Expanded View Figures

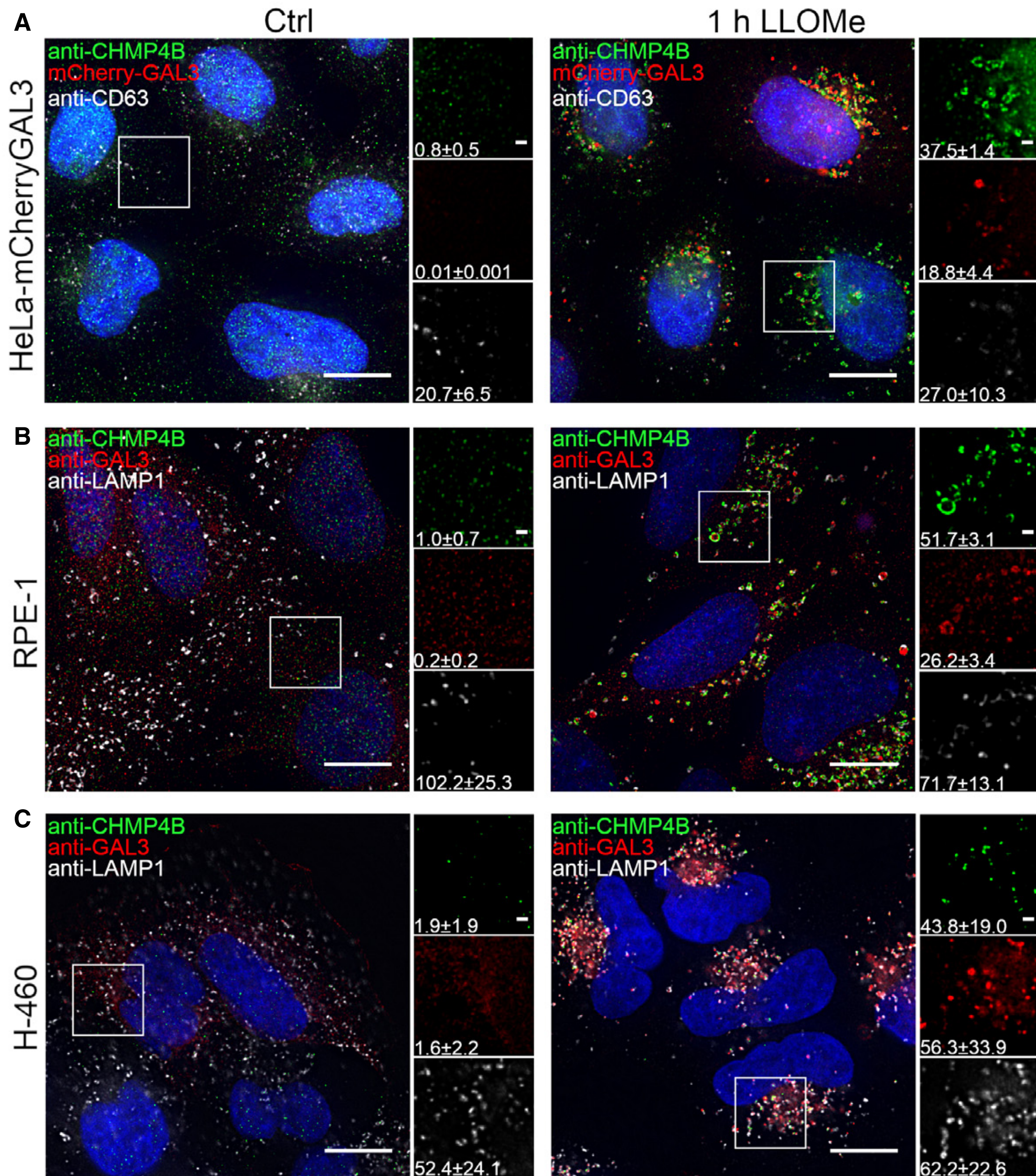

Figure EV1. CHMP4B is recruited to Galectin-3-positive damaged endolysosomal membranes in various cell lines.

A Representative fluorescence images of a HeLa-mCherry-Galectin-3 stable cell line treated with 250 μM lysosomotropic drug LLOMe for 1 h, fixed and labeled with CD63 antibody. To determine the statistical significance, the number of CHMP4B, GAL3, and CD63 foci per cell observed after LLOMe treatment was compared to Ctrl using Student's *t*-test. *P*-values for CHMP4B, GAL3, and CD63 are as follows: $P = 0.000001$, $P = 0.0017$, $P = 0.4160$, respectively.

B Experimental setup as in (A) where RPE-1 cells are labeled with CHMP4B, Galectin-3, and LAMP1 antibodies. *P*-values for CHMP4B, GAL3, and LAMP1 foci per cell (Ctrl versus LLOMe treatment): $P = 0.00001$, $P = 0.00018$, $P = 0.1368$ (Student's *t*-test), respectively.

