Expanded View Figures

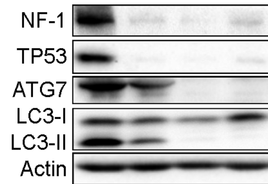

Figure EV1. Confirmation of CRISPR/Cas9-mediated gene editing and autophagy inhibition in cells.

Western blot analyses of glial cells infected with shRNA against *Nf-1* and *Tp53*, and then with Cas9 and sgRNAs against *Atg7*.

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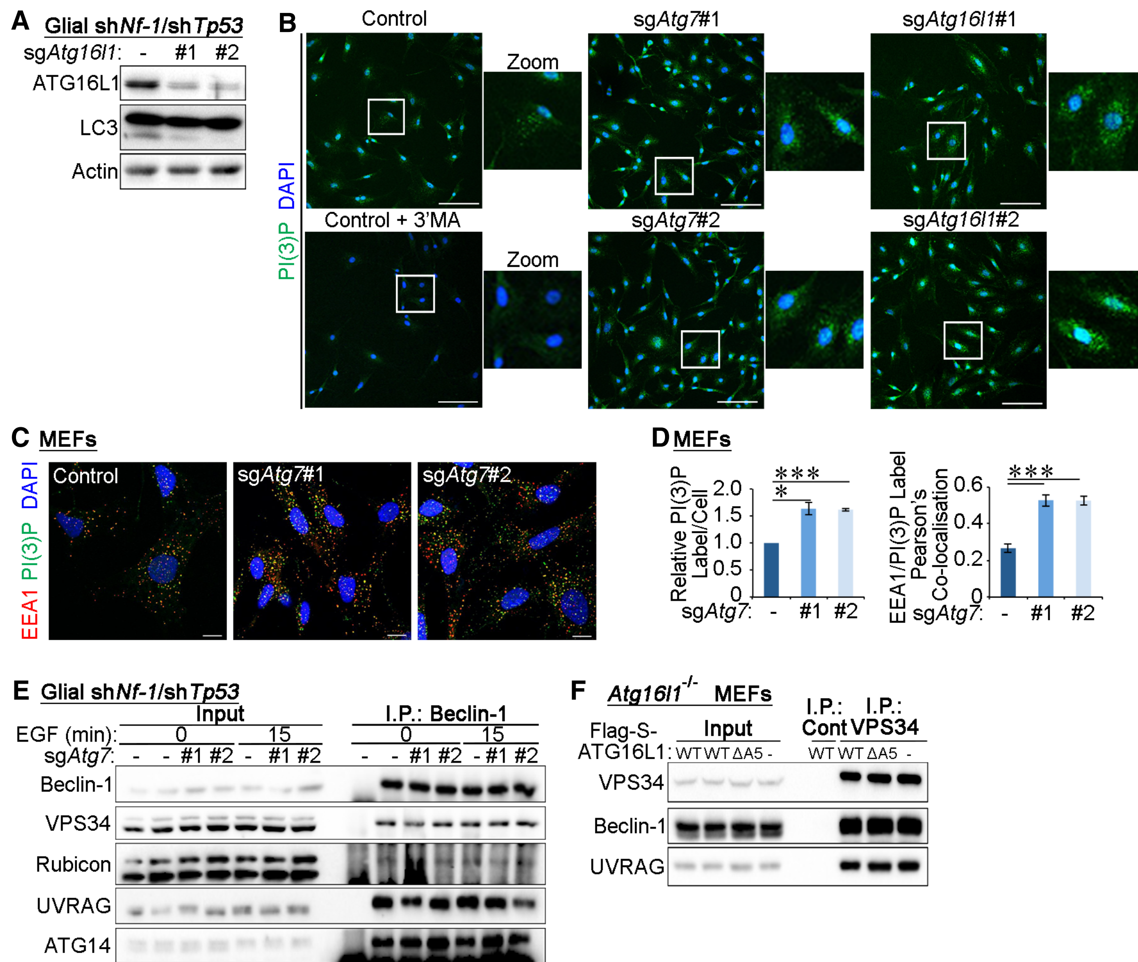

Figure EV2. Total PI(3)P levels increase in autophagy-inhibited cells.

All cells were serum starved for 4 h before assaying.

