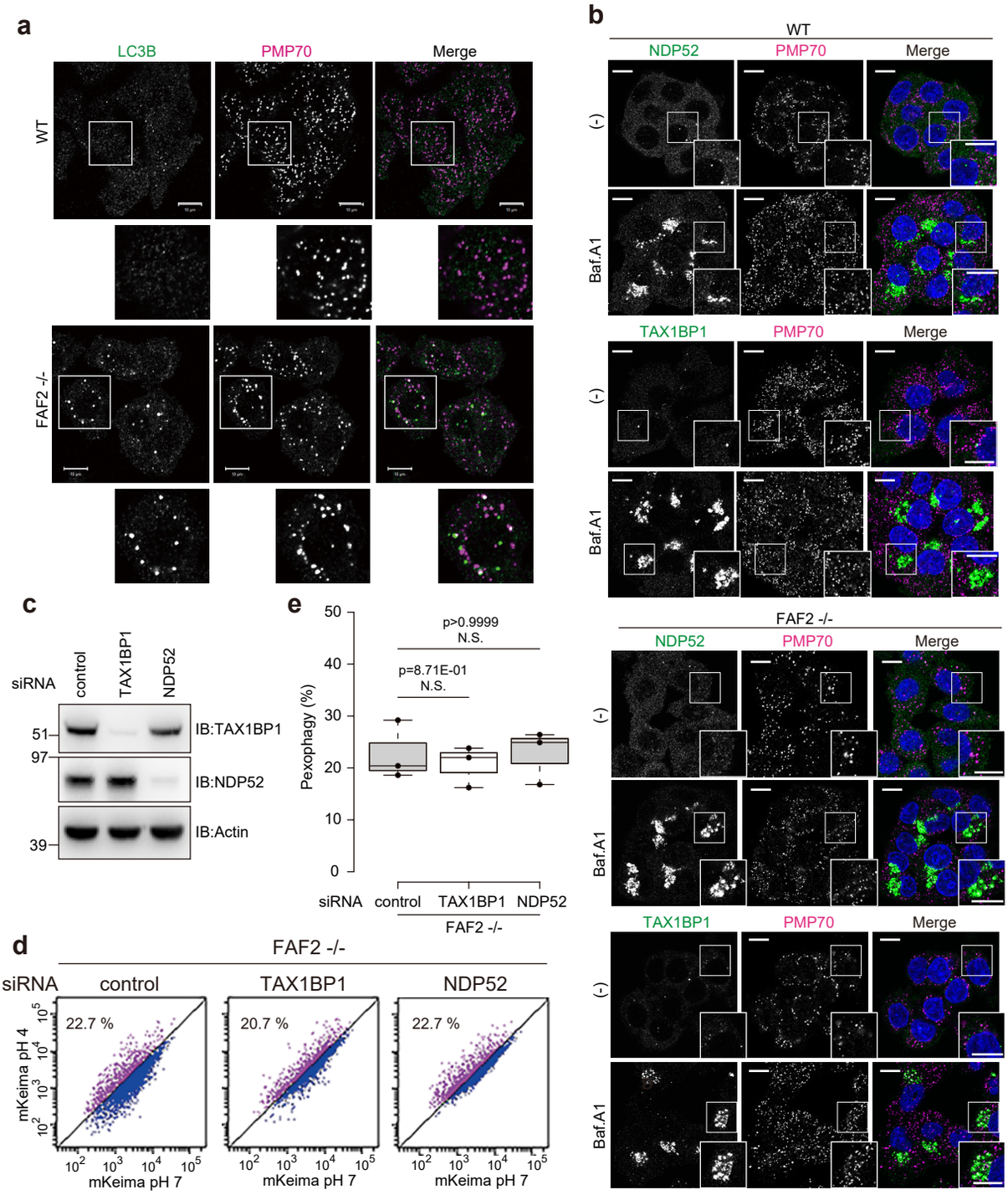
c. N-terminal sequence alignment of select human UBA-UBX-containing proteins. Amino acids highlighted in red correspond to ubiquitin-interacting residues in NSFL1C/p47 and FAF2.



