Expanded View Figures

Figure EV1. TFG regulates ULK1 independently of its function on COPII trafficking.

A HeLa cells were grown for 1 h in starvation (STV) medium, then fixed, permeabilized and stained with TFG (green) and ATG13 (red). Hoechst was used to stain nuclei. Representative micrograph is reported. Scale bar 2 μm.

B HeLa cells were transfected with the indicated siRNAs and grown in nutrient complete (FED) or starvation (STV) media for 1 h. Protein extracts were analysed by WB to detect p-ATG13, ATG13, GADPH, p-ATG14, ATG14, ACTIN and TFG protein levels. Densitometry analysis representing p-ATG13/ATG13 normalized over GADPH and p-ATG14/ATG14 normalized over ACTIN of starved samples of both the conditions analysed was also reported. All data are expressed as mean ± SEM. Statistical analyses were obtained by unpaired Student's *t*-test. ns, not significant ($n = 3$ independent experiments).

C HeLa cells were transfected with the indicated siRNAs and grown in the presence or not of cycloheximide (CHX) for indicated time periods. Protein extracts were analysed by WB to detect FIP200, ATG13, TFG and ACTIN. Densitometry analysis of FIP200 over ACTIN (bottom left) and ATG13 over ACTIN (bottom right) is also displayed. Values are mean ± SEM. Statistical analyses were performed by multiple *t*-test. ns, not significant ($n = 4$ independent experiments).

D Quantification of ULK1 puncta in HeLa cells transfected with the indicated siRNAs and cultured in complete growth or starvation (STV) media for 1 h is shown. TFG downregulation and ULK1 protein levels were analysed by WB as reported. All data are expressed as mean ± SEM. Statistical analysis was obtained by two-way ANOVA followed by Tukey's multiple comparison test ***$P < 0.001$ and ****$P < 0.0001$ ($n = 3$ independent experiments).

E HeLa cells were grown for 1 h in starvation (STV) medium, then fixed, permeabilized and stained with ATG13 (red) and ULK1 (green). Hoechst was used to stain nuclei. Representative micrograph and fluorescence plot are reported. Scale bar 5 μm.

F HeLa cells were co-transfected with control siRNA and an empty vector (EV), or 3'UTR TFG siRNA together with an EV or HA-TFG plasmids. Cells were grown for 1 h in starvation medium, then fixed, permeabilized and labelled with anti-ATG13 (green) antibody. Hoechst was used to stain nuclei. The average of ATG13 puncta number per cell is reported (bottom). All data are expressed as mean ± SEM. Statistical analysis was obtained by two-way ANOVA followed by Tukey's multiple comparison test. *$P < 0.05$ ($n = 3$ independent experiments, $n \geq 13$ fields analysed). Scale bars 5 μm.

G, H HeLa cells were transfected with SEC23A and SEC16 siRNAs (siSEC23A) and (siSEC16), respectively. Cells were cultured with nutrient complete medium (FED) or starvation medium (STV) for 1 h. HeLa cells were fixed, permeabilized and stained with ULK1 (red) antibody. Hoechst was used to stain nuclei. Graphs reporting the average of ULK1 puncta number per cell for the indicated conditions are shown (bottom) ($n = 3$ independent experiments, $n \geq 6$ fields analysed for G; $n = 3$ independent experiments, $n \geq 9$ fields analysed for H). Scale bars 5 μm. All values are expressed as the mean ± SEM. Statistics were performed by two-way ANOVA followed by Tukey's multiple comparison test. ns, not significant.

I HeLa cells were transfected with indicated siRNAs. Protein extracts were analysed by WB to detect SEC16, ULK1, SEC23, TFG and ACTIN protein levels. Quantification of ULK1 over ACTIN is reported in the graph (right). Data are mean ± SEM. Statistics were performed by one-way ANOVA followed by Tukey's multiple comparison test. **$P < 0.01$ and ns, not significant ($n = 3$ independent experiments).

