SUPPLEMENTARY FIGURES, TABLES AND LEGENDS

Figure S1

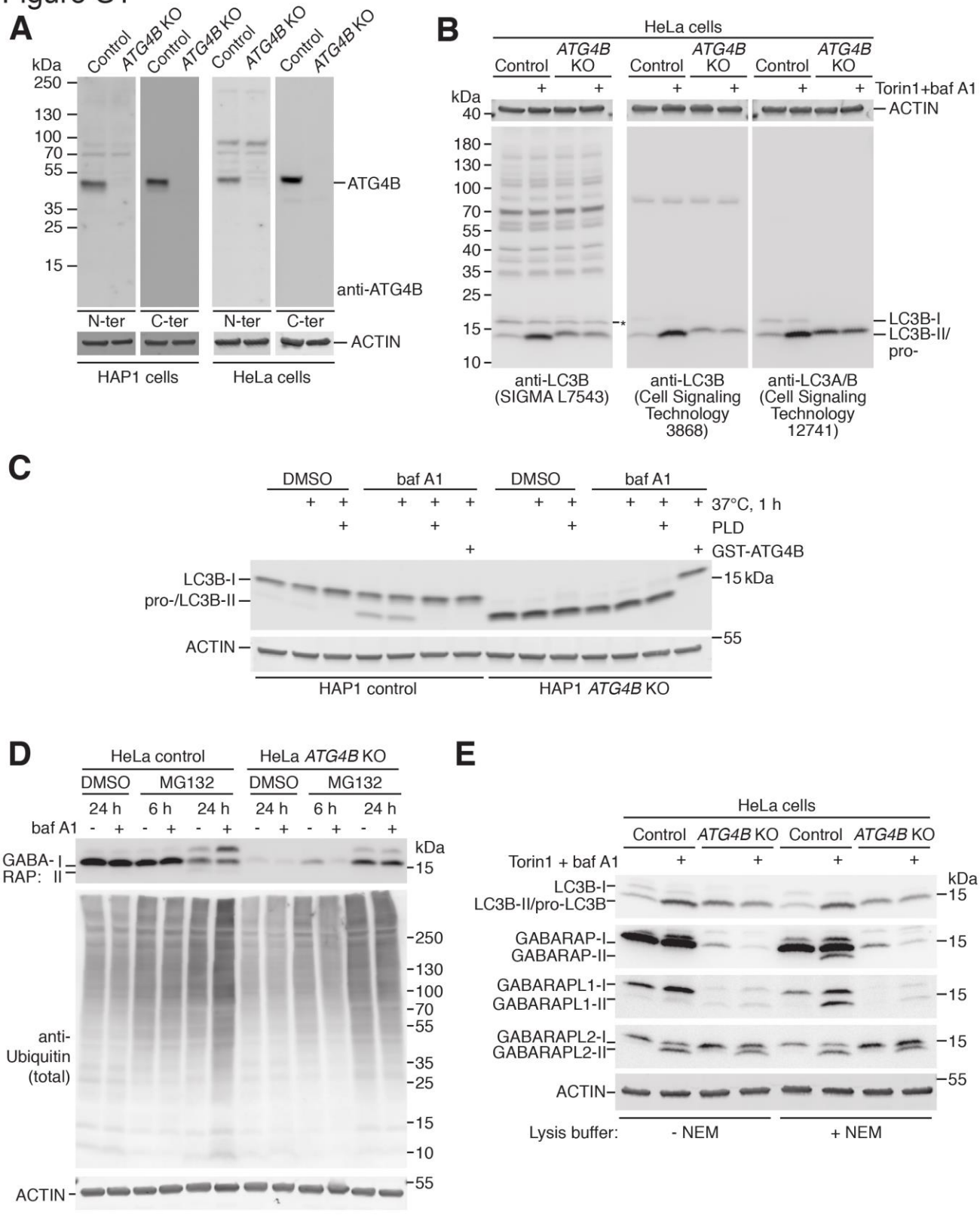


Figure S1. Additional characterization of human *ATG4B* KO cells. (**A**) Protein-level validation of CRISPR-Cas9-mediated *ATG4B* knockout. Lysates from control and *ATG4B* KO HAP1 and HeLa cells were subject to western blotting using polyclonal ATG4B antibodies raised against the N terminus (SIGMA, A2981) and C terminus (Cell Signaling Technology, 5299) of the protein. (**B**) Determination of background bands detected by anti-LC3B (SIGMA, L7543). HeLa control and *ATG4B* KO cells were treated for 3 h with DMSO or 250 nM Torin1 + baf A1 prior to lysis. Samples of the same lysates were run in triplicate on an SDS-PAGE gel before transfer to membrane. The membrane was cut and incubated with 3 different anti-LC3 antibodies as indicated. Asterisk indicates background band detected at the same molecular weight as LC3B-I. (**C**) PLD band shift assay performed on HAP1 control or *ATG4B* KO cells treated for 6 h with DMSO or 10 nM baf A1 and lysed in PLD assay buffer lacking NEM. Lysates were subject to *in vitro* treatment with purified PLD or GST-ATG4B prior to western blotting. (**D**) Western blot of lysates from HeLa control and *ATG4B* KO cells treated with the proteasome inhibitor MG132 (10 µM) for 6 or 24 h, in the presence or absence of 10 nM baf A1 added for the final 3 h of treatment. (**E**) Western blotting of LC3/GABARAP proteins using lysates from HeLa control and *ATG4B* KO cells treated for 3 h with DMSO or 250 nM Torin1 and 10 nM baf A1. Cells were lysed in the presence or absence of 20 mM NEM.