- A Western blot analyses of sh*Nf-1*/sh*Tp53* glial cells expressing gRNA sequences targeting *Atg16l1*.
- B 20× magnification of images in Fig 2A. Control, sg*Atg7*, or sg*Atg16l1* cells were treated with 2 ng/ml EGF for 15 min. Cells were then processed for staining using a PI(3)P probe (Alexa 488-labelled 2XFYVE domain). To ensure the specificity of the probe, control cells were pre-treated with 5 mM 3'MA for 30 min. Scale bar: 100 μm.
- C Control or sg*Atg7* MEF cells were treated with 2 ng/ml EGF for 15 min before fixation and staining using EEA1 antibodies and a PI(3)P probe (Alexa 488-labelled 2XFYVE domains). Scale bar: 10 μm.
- D Quantification of PI(3)P$^+$ vesicles per cell and the Pearson's colocalisation coefficient between PI(3)P and EEA1 (in C).
- E Endogenous Beclin-1 was immunoprecipitated from control and sg*Atg7* cells that were stimulated with 2 ng/ml EGF for 15 min. Cells were lysed in CHAPS buffer followed by immunoprecipitation of Beclin-1 and analyses by Western blotting.
- F *Atg16l1*$^{-/-}$ MEFs were reconstituted with wild type (WT) ATG16L1 or a mutant of ATG16L1 containing a deletion in the ATG5 binding domain (residues 1–39, ΔA5). Endogenous VPS34 was immunoprecipitated from these cells following 15 min of EGF (2 ng/ml). Binding partners were assessed by Western blotting.

Data information: Statistical analyses were performed on at least three independent experiments, where error bars represent SEM and *P* values represent a two-tailed Student's *t*-test: **P* < 0.05, ****P* < 0.001.

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Figure EV3. The canonical autophagy machinery is required for early endosome targeting.

A MEF cells stably expressing GFP-LC3 were serum starved for 4 h, then treated with 100 μM monensin for 1 h and stimulated with 2 ng/ml EGF for 15 min before immunofluorescence staining against EEA1. White arrows indicate colocalisation. Scale bar: 10 μm.

B Quantification of percentage of total EEA1 vesicles that colocalise with GFP-LC3 (in A).

C Box-and-whisker representation of the quantification of the number of EEA1-positive vesicles per cell in control and sg*Atg7* cell lines. No significant differences are observed in early endosome numbers between control and autophagy-inhibited cells (control versus sg*Atg7*#2: $P = 0.29$, control versus sg*Atg7*#3: $P = 0.43$).

D Glial sh*Nf-1*/sh*Tp53* control cells stably expressing Flag-S-ATG16L1 were either serum starved (-FBS) or treated with amino acid-free DMEM (-AA) for 4 h before immunofluorescence staining against Flag tag and EEA1. Scale bar: 10 μm.

E Glial sh*Nf-1*/sh*Tp53* control and sg*Atg7* cells stably expressing wild-type ATG16L1 (Flag-S-ATG16L1^{WT}) or LAP-deficient K490A mutant of ATG16L1 (Flag-S-ATG16L1^{K490A}) were serum starved for 4 h followed by stimulation with 2 ng/ml EGF for 15 min and immunofluorescence staining to detect Flag tag and EEA1. Scale bar: 10 μm.

F Quantification of percentage of total EEA1 vesicles positive for Flag-S-ATG16L1 (in E) with quantification of endogenous ATG16L1 included (see Fig 4E).

G *Atg16l1*$^{-/-}$ MEF cells stably expressing wild-type (Flag-S-ATG16L1^{WT}) or LAP-deficient mutant (Flag-S-ATG16L1^{K490A}) of ATG16L1 were serum starved for 4 h followed by stimulation with 2 ng/ml EGF for 15 min and immunofluorescence staining to detect Flag tag and EEA1. White arrows indicate colocalisation. Scale bar: 10 μm.

H Quantification of the percentage of total EEA1 vesicles that stain positive for wild-type or the K490A point mutant of ATG16L1 expressed in *Atg16l1*$^{-/-}$ MEFs (in G).

I *Atg13*$^{-/-}$ MEF cells were treated for 1 h with 100 μM monensin and then stimulated with 2 ng/ml EGF for 15 min. ATG16L1 and EEA1 were then detected by immunofluorescence staining. White arrows indicate colocalisation. Scale bar: 10 μm.