J HeLa cells were transiently transfected with the indicated siRNAs and grown in complete or starvation (STV) media, in the presence or absence of CLQ for 1 h. Protein extracts were analysed by WB to detect LC3B-II and ACTIN protein levels and SEC23A downregulation as indicated. Densitometry analysis of LC3B-II normalized over ACTIN is reported (right). All data are reported as mean ± SEM. Significance was assigned by two-way ANOVA followed by Tukey's multiple comparison test. *$P < 0.05$ ($n = 3$ independent experiments).

K HeLa cells were transfected with indicated siRNAs, fixed and immunolabelled with ULK1 (red) antibody. Hoechst was used to stain nuclei. ULK1 puncta number was quantitated and reported in the graph (bottom). All data are mean ± SEM. Statistics were performed by two-way ANOVA followed by Tukey's multiple comparison test. **$P < 0.01$; ***$P < 0.001$ ($n = 3$ independent experiments, $n \geq 7$ fields analysed). Scale bar 5 μm.

L HeLa cells were transfected with indicated siRNAs. Protein extracts were analysed by WB for indicated antibodies. ULK1 over ACTIN protein levels is reported in the graph (right). All values are expressed as the mean ± SEM. Statistics were performed by one-way ANOVA followed by Dunnett's multiple comparison test. *$P < 0.05$ ($n = 3$ independent experiments).