Figure S2

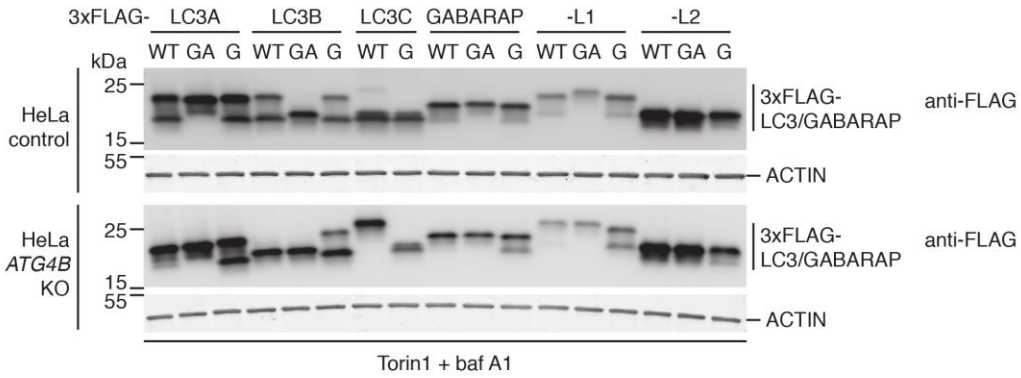


Figure S2. Original blots for Figure 2D**.**

Figure S3

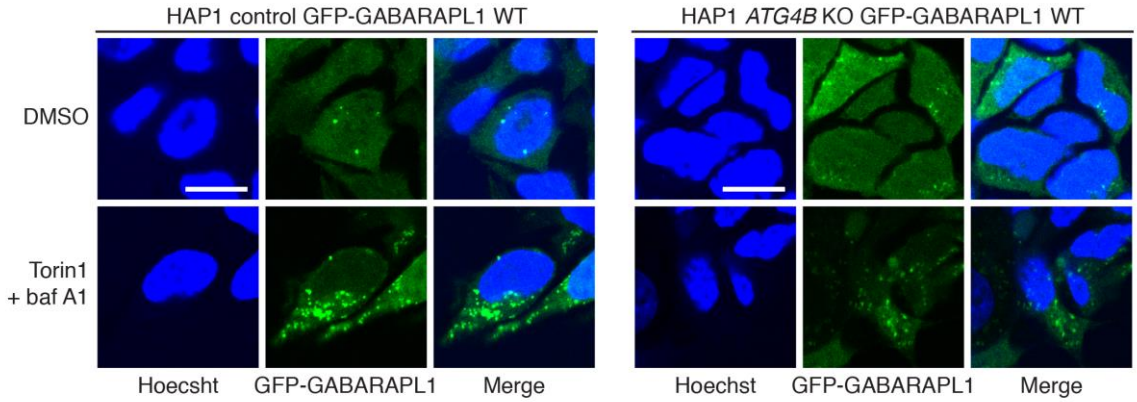


Figure S3. Stably expressed GFP-GABARAPL1 WT localizes to puncta in the absence of ATG4B. HAP1 control and *ATG4B* KO cells stably expressing GFP-GABARAPL1 WT were treated for 3 h with DMSO or 250 nM Torin1 + 10 nM baf A1 prior to fixation and confocal imaging. Scale bar: 10 µm.

Figure S4

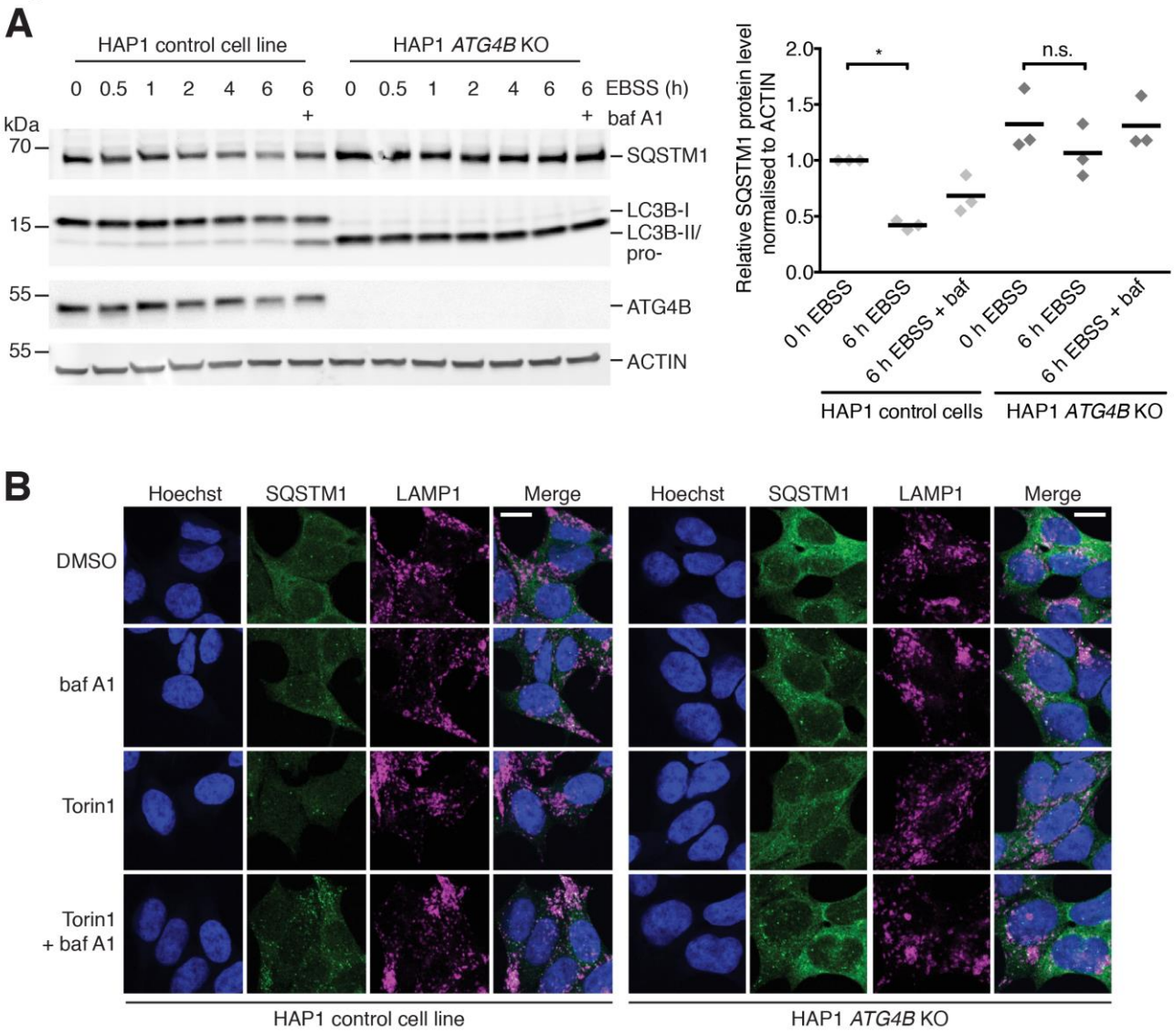


Figure S4. Defect in starvation and Torin1-induced SQSTM1 degradation in HAP1 cells lacking ATG4B. (**A**) Western blot of lysates (lacking NEM) from HAP1 control and *ATG4B* KO cells starved for incremental time points by washing twice with PBS and incubating with EBSS (or in the presence of 10 nM baf A1). Representative blots are shown on the left hand side. Protein level of SQSTM1 from 3 independent experiments was quantified by densitometry and plotted (right panel) after normalizing against the ACTIN protein level. Individual data points are shown and mean is displayed as a line. * $P \leq 0.05$, n.s. $P > 0.05$ (Sidak's multiple comparison test). (**B**) Immunocytochemistry of endogenous SQSTM1 and LAMP1 in control and *ATG4B* KO HAP1 cells. Cells were treated for 3 h with DMSO or 250 nM Torin1 + 10 nM baf A1 prior to fixation and staining. Scale bar: 10 µm.