C Representative images of fixed H-460 cells after 1-h treatment with 250 μM LLOMe. Cells were stained for endogenous CHMP4B, GAL3, and LAMP1. CHMP4B, GAL3, and LAMP1 foci per cell (Ctrl versus LLOMe treatment): $P = 0.0192$, $P = 0.0493$, $P = 0.6333$ (Student's *t*-test), respectively.

Data information: For all conditions, the number of foci per cell was quantified from >70 cells per condition from three independent experiments. Data are presented as mean ± SD. Scale bars: 10 and 1 μm (inset).

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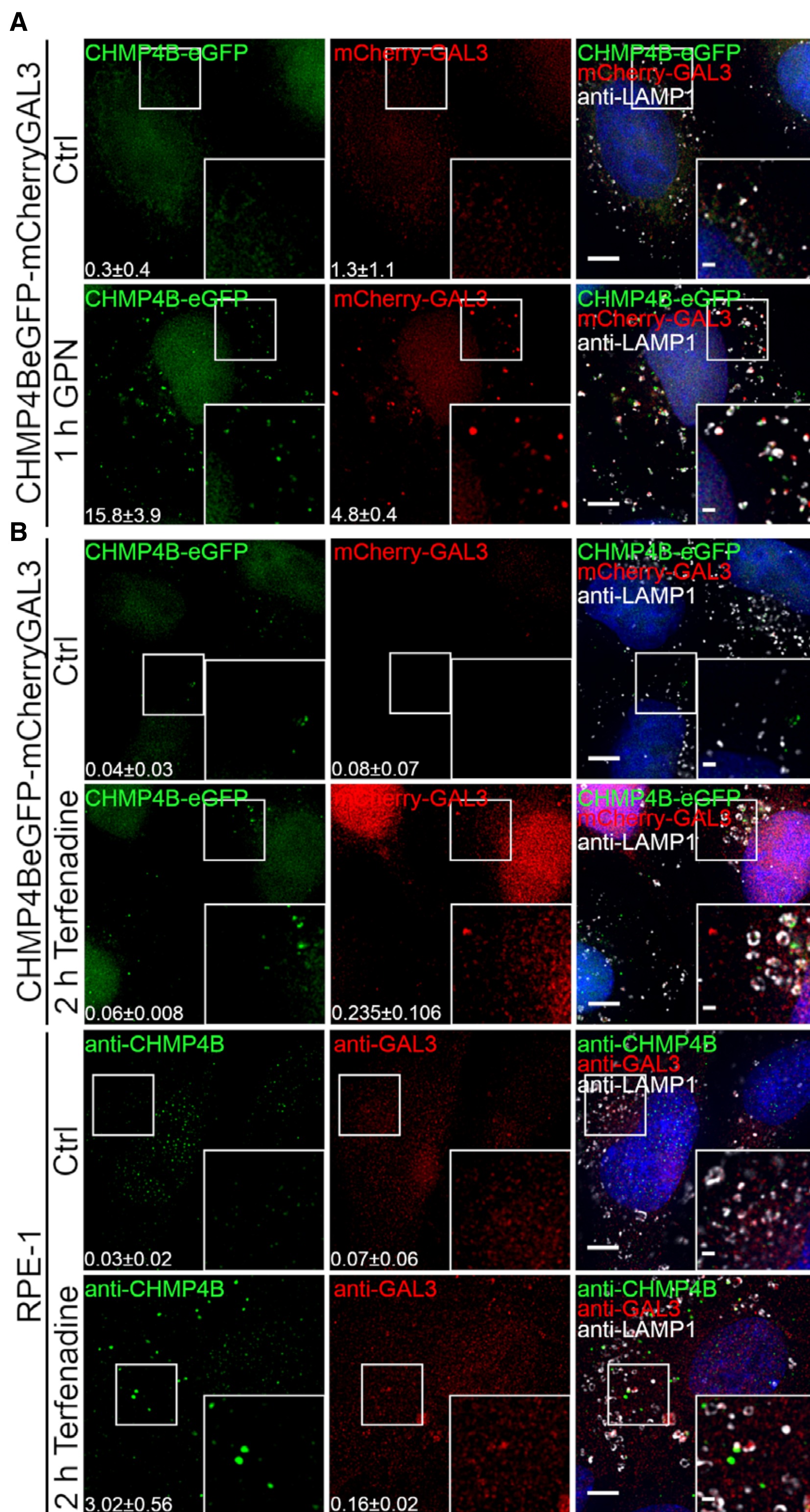

Figure EV2. CHMP4B is recruited to damaged endolysosomal membranes after treatment with different agents.

A HeLa cells stably expressing CHMP4B-eGFP and mCherry-Galectin-3 were treated with 250 µM lysosomotropic drug GPN for 1 h, fixed and imaged. Representative images showing recruitment of CHMP4B to damaged endolysosomal membranes are presented. Number of foci per cell (>90 cells per condition from four independent experiments) was quantified and is indicated as mean ± SD. *P*-values for comparisons CHMP4B, GAL3, and LAMP1 foci per cell (Ctrl versus GPN treatment) are as follows: $P = 0.0002$, $P = 0.0008$, $P = 0.1113$ (Student's *t*-test), respectively.