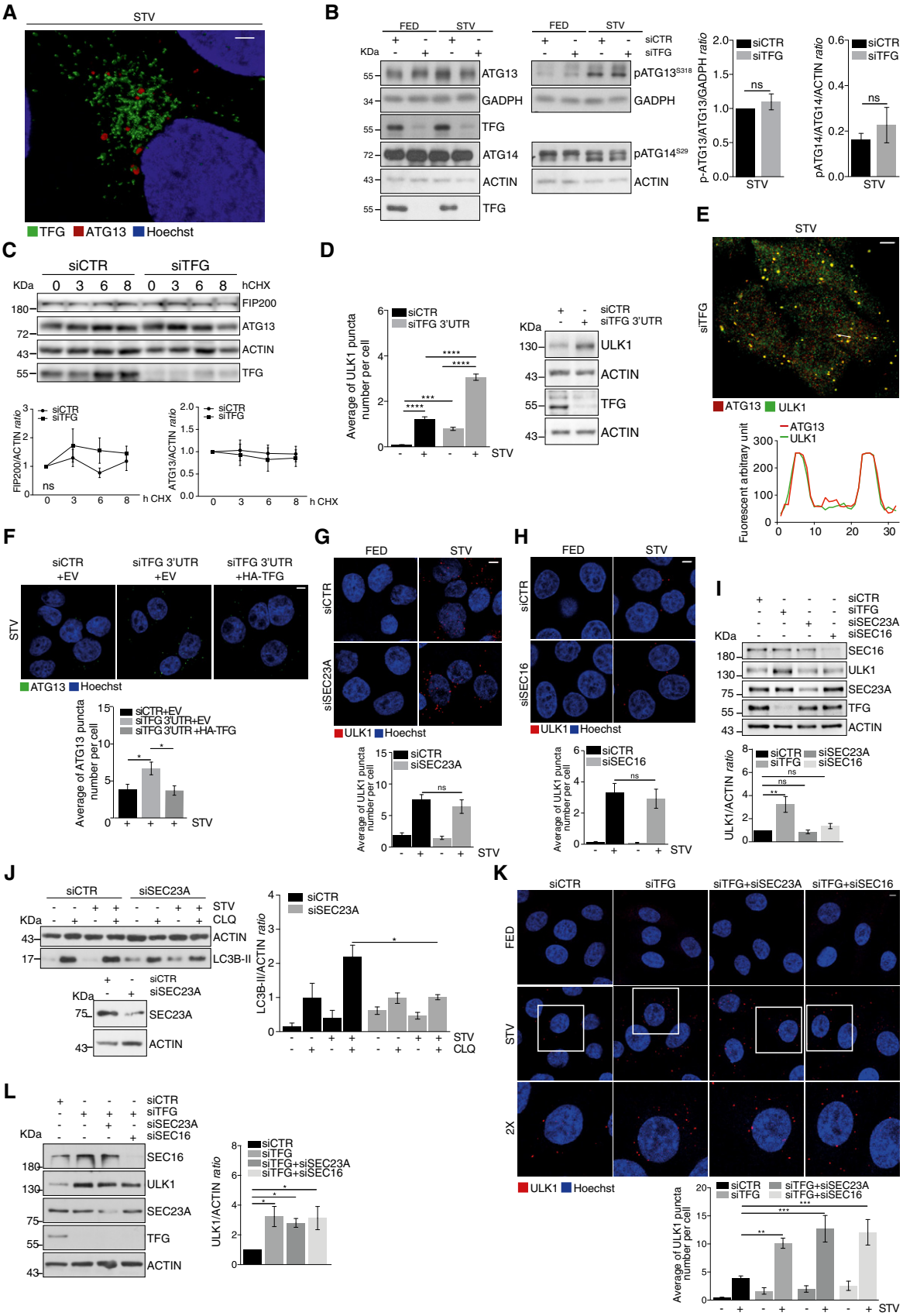


Figure EV1.

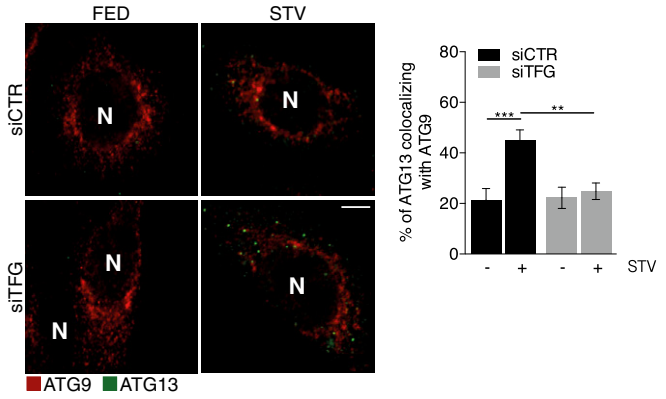


Figure EV2. TFG downregulation affects ULK1-ATG9 co-localization.

HEK293 cells stably expressing GFP-ATG13 were interfered or not with TFG siRNA (siTFG) and starved for 1 h. Cells were fixed and immunolabelled with ATG9 (red) antibody. Co-localization analysis was performed by Jacop plugin. Values of Mander's coefficient for ULK1 are reported as percentage and represented as mean ± SEM. Significance was assigned by two-way ANOVA followed by Tukey's multiple comparison test. $^{**}P < 0.01$ and $^{***}P < 0.001$. Scale bar 5 μm. N, nucleus ($n = 3$ independent experiments, $n \geq 20$ fields analysed).