Figure S5

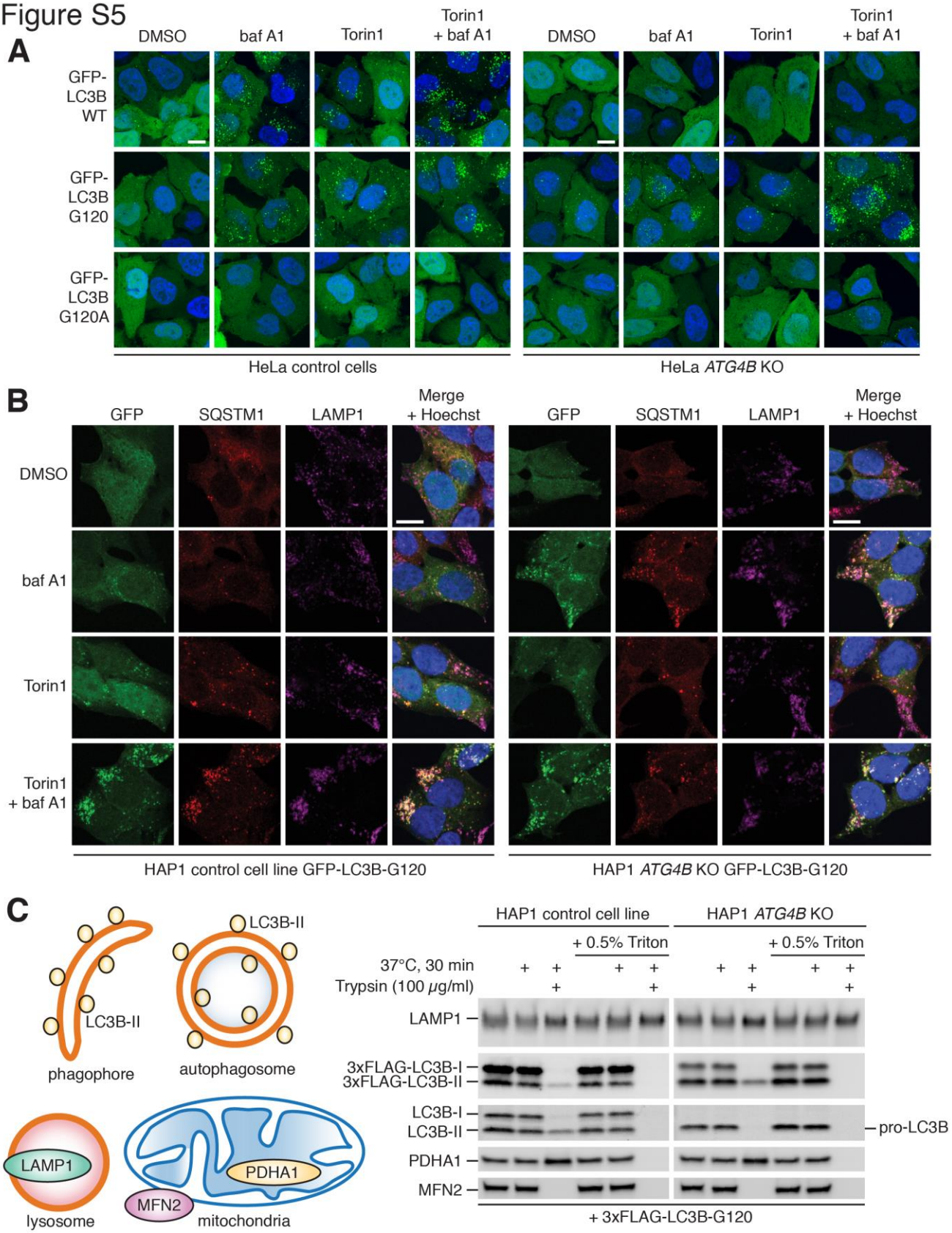
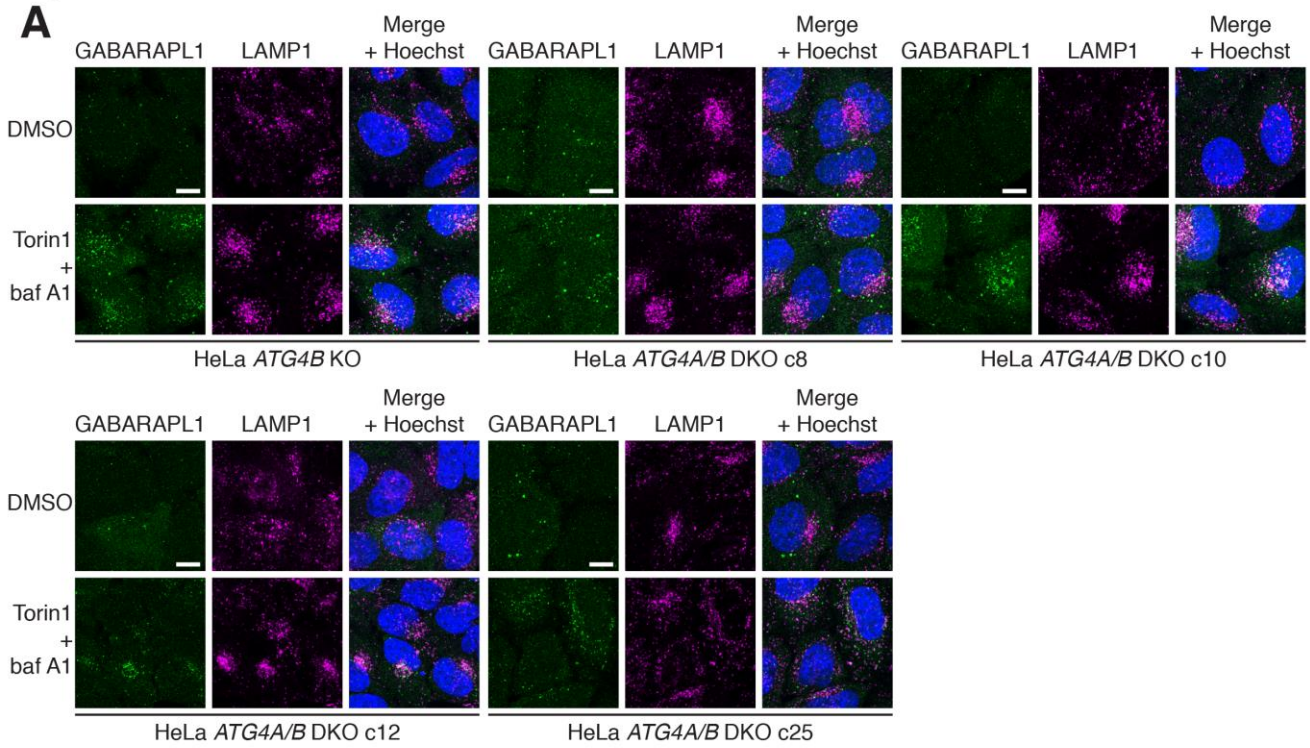