J Quantification of percentage of total EEA1 vesicles positive for Flag-S-ATG16L1 (in I).

Data information: Statistical analyses were performed on at least three independent experiments, where error bars represent SEM and P values represent a two-tailed Student's t-test: NS $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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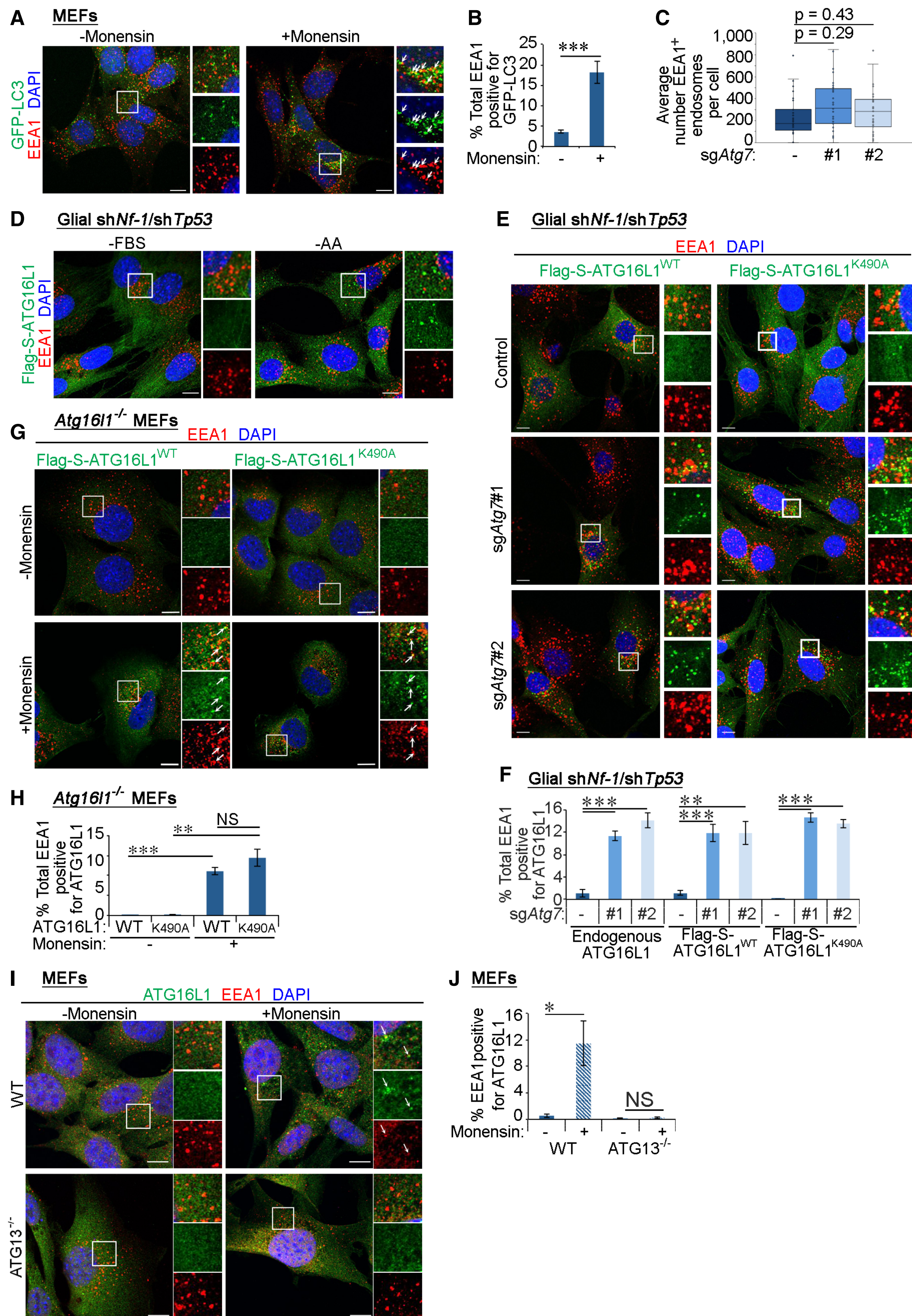

Figure EV3.