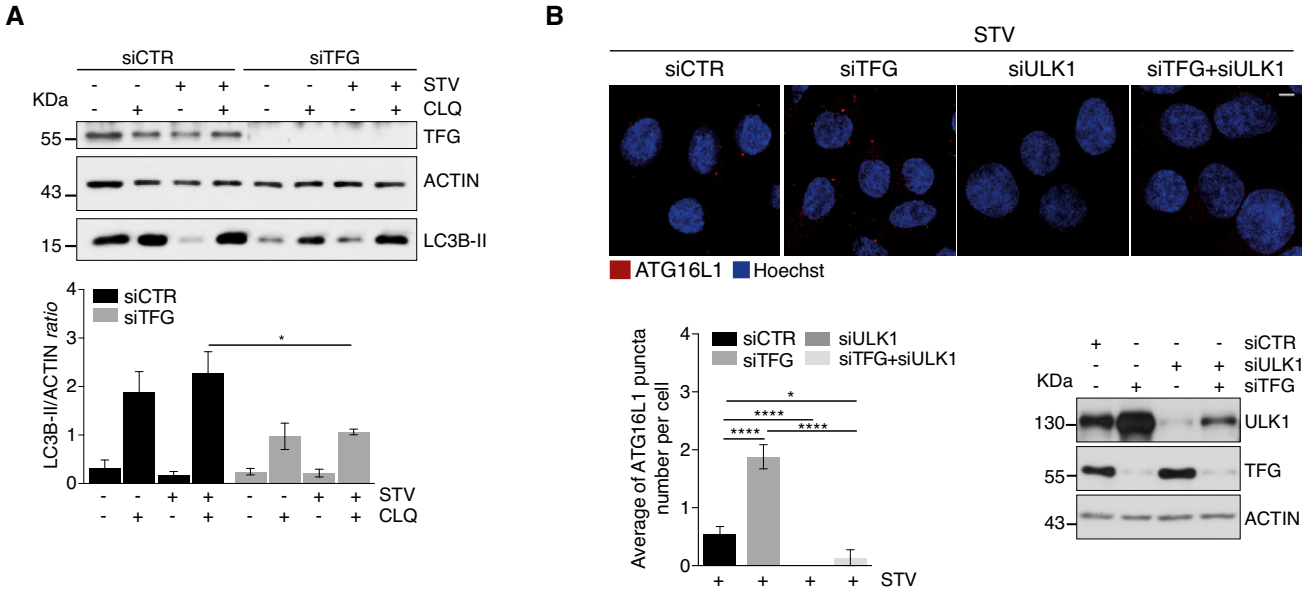


Figure EV3. ATG16L1 puncta accumulation depends on ULK1 in TFG-depleted cells.

A HeLa cells were transfected with indicated siRNAs and cultured in fed or starvation conditions (STV) in the presence or absence of CLQ for 1 h. Protein extracts were analysed by WB for the expression of TFG, ACTIN and LC3B-II. Densitometry analysis of LC3B-II over ACTIN is shown (bottom). Values are expressed as mean ± SEM. Statistical analysis was performed by Tukey's multiple comparison test. $^{*}P < 0.05$ ($n = 3$ independent experiments).

B HeLa cells were transfected with indicated siRNAs. Cells were then cultured in starvation medium for 1 h, fixed and immunolabelled with ATG16L1 (red) antibody. Hoechst was used to stain nuclei. Analysis of ATG16L1 puncta is reported in the graph (bottom left). Values are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test. $^{*}P < 0.05$ and $^{****}P < 0.0001$. Scale bar 5 μm ($n = 5$ independent experiments). WB analysis to detect ULK1 and TFG downregulations is also shown (bottom right).

Figure EV4. Both TFG depletion and TFGR106C mutation induce autophagy impairment.

A HeLa cells were co-transfected with TFG or control siRNA together with vectors encoding for Myc-ULK1 and HA-LC3B. After 30' of starvation, cells were lysed, and protein extracts were immunoprecipitated by using an anti-HA antibody or IgG as negative control. ULK1 LC3B and TFG protein levels were analysed by WB.