A
DMSO
baf A1
Torin1
Torin1 + baf A1
GFP-LC3B WT
GFP-LC3B G120
GFP-LC3B G120A
HeLa control cells
HeLa ATG4B KO
B
GFP
SQSTM1
LAMP1
Merge + Hoechst
DMSO
baf A1
Torin1
Torin1 + baf A1
HAP1 control cell line GFP-LC3B-G120
HAP1 ATG4B KO GFP-LC3B-G120
C
LC3B-II
phagophore
autophagosome
LAMP1
lysosome
PDHA1
MFN2
mitochondria
HAP1 control cell line
HAP1 ATG4B KO
+ 0.5% Triton
37°C, 30 min
Trypsin (100 μg/ml)
LAMP1
3xFLAG-LC3B-I
3xFLAG-LC3B-II
LC3B-I
LC3B-II
pro-LC3B
PDHA1
MFN2
+ 3xFLAG-LC3B-G120

Figure S5. Additional characterization of primed LC3B expression in ATG4B deficient HAP1 and HeLa cells. (**A**) Confocal microscopy of GFP-tagged LC3B mutants stably expressed in HeLa control and *ATG4B* KO cells treated for 3 h with combinations of DMSO or 250 nM Torin1 with or without 10 nM baf A1. Scale bar: 10 µm. (**B**) Immunocytochemistry of endogenous SQSTM1 and LAMP1 in HAP1 control and *ATG4B* KO cells stably expressing GFP-LC3B-G120. Cells were treated for 3 h with combinations of DMSO or 250 nM Torin1 with or without 10 nM baf A1 for 3 h prior to fixation and staining. Scale bar: 10 µm. (**C**) Protease protection assay of homogenates from HAP1 control and *ATG4B* KO cells transfected with a plasmid encoding 3xFLAG-LC3B-G120 and treated for 3 h with 250 nM Torin1 and 10 nM baf A1. Diagrams (right hand side) show subcellular localization of target proteins detected by western blotting (left hand side).

Figure S6



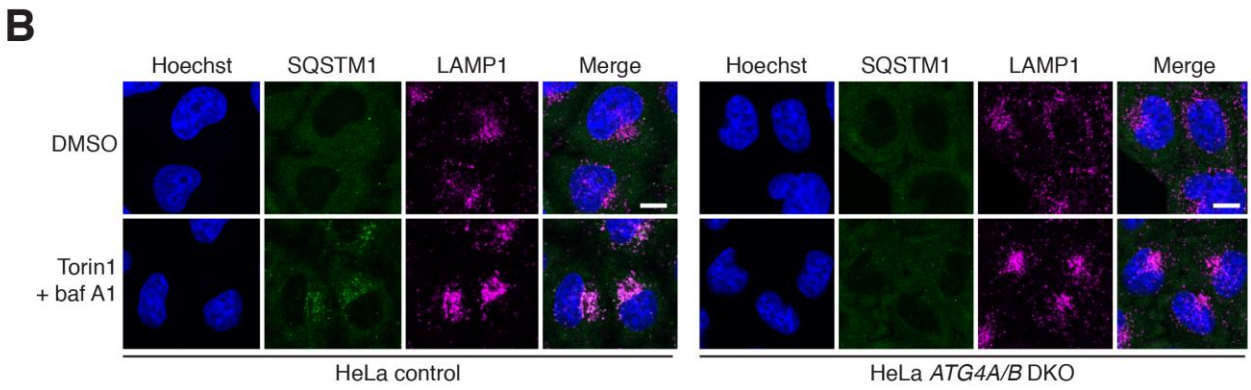


Figure S6. Additional characterization of HeLa *ATG4A/B* DKO cells. (**A**) Immunocytochemistry of endogenous GABARAPL1 and LAMP1 in HeLa *ATG4B* KO and different clones of *ATG4A/B* DKO cells treated for 3 h with DMSO or 250 nM Torin1 + 10 nM baf A1. Scale bar: 10 µm. (**B**) Immunocytochemistry of endogenous SQSTM1 and LAMP1 in HeLa control and *ATG4A/B* DKO (c25) cells treated with DMSO or 250 nM Torin1 + 10 nM baf A1 for 3 h. Scale bar: 10 µm.