Supplementary Figure 11. The HP and UBA domains of FAF2 are crucial for membrane anchoring and ubiquitin recognition.

a. The FAF2 ΔHP mutant is recruited to ubiquitylated lysosomes. HeLa cells expressing HA-FAF2 WT, ΔUBA, ΔUBX, or ΔHP were treated with or without LLOMe for 1.5 hours and were then immunostained with anti-HA, anti-Flag, and anti-LAMP1 antibodies. Higher magnification images of the boxed regions are shown below each panel. Scale bars, 10 μm. deletion; Δ. n=2 assays.

b. The FAF2 UBA domain is required for translocation to ubiquitin-coated lysosomes. HeLa cells expressing HA-FAF2 ΔUBA, ΔHP, or ΔUBA/ΔHP mutans were treated with LLOMe for 1.5 hours and were then immunostained with anti-HA and anti-LAMP1 antibodies. Higher magnification images of the boxed regions are shown below each panel. Scale bars, 10 μm. deletion; Δ. n=2 assays.



Supplementary Figure 12. TAX1BP1 and NDP52 do not mediate pexophagy in FAF2-deficient cells.

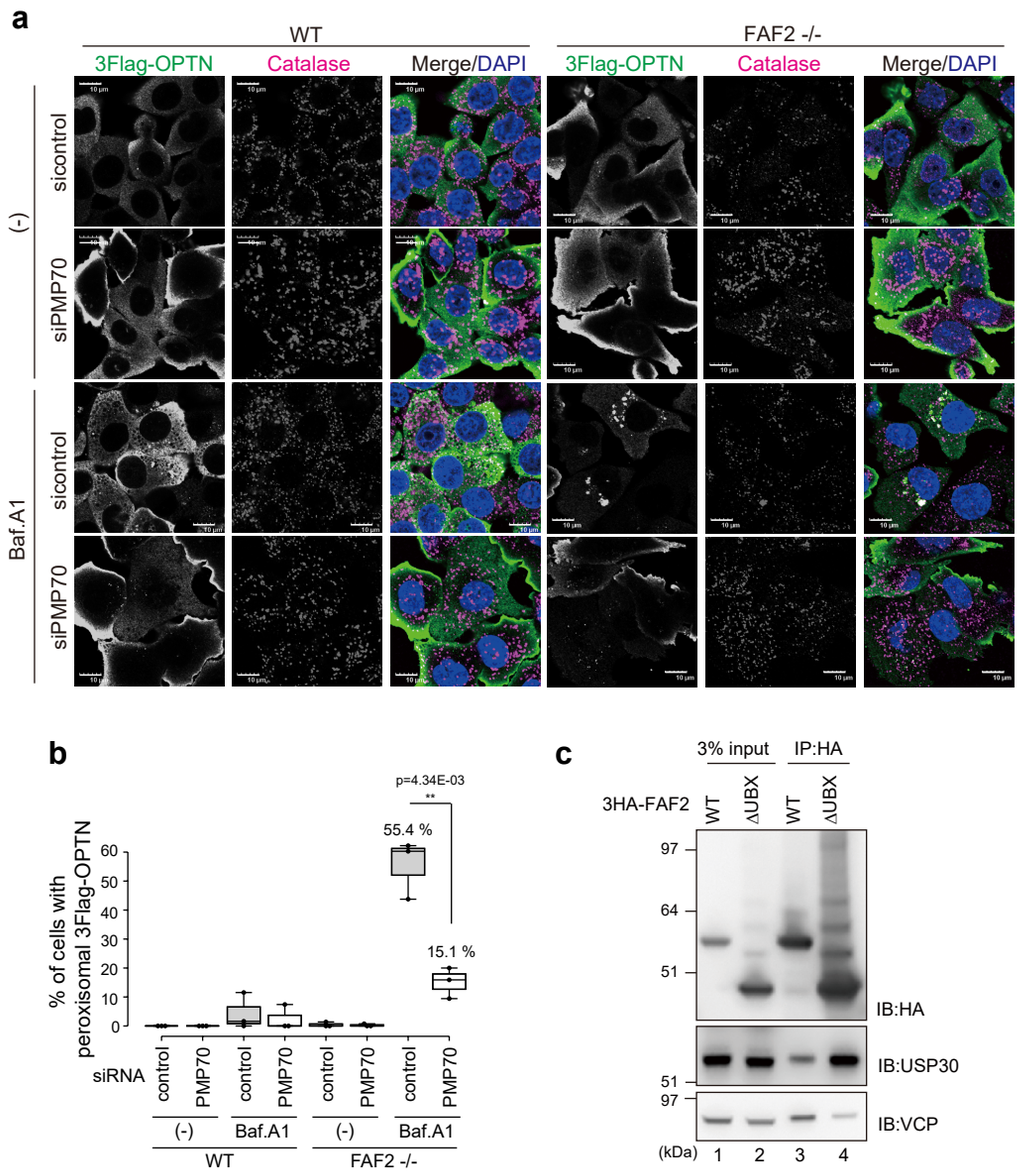
a. LC3B-positive signals partially colocalize with PMP70-positive peroxisomes. WT or FAF2 -/- cells were immunostained with the indicated antibodies. Higher magnification images of the boxed regions are shown below each panel. Scale bars, 10 μm.