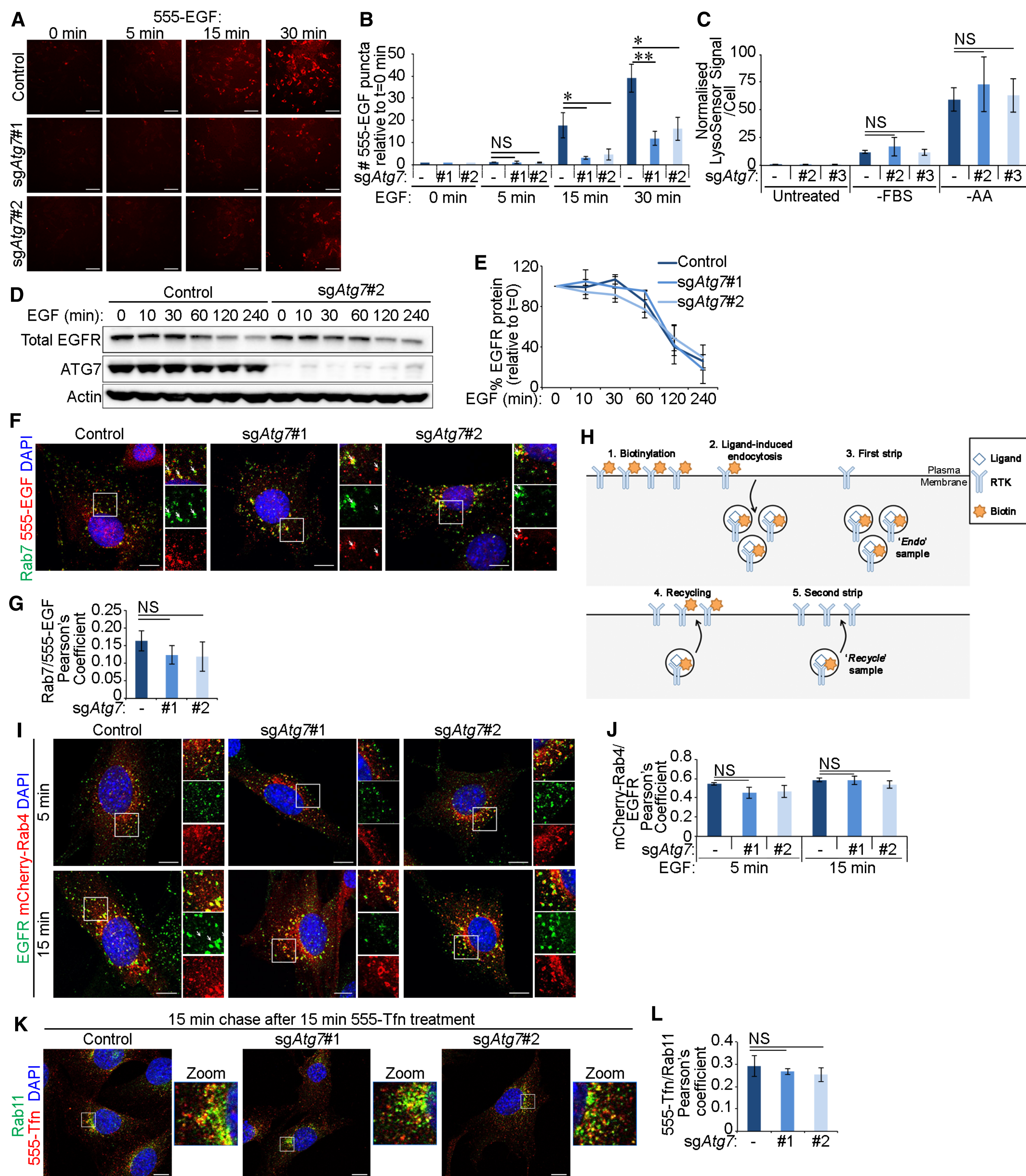

Figure EV4.

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Figure EV4. Targeting of EGFR to late endosomes and Rab4^{+} recycling endosomes remain intact in the absence of autophagy.

The following experiments were performed in sh*Nf-1*/sh*Tp53* glial cells serum starved for 4 h before assaying. Cells were expressing either Cas9 alone (control) or Cas9 and sgRNA targeting *Atg7* (sg*Atg7* #1 or #2).