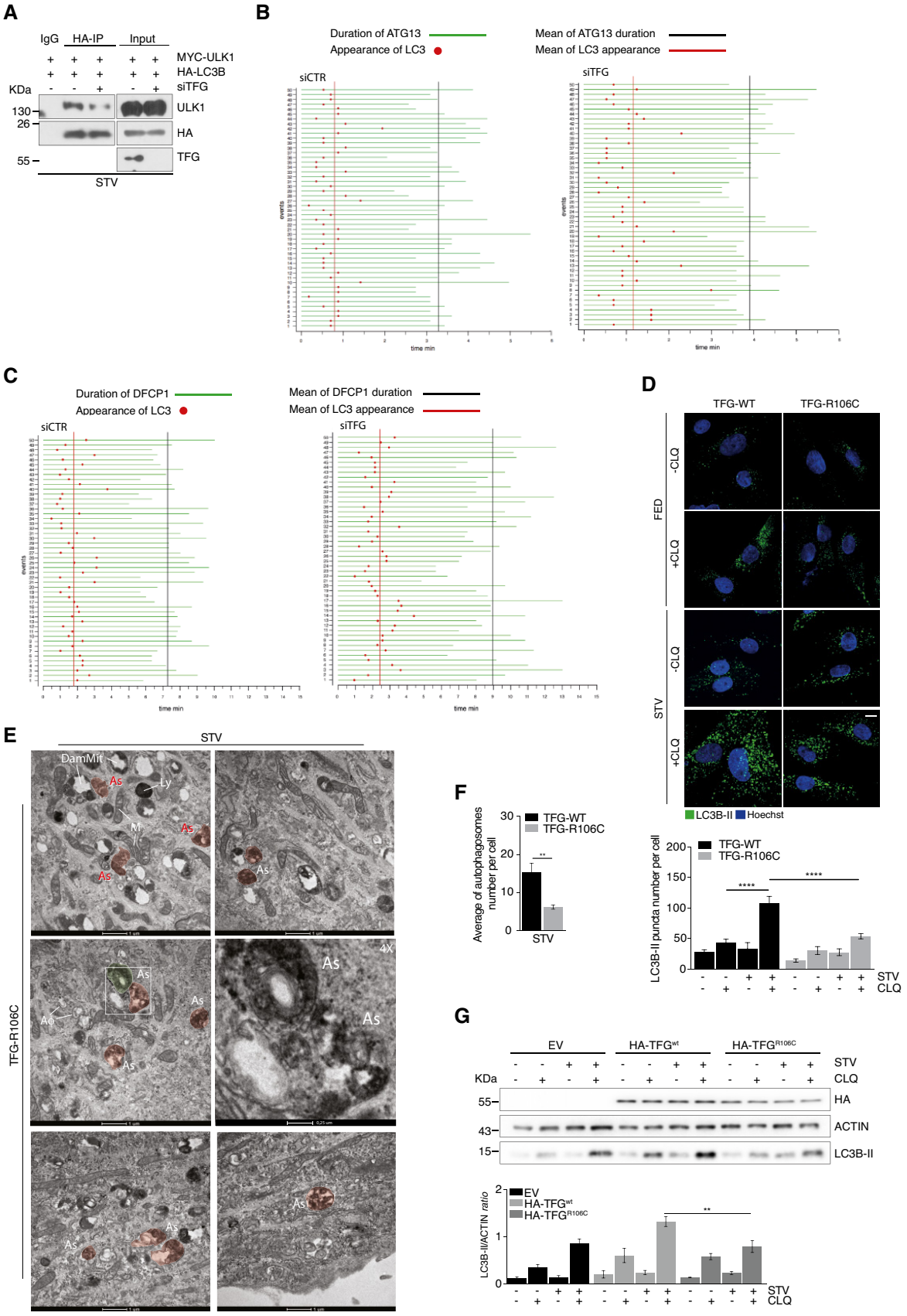
B, C Temporal relationship of LC3B and ATG13 (B) or LC3B and DFCP1 (C). Starved HEK293 cells stably expressing GFP-ATG13 or GFP-DFCP1 together with CFP-LC3B were analysed by live-cell imaging. 50 unique events showing the moment at which LC3 appears on ATG13 (B) or DFCP1 (C) (red point) are reported; green lines depict the lifespan of ATG13 particles (B) or DFCP1 structures (C), respectively ($n = 3$ independent experiments, $n = 50$ events analysed).

D Control (TFG-WT) and patient's fibroblasts (TFG-R106C) were grown in fed or starvation conditions in the presence or absence of CLQ for 1 h. Cells were fixed permeabilized and labelled with LC3B-II antibody. Hoechst was used to stain nuclei. LC3B-II puncta number was analysed (bottom). Data are expressed as the mean value ± SEM. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparison test. ****$P < 0.0001$ ($n = 5$ independent experiments). Scale bar 5 μm.

E Patient's fibroblasts (TFG-R106C) were cultured in STV medium for 1 h. Cells were processed and imaged by TEM. Representative electron micrographs are reported. AΦ, autophagosome; As, abnormal structures; M, mitochondrion; DamMit, damaged mitochondrion; and Ly, lysosome. Scale bar 1 μm or 0,25 μm as indicated.