b. Neither NDP52 nor TAX1BP1 localize to peroxisomes in FAF2-/- cells. WT or FAF2-/- HCT116 cells were treated with or without Baf.A1 for 24 hours and were then immunostained with the indicated antibodies. FAF2-/- cells that did not have complete peroxisomal loss were used to generate representative images of the cell line. Higher magnification images of the boxed regions are shown in the smaller panels. Scale bars, 10 μm. n=2 assays.

c. Immunoblot confirmation of TAX1BP1 and NDP52 knockdown. Cells transfected with siRNAs were immunoblotted using the indicated antibodies.

d. FACS-based analysis of the pexophagy flux. Representative FACS data (mKeima-SKL 561/488) for the siRNA-transfected FAF2-/- cells are shown along with the percentage of pexophagy-positive cells indicated.

e. Quantitative analysis of the pexophagy flux for cells in (d). Dots represent individual data points from three independent experiments. Statistical significance was calculated using one-way ANOVA; N.S.- not significant. The center lines correspond to the medians and the box limits indicate the 25th and 75th percentiles as determined using R. The box plot whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Statistical significance was calculated using a one-tailed Welch’ s t-test; * $p < 0.05$. Source data are provided as a Source Data file.



Supplementary Figure 13. OPTN recruitment to peroxisomes is diminished in PMP70-depleted cells.

a. WT or FAF2 -/- cells stably expressing 3Flag-OPTN were transfected with control or PMP70 siRNAs and were subsequently immunostained using anti-Flag and anti-catalase antibodies. Scale bars, 10 μm. n=3 assays.

b. Quantitative analysis of cells in (a). The percentage of cells with peroxisomal 3Flag-OPTN was determined. Dots represent individual data points from three independent experiments. Statistical significance was calculated using a one-tailed Welch's t-test; ** $p < 0.01$. The center lines correspond to the medians and the box limits indicate the 25th and 75th percentiles as determined using R. The box plot whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

c. The FAF2 ΔUBX mutation stabilizes associations between FAF2 and USP30. WT cells stably expressed 3HA-FAF2 WT or ΔUBX were immunoprecipitated with anti-HA beads. The samples (IP products and 3% of input) were immunoblotted with the indicated antibodies. deletion; Δ. n=2 assays. Source data are provided as a Source Data file.

Supplementary Figure 14. Antibodies used in this study

<For immunoblotting>

Antibody		clone	Catalogue no.	Source	RRID	Dilution
Anti-PMP70	Mouse monoclonal	70-18	SAB4200181	SIGMA	AB_10639362	1:500
Anti-PEX14	Rabbit polyclonal	(-)	ABC142	Millipore	(-)	1:1000
Anti-PEX16	Rabbit polyclonal	(-)	14816-1-AP	Proteintech	AB_2162250	1:250
Anti-PEX19	Rabbit polyclonal	(-)	14713-1-AP	Proteintech	AB_2162265	1:500
Anti-Tubulin	Rat monoclonal	YL1/2	ab6160	abcam	AB_305328	1:3000
Anti-β-Actin	Mouse monoclonal	6D1	M177-3	MBL	AB_10697039	1:2000
Anti-USP30	Rabbit polyclonal	(-)	NBP1-81914	Novus Biologicals	AB_11011210	1:500
Anti-FAF2	Rabbit Polyclonal	(-)	16251-1-AP	ProteinTech	AB_2262469	1:1000
Anti-Ubiquitin	Mouse monoclonal	P4D1	sc-8017	SantaCruz	AB_628423	1:1000
Anti-p97//VCP	Mouse monoclonal	5	ab11433	abcam	AB_298039	1:1000
Anti-HA	Mouse monoclonal	TANA-2	M180-3	MBL	AB_10951811	1:1000
Anti-HA	Rat monoclonal	3F10	12158167001	Roche	AB_390915	1:1000
Anti-Flag	Rabbit Polyclonal	(-)	PM020	MBL	AB_591224	1:1000
Anti-Flag	Mouse monoclonal	FLA-1	M185-3L	MBL	AB_11123930	1:2000
Anti-p62	Rabbit Polyclonal	(-)	PM066	MBL	AB_10896692	1:1000
Anti-OPTN	Rabbit Polyclonal	(-)	10837-1-AP	Proteintech	AB_2156665	1:2000
Anti-NBR1	Mouse monoclonal	4BR	sc-130380	SantaCruz	AB_2149402	1:200
Anti-NPLOC4	Rabbit Polyclonal	(-)	11638-1-AP	Proteintech	AB_10597107	1:1000
Anti-UFD1	Rabbit Polyclonal	(-)	10615-1-AP	Proteintech	AB_2213944	1:1000
Anti-NSFL1C/p47	Rabbit Polyclonal	(-)	15620-1-AP	Proteintech	AB_2878158	1:1000
Anti-PEX26	Rabbit polyclonal	(-)	NBP1-32743	Novus Biologicals	AB_2268086	1:500
Anti-HaloTag	Mouse monoclonal	(-)	G9211	Promega	AB_2688011	1:2000
Anti-TAX1BP1	Rabbito monoclonal	D1D5	5105S	Cell Signaling Technology	AB_11178939	1:500
Anti-NDP52	Rabbito monoclonal	D1E4A	60732	Cell Signaling Technology	AB_2732810	1:1000
HRP-conjugated goat anti-mouse						
	Goat polyclonal		115-035-003	Jackson ImmnoResearch Inc.	AB_10015289	1:5000
HRP-conjugated goat anti-rabbit						
	Goat polyclonal		111-035-144	Jackson ImmnoResearch Inc.	AB_2307391	1:5000
HRP-conjugated donkey anti-rat						
	Donkey polyclonal		712-035-153	Jackson ImmnoResearch Inc.	AB_2340639	1:5000