A Cells were treated with 20 ng/ml Alexa 555-EGF (555-EGF) for the indicated times before fixation. 555-EGF was detected using the ImageXpress high-content imaging platform, and representative images of 555-EGF are shown. Scale bar: 100 μm.
B Following high-content imaging of 555-EGF and DAPI, 555-EGF uptake relative to cell number was quantified and made relative in each cell line to background readings from unstimulated cells (t = 0 min) (in A).
C Control or sg*Atg7* glial sh*Nf-1*/sh*Tp53* cells were either untreated, serum starved (-FBS) for 4 h, or amino acid starved (-AA) for 2 h and LysoSensor probe added for 1 h. Shown are quantifications of LysoSensor fluorescence intensity per cell normalised to untreated control cells.
D Cells were stimulated with 20 ng/ml EGF for the indicated times before EGFR levels were analysed by Western blotting.
E Densitometry analyses of Western blotting showing EGFR degradation with measurements made relative to EGFR levels in unstimulated cells (t = 0 min) (in D).
F Cells were stimulated with 20 ng/ml 555-EGF for 15 min before immunofluorescence staining against endogenous Rab7. White arrows indicate colocalisation. Scale bar: 10 μm.
G Quantification of Pearson's colocalisation coefficient between 555-EGF and Rab7 (in F).
H Schematic representation of cell surface protein biotinylation assay to assess endocytosis and recycling rates (see Fig 6C and D).
I Cells stably expressing mCherry-Rab4 were stimulated with 20 ng/ml EGF for 5 or 15 min and then fixed and stained by immunofluorescence against EGFR. White arrows indicate colocalisation. Scale bar: 10 μm.
J Quantification of Pearson's colocalisation coefficient between mCherry-Rab4 and EGFR (in I).
K Cells were stimulated with Alexa 555-transferrin (555-Tfn, 20 ng/ml) for 15 min and chased with media without transferrin for 15 min. Cells were then stained by immunofluorescence against Rab11. Scale bar: 10 μm.
L Quantification of Pearson's colocalisation coefficient between 555-Tfn and Rab11 (in K).

Data information: Statistical analyses were performed on at least three independent experiments, where error bars represent SEM and P values represent a two-tailed Student's t-test: NS $P > 0.05$, *$P < 0.05$, **$P < 0.01$.