Figure S7

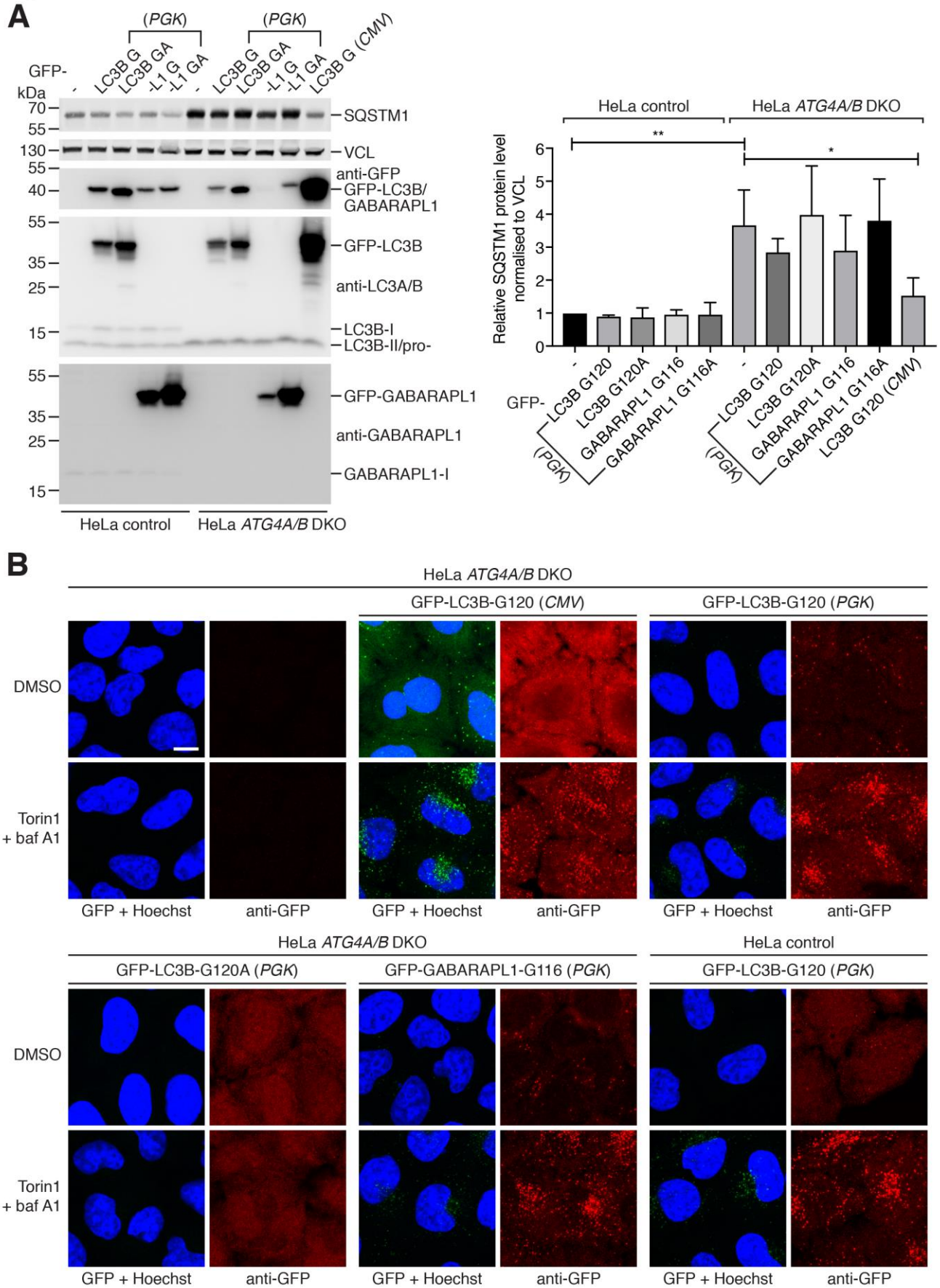

A
(PGK)
(PGK)
GFP-
-
LC3B G
LC3B GA
-L1 G
-L1 GA
-
LC3B G
LC3B GA
-L1 G
-L1 GA
LC3B G (CMV)
kDa
70
55
SQSTM1
130
VCL
anti-GFP
GFP-LC3B/
GABARAPL1
GFP-LC3B
anti-LC3A/B
LC3B-I
LC3B-II/pro-
GFP-GABARAPL1
anti-GABARAPL1
GABARAPL1-I
HeLa control
HeLa ATG4A/B DKO
HeLa control
HeLa ATG4A/B DKO
Relative SQSTM1 protein level normalised to VCL
GFP-
LC3B G120
LC3B G120A
GABARAPL1 G116
GABARAPL1 G116A
LC3B G120 (CMV)
B
HeLa ATG4A/B DKO
GFP-LC3B-G120 (CMV)
GFP-LC3B-G120 (PGK)
DMSO
Torin1
+ baf A1
GFP + Hoechst
anti-GFP
HeLa ATG4A/B DKO
HeLa control
GFP-LC3B-G120A (PGK)
GFP-GABARAPL1-G116 (PGK)
GFP-LC3B-G120 (PGK)

Figure S7. Validation of HeLa cells stably expressing reduced levels of *PGK* promoter-driven GFP-LC3B or GABARAPL1 bypass mutants. (**A**) Western blot analysis of HeLa control and *ATG4A/B* DKO (c25) cells, either untransduced (-) or stably expressing lentiviral-delivered GFP-LC3B-G120/-G120A (G/GA) or GFP-GABARAPL1-G116/-G116A (G/GA) under control of the low expression *PGK* promoter. As a positive control, *ATG4A/B* DKO (c25) cells stably expressing *CMV*-driven GFP-LC3B-G120 (as shown in Figures 7C-E) were assessed in parallel. Densitometry quantification of basal SQSTM1 protein level in each cell line from 3 independent experiments is shown on the right hand side. ** $P \leq 0.01$, * $P \leq 0.05$, differences between other groups were not significant ($P > 0.05$, Sidak's multiple comparison test). (**B**) Immunocytochemistry of GFP localization in cells shown in Figure S7A treated with DMSO or 250 nM Torin1 + 10 nM baf A1 for 3 h. Native GFP fluorescence (shown in green) was difficult to detect at reduced expression levels. To reveal GFP construct localization, cells were stained using anti-GFP antibody that was detected on a separate channel shown in red. Scale bar: 10 µm.