F Quantification of the autophagosomes number per cell in control (TFG-WT) and patient's fibroblasts (TFG-R106C) grown in starvation (STV) conditions is shown. Data are expressed as the mean value ± SEM. Statistical analysis were performed by unpaired Student's *t*-test. **$P < 0.01$. A minimum of 10 cells for condition were observed ($n = 3$ independent experiments, $n \geq 10$ cells/condition analysed).

G HeLa cells were transfected with an empty vector (EV) or HA-TFG^{wt}, or HA-TFG^{R106C} and grown in fed or starvation conditions (STV) in the presence or absence of CLQ for 1 h. Protein extracts were analysed by WB to detect HA-TFG ACTIN and LC3B-II as indicated. Densitometry analysis of LC3B-II over ACTIN is shown (bottom). All data are expressed as the mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparison test. **$P < 0.01$ ($n = 3$ independent experiments).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Appearance of LC3
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Appearance of LC3
Mean of DFCP1 duration
Mean of LC3 appearance
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D
TFG-WT
TFG-R106C
FED
STV
-CLQ
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Hoechst
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Average of autophagosomes number per cell
TFG-WT
TFG-R106C
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ACTIN
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LC3B-II/ACTIN ratio
EV
HA-TFGwt
HA-TFGR106C
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STV
CLQ

Figure EV4.

Figure EV5. TFG-LC3C interaction is important for ULK1 activity in autophagy induction.

A HeLa cells were co-transfected with untagged TFG and HA-tagged LC3A, LC3B or LC3C. After 1 h of starvation, protein extracts were immunoprecipitated by using an anti-TFG antibody or IgG as negative control. LC3 subfamily and TFG were analysed by WB.

B HeLa cells were nutrient-starved for the indicated time periods in the presence or absence of NH_4Cl for the indicated timepoints of starvation. TFG, ACTIN and LC3B-II (as indicator of NH_4Cl activity) were shown in the WB. Densitometry analysis of TFG over ACTIN protein levels was analysed in the graph (right). Data are expressed by mean ± SEM. Statistical analysis was obtained by two-way ANOVA followed by Tukey's multiple comparison test. ns, not significant ($n = 3$ independent experiments).

C TFG LIR motives, obtained from the autophagy database iLIR with their sequence and position, are shown.

D Schematic illustration of TFG wild type (TFG^{wtLIR}) and TFG mutants ($TFG^{mutLIR2}$ and $TFG^{mutLIR3}$) is shown. The aromatic F and the hydrophobic L residues of the TFG^{wtLIR} were changed into two alanine residues for both the TFG mutants.

E Pulldown assays were performed by using purified GST-GABARAP, GST-GABARAPL1 and GST-GABARAPL2 isoforms, each incubated together with HA-TFG^{wtLIR}- or HA-$TFG^{mutLIR3}$- or Myc-ULK1-transfected HeLa cell lysates, respectively. Myc-ULK1 was used as positive control. Ponceau staining reports GST-GABARAP isoforms and GST-GFP used as negative control. WB analyses show Myc-ULK1, HA-TFG^{wtLIR} and HA-$TFG^{mutLIR3}$. Densitometry analysis of HA-TFG normalized over GST-tagged GABARAP isoforms (Ponceau) was performed and reported (right). Data are mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparison test. ns, not significant ($n = 3$ independent experiments).

F HeLa cells were transfected with the indicated siRNA, and LC3C mRNA relative levels were analysed by qPCR. Values are expressed as mean ± SEM. Statistical analysis was performed by unpaired *t*-test. $^{****}P < 0.0001$ ($n = 3$ independent experiments).

G Pulldown assays were performed by using purified GST-LC3A, GST-LC3B and GST-LC3C isoforms, each incubated together with HA-TFG^{wtLIR}- or HA-$TFG^{mutLIR2}$- or HA-NDP52-transfected HeLa cell lysates, respectively. HA-NDP52 was used as positive control. Ponceau staining reports the indicated GST-LC3 isoforms and GST-GFP used as negative control. WB analyses show HA-NDP52, HA-TFG^{wtLIR} and HA-$TFG^{mutLIR2}$ in association with LC3A and LC3C (left) and HA-NDP52, HA-TFG^{wtLIR} and HA-$TFG^{mutLIR2}$ in association with LC3B (right) are shown. Densitometry analysis of HA-TFG normalized over GST-tagged LC3 isoforms (Ponceau) was performed and reported as indicated (right). All values are expressed as mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparison test. ns, not significant ($n = 3$ independent experiments).