<For immunocytochemistry>

Antibody		clone	Catalogue no.	Source	RRID	Dilution
Anti-Catalase	Mouse monoclonal	1A1	LF-MA0003	AbFrontier	AB_1611839	1:1000
Anti-PMP70	Mouse monoclonal	70-18	SAB4200181	SIGMA	AB_10639362	1:200
Anti-PEX14	Rabbit polyclonal	(-)	ABC142	Millipore	(-)	1:500
Anti-Hsp60	Goat polyclonal	(-)	sc1052(N-20)	santa cruz	AB_631683	1:250
Anti-LC3B	Rabbit polyclonal	(-)	PM036	MBL	AB_2274121	1:1000
Anti-p62	Rabbit Polyclonal	(-)	PM066	MBL	AB_10896692	1:1000
Anti-OPTN	Rabbit Polyclonal	(-)	10837-1-AP	Proteintech	AB_2156665	1:400
Anti-NBR1	Mouse monoclonal	5C3	ab55474	abcam	AB_2149404	1:100
Anti-NDP52	Rabbit Polyclonal	(-)	GTX115378	GeneTex	AB_10620266	1:400
Anti-TAX1BP1	rabbit monoclonal	D1D5	5105S	Cell Signaling Technology	AB_11178939	1:250
Anti-Flag	Rabbit Polyclonal	(-)	PM020	MBL	AB_591224	1:1000
Anti-HA	Rat monoclonal	3F10	12158167001	Roche	AB_390915	1:500
Anti-LAMP1	Mouse monoclonal	H4A3	sc-20011	santa cruz	AB_626853	1:200
Anti-FAF2	Rabbit Polyclonal	(-)	16251-1-AP	ProteinTech	AB_2262469	1:500
Anti-Ubiquitin	Mouse monoclonal	P4D1	sc-8017	SantaCruz	AB_628423	1:100
Goat anti-Rabbit IgG Alexa Fluor 488 conjugated			A-11034	Invitrogen	AB_2576217	1:2000
Goat anti-Rabbit IgG Alexa Fluor 568 conjugated			A-11036	Invitrogen	AB_10563566	1:2000
Goat anti-Rabbit IgG Alexa Fluor 647 conjugated			A-21245	Invitrogen	AB_2535813	1:2000
Goat anti-Mouse IgG Alexa Fluor 488 conjugated			A-11029	Invitrogen	AB_2534088	1:2000
Goat anti-Mouse IgG Alexa Fluor 568 conjugated			A-11031	Invitrogen	AB_144696	1:2000
Goat anti-Mouse IgG Alexa Fluor 647 conjugated			A-21236	Invitrogen	AB_2535805	1:2000
Donkey anti-Goat igG Alexa Fluor 647 conjugated			A-21447	Thermo Fisher Scientific	AB_2535864	1:2000