Figure S8

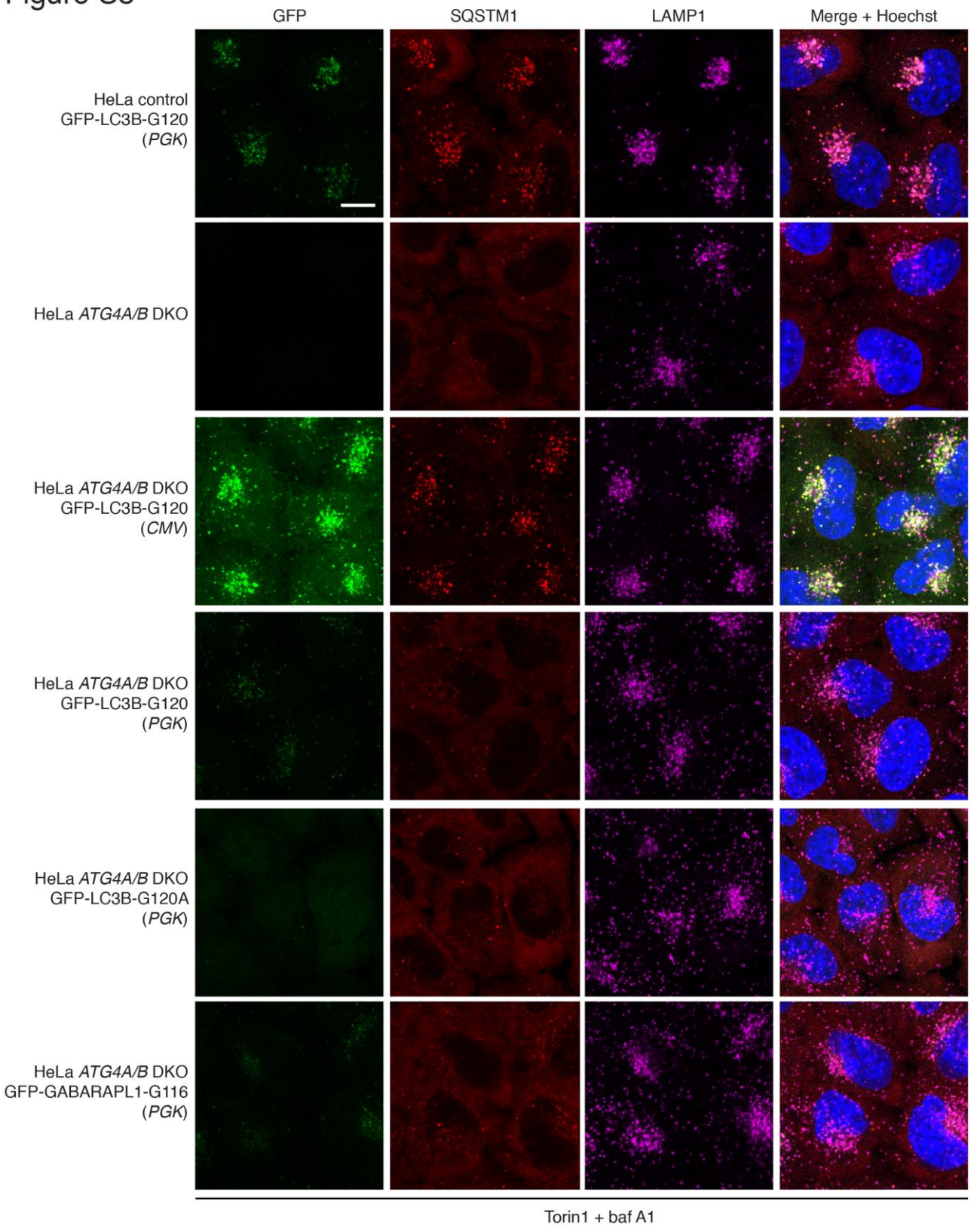

GFP
SQSTM1
LAMP1
Merge + Hoechst
HeLa control
GFP-LC3B-G120
(PGK)
HeLa ATG4A/B DKO
HeLa ATG4A/B DKO
GFP-LC3B-G120
(CMV)
HeLa ATG4A/B DKO
GFP-LC3B-G120
(PGK)
HeLa ATG4A/B DKO
GFP-LC3B-G120A
(PGK)
HeLa ATG4A/B DKO
GFP-GABARAPL1-G116
(PGK)
Torin1 + baf A1

Figure S8. Reduced efficiency of SQSTM1 lysosome delivery in *ATG4A/B* DKO cells expressing low levels of GFP-tagged LC3B or GABARAPL1 bypass mutants compared to high expression of GFP-LC3B-G120. Immunocytochemistry of endogenous SQSTM1 and LAMP1 in cells shown in Figure S7A treated for 3 h with 250 nM Torin1 + 10 nM baf A1 prior to fixation and staining. Native GFP fluorescence is shown. Scale bar: 10 µm.

Figure S9

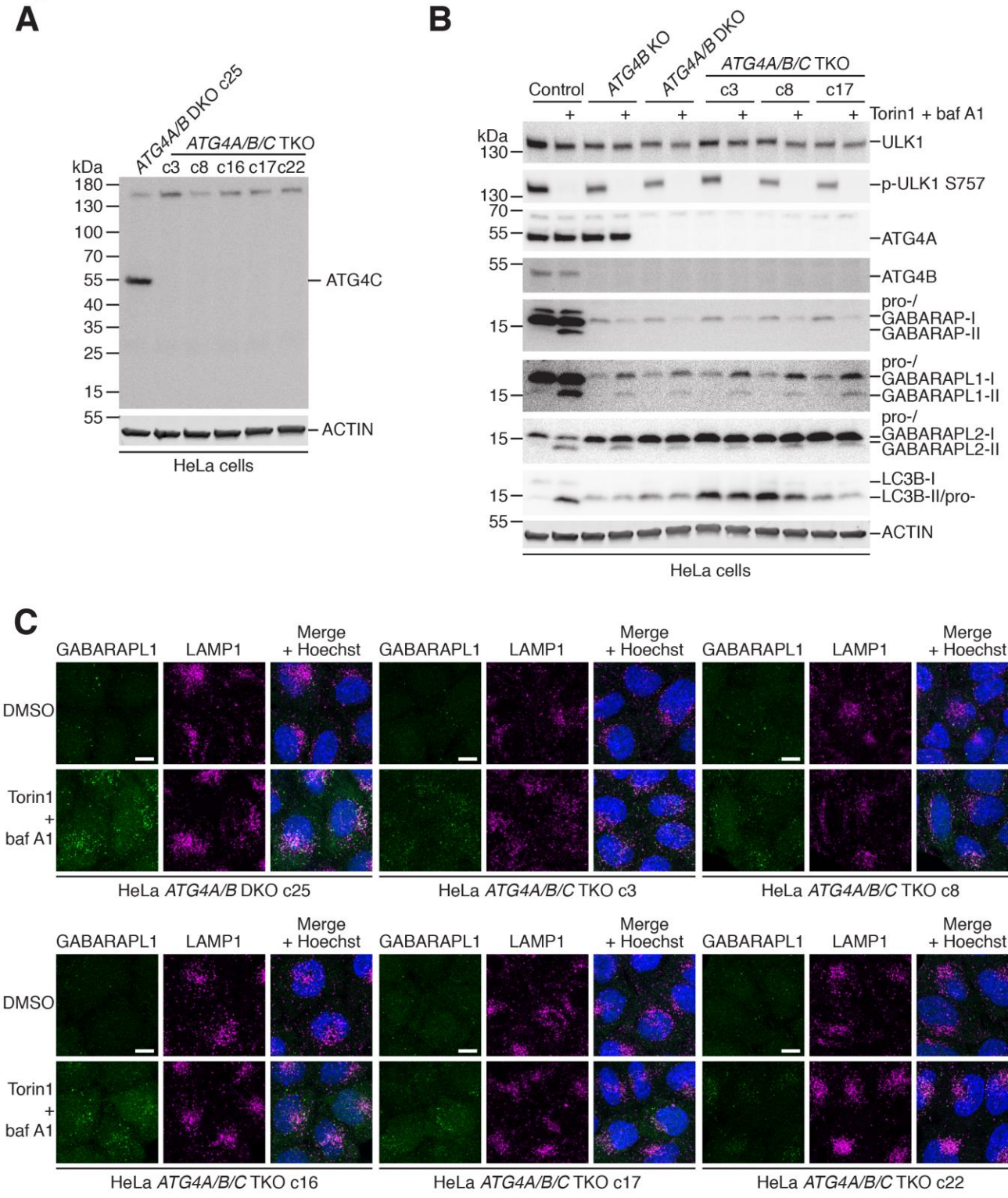

A
ATG4A/B DKO c25
ATG4A/B/C TKO
kDa
c3 c8 c16 c17 c22
180
130
100
70
55
40
35
25
15
55
ATG4C
ACTIN
HeLa cells
B
Control
ATG4B KO
ATG4A/B DKO
ATG4A/B/C TKO
c3 c8 c17
Torin1 + baf A1
kDa
130
ULK1
130
p-ULK1 S757
70
55
ATG4A
55
ATG4B
15
pro-/
GABARAP-I
GABARAP-II
15
pro-/
GABARAPL1-I
GABARAPL1-II
15
pro-/
GABARAPL2-I
GABARAPL2-II
15
LC3B-I
LC3B-II/pro-
55
ACTIN
HeLa cells
C
GABARAPL1
LAMP1
Merge
+ Hoechst
DMSO
Torin1
+
baf A1
HeLa ATG4A/B DKO c25
HeLa ATG4A/B/C TKO c3
HeLa ATG4A/B/C TKO c8
HeLa ATG4A/B/C TKO c16
HeLa ATG4A/B/C TKO c17
HeLa ATG4A/B/C TKO c22