H HeLa cells harbouring endogenously HA-tagged LC3C were transfected with HA-TFG^{wtLIR} or HA-$TFG^{mutLIR2}$. After 30' of starvation, cells were lysed, and protein extracts were immunoprecipitated by using an anti-TFG antibody or IgG as negative control. LC3C and TFG were analysed by WB.

I HeLa cells stably expressing GFP-ATG13 together with BFP-empty vector (EV) or BFP-TFG^{wtLIR} or BFP-$TFG^{mutLIR3}$ were co-transfected with mCherry-SEC16 together with siRNA targeting endogenous TFG (siTFG 3'UTR) or with a non-targeting control (siCTR). Endogenously interfered cells were used to perform live-cell imaging analysis (Fig 5I). WB analysis to detect endogenous or BFP-TFG protein levels is reported.

J HeLa cells stably expressing GFP-ATG13 together with BFP-TFG^{wtLIR} or BFP-$TFG^{mutLIR3}$ were transfected with siRNA targeting endogenous TFG (siTFG 3'UTR) or with a non-targeting control (siCTR) as indicated. Endogenously interfered cells were used to perform live-cell imaging analysis (Fig 5J). WB analysis to detect endogenous or BFP-TFG protein levels is reported.

K HeLa cells harbouring endogenously HA-tagged LC3C were starved for 30'. Protein extracts were immunoprecipitated by using an anti-HA antibody or IgG as negative control. ULK1, TFG and HA-LC3C protein levels were analysed by WB.

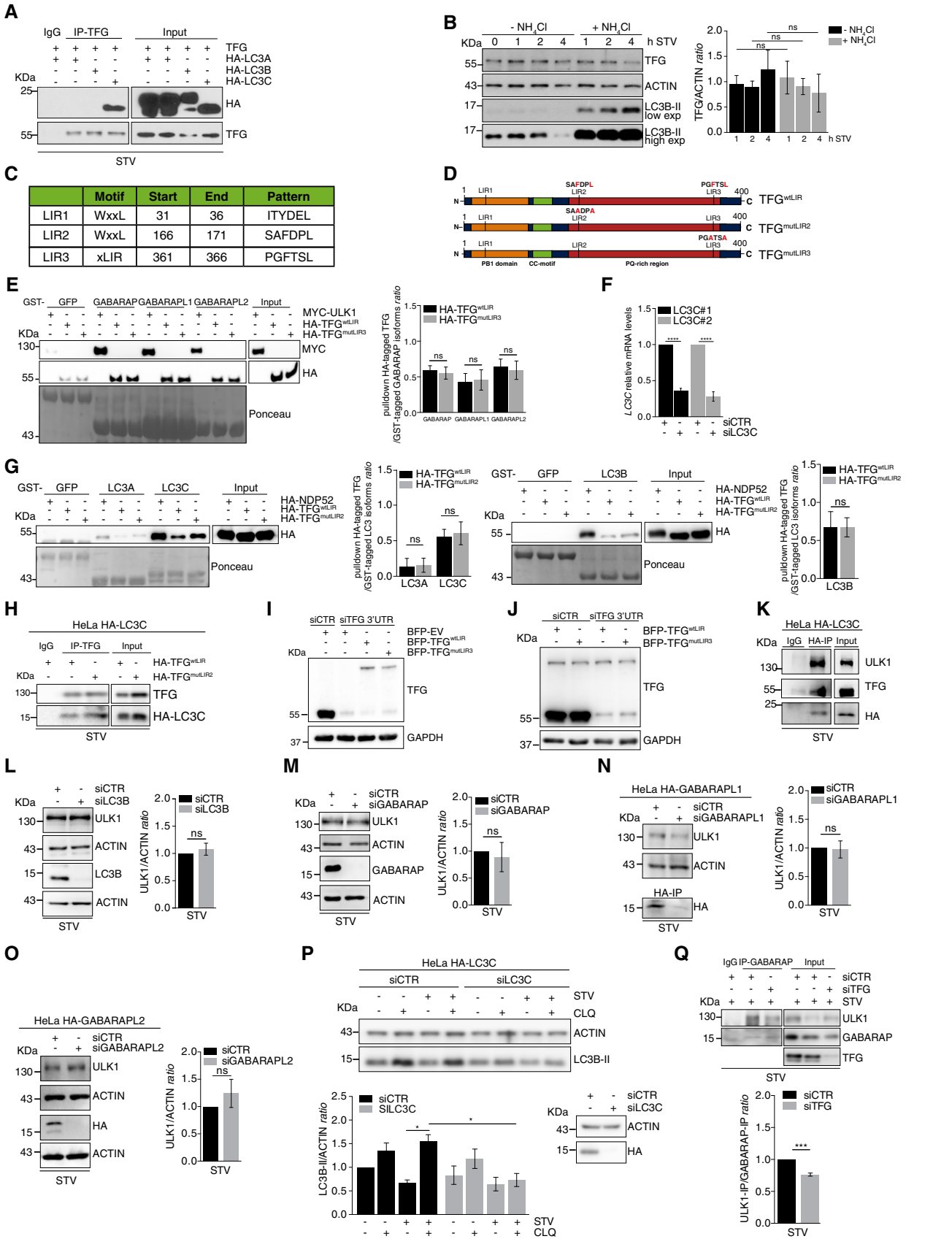
L, M HeLa cells were transfected with unrelated siRNA (siCTR) or siRNA targeting LC3B (siLC3B) (L) or GABARAP (siGABARAP) (M). (L-M) After 30' of starvation, cells were lysed and protein extracts were analysed by WB for indicated markers. ULK1 over actin is reported in the graphs (right). Data are shown as mean value ± SEM. Statistical analysis was performed by unpaired Student's *t*-test. ns, not significant ($n = 3$ independent experiments).