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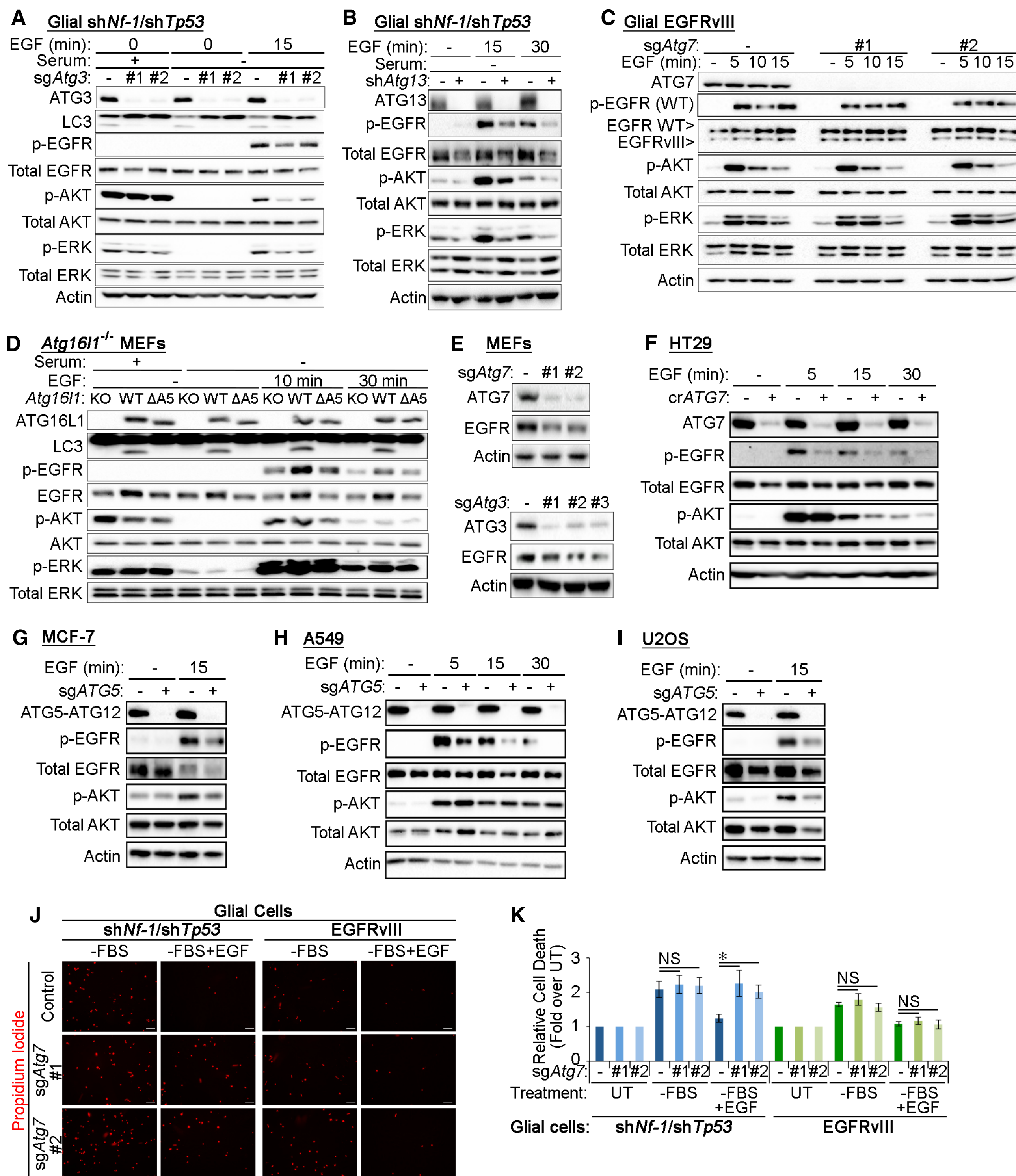

Figure EV5.

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Figure EV5. Analyses of EGFR signalling and levels in various cell lines upon autophagy inhibition.

A Glial sh*Nf-1*/sh*Tp53* sg*Atg3* cells were either left untreated (+serum) or serum starved for 4 h and then stimulated with 2 ng/ml EGF for 15 min before analysis by Western blotting.

B Glial sh*Nf-1*/sh*Tp53* sh*Atg13* cells were serum starved for 4 h and then stimulated with 2 ng/ml EGF for 15 or 30 min before analysis by Western blotting.

C Glial EGFRvIII-expressing control and sg*Atg7* cells were serum starved for 4 h and then stimulated with 2 ng/ml EGF before analysis by Western blotting.

D *Atg16l1*$^{-/-}$ MEFs were reconstituted with wild-type (WT) ATG16L1 or with a mutant of ATG16L1 with deletion of the ATG5-binding domain (residues 1-39, ΔA5). Cells were either untreated or serum starved for 4 h and then stimulated with 2 ng/ml EGF for 10 or 30 min before signalling pathway analyses by Western blotting.

E Total EGFR levels in untreated sg*Atg7* and sg*Atg3* MEFs were analysed by Western blotting.

F–I The following cells were serum starved for 4 h followed by stimulation with 2 ng/ml EGF before Western blot analyses: HT29 control and cr*ATG7* (F); MCF-7 control and sg*ATG5* (G); A549 control and sg*ATG5* (H); and U2OS control and sg*ATG5* (I).

J Representative images of propidium iodide staining in control or sg*Atg7* glial sh*Nf-1*/sh*Tp53* or EGFRvIII-expressing cells that were serum starved for 24 h in the presence or absence of 20 ng/ml EGF. Scale bar: 100 μm.

K Quantification of the experiment in J with propidium iodide relative fluorescence units (RFU) made relative to cell number as assessed by DAPI RFU measured by a fluorescence plate reader. These values were then made relative to the values in the negative control setting (untreated cells, UT).

Data information: Statistical analyses were performed on at least three independent experiments, where error bars represent SEM and *P* values represent a two-tailed Student's *t*-test: NS $P > 0.05$, *$P < 0.05$.

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