Figure S9. Additional characterization of HeLa *ATG4A/B/C* TKO cells. (**A**) Western blot analysis of HeLa *ATG4A/B* DKO (c25) and different clones of HeLa *ATG4A/B/C* TKO cells lysed without NEM and probed with anti-ATG4C. (**B**) Western blot analysis of HeLa control, *ATG4B* KO, *ATG4A/B* DKO (c25) cells and different *ATG4A/B/C* TKO clones treated for 3 h with DMSO or 250 nM Torin1 + 10 nM baf A1. (**C**) Immunocytochemistry of endogenous GABARAPL1 and LAMP1 in HeLa *ATG4A/B* DKO cells and different *ATG4A/B/C* TKO cell clones treated with DMSO or 250 nM Torin1 + 10 nM baf A1 for 3 h. Scale bar: 10 µm.

Figure S10

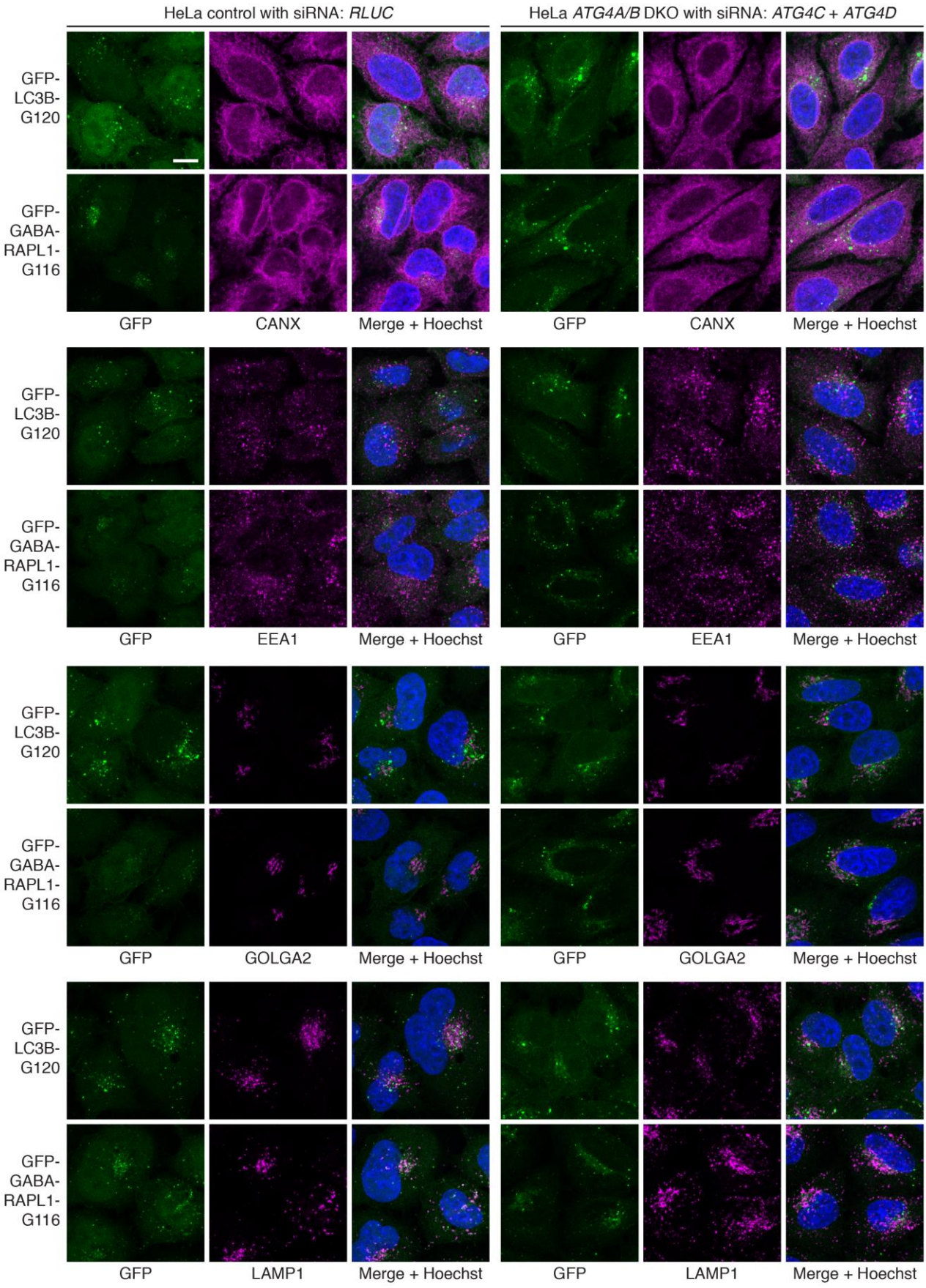

HeLa control with siRNA: *RLUC*
HeLa *ATG4A/B* DKO with siRNA: *ATG4C* + *ATG4D*
GFP-LC3B-G120
GFP-GABA-RAPL1-G116
GFP
CANX
Merge + Hoechst
EEA1
GOLGA2
LAMP1

Figure S10. Delipidation by ATG4 isoforms is not necessary to prevent mislocalization of LC3/GABARAP to non-autophagic organelles. HeLa control and *ATG4A/B* DKO (c25) cells stably expressing GFP-LC3B-G120 or GFP-GABARAPL1-G116 were transfected with siRNA targeting *RLUC* or *ATG4C* + *ATG4D* prior to immunocytochemistry and confocal microscopy to assess GFP localization. Cells were stained with antibodies targeting different organelle markers: CANX (calnexin; endoplasmic reticulum), EEA1 (early endosome antigen 1), GOLGA2 (golgin A2; Golgi) and LAMP1 (lysosomal associated membrane protein 1). Scale bar: 10 μm.

Table S1. Details of genomic loci and oligonucleotides used for CRISPR-Cas9 genome editing in HeLa cells, as well as PCR primers used for genomic validation by Sanger sequencing.