N HeLa cells harbouring endogenously HA-tagged GABARAPL1 were transfected with siRNA targeting GABARAPL1 or unrelated siRNA (siCTR). After 30' of starvation, cells were lysed. Part of protein extract was immunoprecipitated with anti-HA antibody to evaluate the downregulation of GABARAPL1. Remaining protein extract was analysed by WB as shown. ULK1 over ACTIN is also reported (right). Data are shown as mean value ± SEM. Statistical analysis was performed by unpaired Student's *t*-test. ns, not significant ($n = 3$ independent experiments).

O HeLa cells harbouring endogenously HA-tagged GABARAPL2 were transfected with siRNA targeting GABARAPL2 or unrelated siRNA (siCTR). Cells were starved for 30' and lysed. Protein extract was analysed by WB to detect the indicated markers. ULK1 over ACTIN is also reported (right). Data are as mean ± SEM. Statistical analysis was performed by unpaired Student's *t*-test. ns, not significant ($n = 3$ independent experiments).

P HeLa cells harbouring endogenously HA-tagged LC3C were transiently transfected with siLC3C or siCTR and grown in complete or starved (STV) media, in the presence or absence of CLQ for 1 h. LC3B-II and ACTIN were analysed by WB. Densitometry analysis of LC3B-II normalized over ACTIN is reported (bottom left). LC3C downregulation is also shown (bottom right). All data are reported as mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparison test. $^{*}P < 0.05$ ($n = 3$ independent experiments).

Q HeLa cells were co-transfected with indicated siRNAs and cultured in starvation medium for 30'. Cells were lysed, and protein extracts were immunoprecipitated by using an anti-GABARAP antibody or IgG as negative control. ULK1, GABARAP and TFG protein levels were analysed by WB. ULK1-IP over GABARAP-IP is reported in the graph (bottom). Data are mean ± SEM. Statistical analysis was performed by unpaired Student's *t*-test. $^{***}P < 0.001$ ($n = 3$ independent experiments).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siCTR
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Figure EV5.