Gene targeted (exon targeted)	**Target location (Reference assembly GRCh38)**	**CRISPR ID/(source)**	**19 nucleotide guide sequence**	**Guide oligo (+) (5' to 3')**	**Guide oligo (-) (5' to 3')**	**Forward genomic primer (5' to 3')**	**Reverse genomic primer (3' to 5')**
ATG4A (exon 2)	X:108126133-108126155	ID: 1195746731 (Sanger CRISPR finder)	ATACCAGCTCATCTGTATC	CACCGATACCAGCTCATCTGTATC	AAACGATACAGATGAGCTGGTATC	GGGGACAAGTTTGTACAAAAAAGCAGGCTACAAAGAAGGCCCCATGGTA	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAGAATTTGGGGATTCTCCTTT
ATG4B (exon 4)	2:241653584-241653606	ID: 949006718 (Sanger CRISPR finder)	CCTAGGTGCCGGCACACCA	CACCGCCTAGGTGCCGGCACACCA	AAACTGGTGTGCCGGCACCTAGGC	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGTTACTGAGTCCTAAGAGGTGTGTGTTG	GGGGACCACTTTGTACAAGAAAGCTGGGTACATTACTCAAGAAACCCCCAAGTTC
ATG4C (exon 4)	1:62816613-62816635	ID: 908305971 (Sanger CRISPR finder)	TAGAGGATCACGTAATTGC	CACCGTAGAGGATCACGTAATTGC	AAACGCAATTACGTGATCCTCTAC	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGCAGTCAGGAAAATCTAACT	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGGGAATTTCATGCAGTTTGT
Non-targeting	N/A	rg_0311 (GeCKO library v2) (Sanjana et al., 2014)	GGCCCGCATAGGATATCGC	CACCGGGCCCGCATAGGATATCGC	AAACGCGATATCCTATGCGGGCCC	N/A	N/A

Table S2. Sequences of primers and DNA templates used to generate Gateway entry clones for LC3/GABARAP coding sequences.

Gene/entry clone	**RefSeq of PCR template**	**Forward primer sequence (5' to 3')**	**Reverse primer sequence (5' to 3')**
MAP1LC3A	NM_032514.3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCCTCAGACCGGCCTTTC	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGAAGCCGAAGGTTTCCTGGGAG
MAP1LC3A (no stop codon)	NM_032514.3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCCTCAGACCGGCCTTTC	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAAGCCGAAGGTTTCCTGG
MAP1LC3A (G120A)	NM_032514.3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCCTCAGACCGGCCTTTC	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGAAGGCGAAGGTTTCCTGGGAG
MAP1LC3A (G120)	NM_032514.3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCCTCAGACCGGCCTTTC	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGCCGAAGGTTTCCTGGGAGG
MAP1LC3B	NM_022818.4	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCGTCGGAGAAGACC	GGGGACCACTTTGTACAAGAAAGCTGGGTATTACACTGACAATTTCATCCCGAAC
MAP1LC3B (no stop codon)	NM_022818.4	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCGTCGGAGAAGACC	GGGGACCACTTTGTACAAGAAAGCTGGGTACACTGACAATTTCATCCCG
MAP1LC3B (G120A)	NM_022818.4	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCGTCGGAGAAGACC	GGGGACCACTTTGTACAAGAAAGCTGGGTATTACACTGACAATTTCATCGCGAACGTC
MAP1LC3B (G120A; no stop codon)	NM_022818.4	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCGTCGGAGAAGACC	GGGGACCACTTTGTACAAGAAAGCTGGGTACACTGACAATTTCATCGCG
MAP1LC3B (G120)	NM_022818.4	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCGTCGGAGAAGACC	GGGGACCACTTTGTACAAGAAAGCTGGGTATTACCCGAACGTCTCCTGGG
MAP1LC3B2 (no stop codon)	NM_001085481.1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCGTCGGAGAAGACC	GGGGACCACTTTGTACAAGAAAGCTGGGTACACTGACAATTTCATCCCG
MAP1LC3C	NM_001004343.2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCGCCTCCACAGAAAATCCC	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGAGAGGATTGCAGGGTCTGTCC
MAP1LC3C (no stop codon)	NM_001004343.2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCGCCTCCACAGAAAATCCC	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAGAGGATTGCAGGGTCTG
MAP1LC3C (G126)	NM_001004343.2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCGCCTCCACAGAAAATCCC	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGCCAAATGTCTCCTGGGAGGC

GABARAP	NM_007278.1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTTCGTGTACAAAGAAGAG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTACAGACCGTAGACACTTTCGTC
GABARAP (no stop codon)	NM_007278.1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTTCGTGTACAAAGAAGAG	GGGGACCACTTTGTACAAGAAAGCTGGGTACAGACCGTAGACACTTTCG
GABARAP (G116A)	NM_007278.1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTTCGTGTACAAAGAAGAG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTACAGAGCGTAGACACTTTCGTC
GABARAP (G116)	NM_007278.1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTTCGTGTACAAAGAAGAG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAACCGTAGACACTTTCGTCAC
GABARAPL1	NM_031412.2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTTCCAGTACAAGGAGG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTATTTCCCATAGACACTCTCATCAC
GABARAPL1 (no stop codon)	NM_031412.2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTTCCAGTACAAGGAGG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTTCCCATAGACACTCTCATC
GABARAPL1 (G116A)	NM_031412.2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTTCCAGTACAAGGAGG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTATTTCGCATAGACACTCTCATCAC
GABARAPL1 (G116)	NM_031412.2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTTCCAGTACAAGGAGG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTACCCATAGACACTCTCATCACTG
GABARAPL2	NM_007285.6	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTGGATGTTCAAGGAGG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGAAGCCAAAAGTGTTCTCTCC
GABARAPL2 (no stop codon)	NM_007285.6	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTGGATGTTCAAGGAGG	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAAGCCAAAAGTGTTCTCTC
GABARAPL2 G116A	NM_007285.6	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTGGATGTTCAAGGAGG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGAAGGCAAAAGTGTTCTCTCC
GABARAPL2 (G116)	NM_007285.6	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTGGATGTTCAAGGAGG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGCCAAAAGTGTTCTCTCCGC

Table S3. Mutations detected by Sanger sequencing of PCR-amplified genomic DNA in main CRISPR KO HeLa clones used in this study.

Cell name	**Main clone**	**Parental cell**	**Gene targeted using CRISPR**	**Mutations detected in targeted gene (no. of Sanger sequence reads detected/total)**
HeLa *ATG4B* KO	N/A	HeLa	*ATG4B*	NM_013325.4:c.262_264del (13/19) NM_013325.4:c.263_264insCT (6/19)
HeLa *ATG4A/B* DKO	c25	HeLa *ATG4B* KO	*ATG4A*	NM_052936.4:c.73_83del (5/5)
HeLa *ATG4A/B/C* TKO	c22	HeLa *ATG4A/B* DKO c25	*ATG4C*	NM_032852.3:c.216delT;223A>C (1/4) NM_032852.3:c.181_247del (1/4) NM_032852.3:c.216_217insT (1/4) NM_032852.3:c.213_215del (1/4)