B Representative fluorescence images showing recruitment of CHMP4B to damaged membranes in HeLa cells stably expressing CHMP4B-eGFP and mCherry-Galectin-3 and RPE-1 cells after 2-h treatment with 7 µm cationic amphiphilic drug terfenadine. In HeLa cells, there is a slight but not significant increase in CHMP4B-positive foci. On the other hand, RPE-1 cells labeled with CHMP4B, GAL3, and LAMP1 antibodies have elevated number of CHMP4 and GAL3 foci per cell. For HeLa-CHMP4BeGFP-mCherry-Galectin-3: CHMP4B, GAL3 foci per cell (Ctrl versus terfenadine treatment): $P = 0.2679$, $P = 0.2266$ (Student's *t*-test), respectively. For RPE-1 cell line: CHMP4B, GAL3 foci per cell (Ctrl versus terfenadine treatment): $P = 0.0169$, $P = 0.1806$ (Student's *t*-test), respectively. Number of foci per cell was quantified from >75 cells per condition from two independent experiments and is indicated in the figure as mean ± SD.

Data information: Scale bars: 5 µm and 1 µm (inset).

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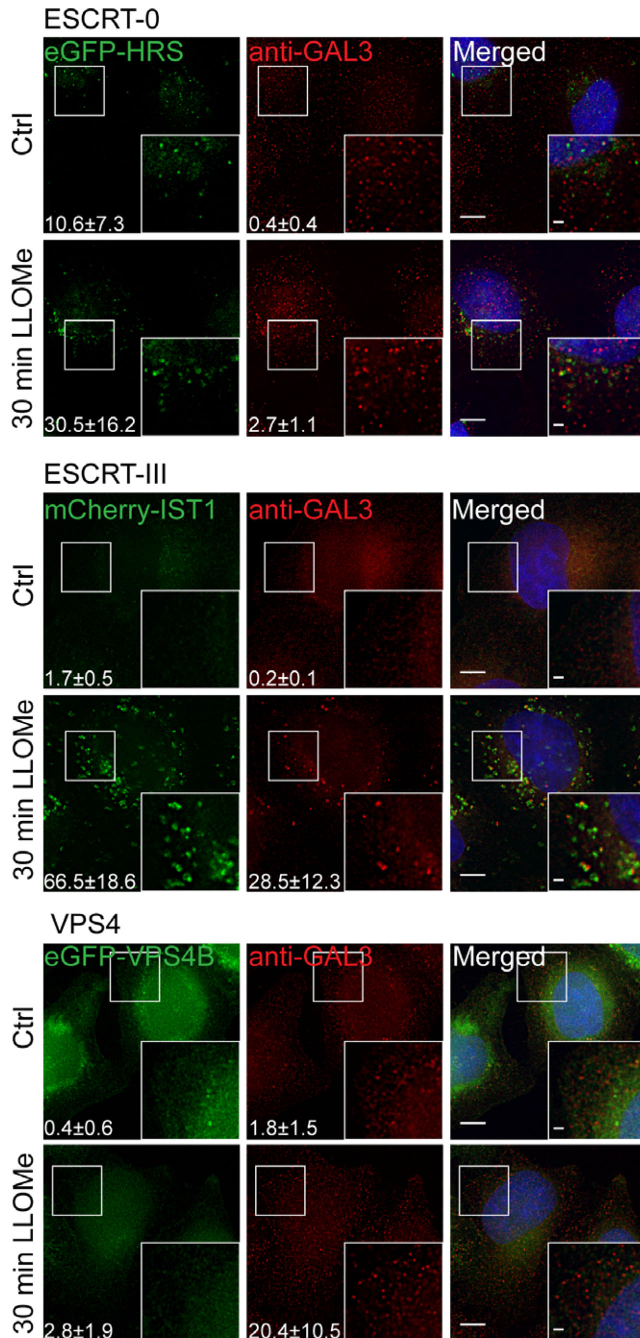

Figure EV3. Screening for additional ESCRT proteins that are involved in the lysosomal repair process.

ESCRT-0: To clarify the role of HRS, a cell line stably expressing eGFP-HRS was incubated with 250 μm LLOMe for 30 min, pre-permeabilized with PEM buffer (for details see Materials and Methods), and fixed. HRS shows no significant changes upon lysosomal damage when compared to untreated cells. ESCRT-III: After treatment with 250 μm LLOMe for 30 min, HeLa cells stably expressing mCherry-IST1 or eGFP-VPS4B were fixed and imaged. As shown in the representative fluorescence images, IST1 is recruited to damaged lysosomes whereas VPS4B shows no change in localization upon endolysosomal membrane damage. Number of foci per cell was quantified and is indicated as mean ± SD. Data were obtained from >71 cells per condition from three independent experiments. Number of foci per cell (Ctrl versus LLOMe treatment): HRS $P = 0.1241$, GAL3 $P = 0.0309$ (Student's *t*-test); IST1 $P = 0.0038$, GAL3 $P = 0.0164$ (Student's *t*-test); VPS4B $P = 0.1102$, GAL3 $P = 0.0387$ (Student's *t*-test). Scale bars: 5 μm and 1 μm (inset).

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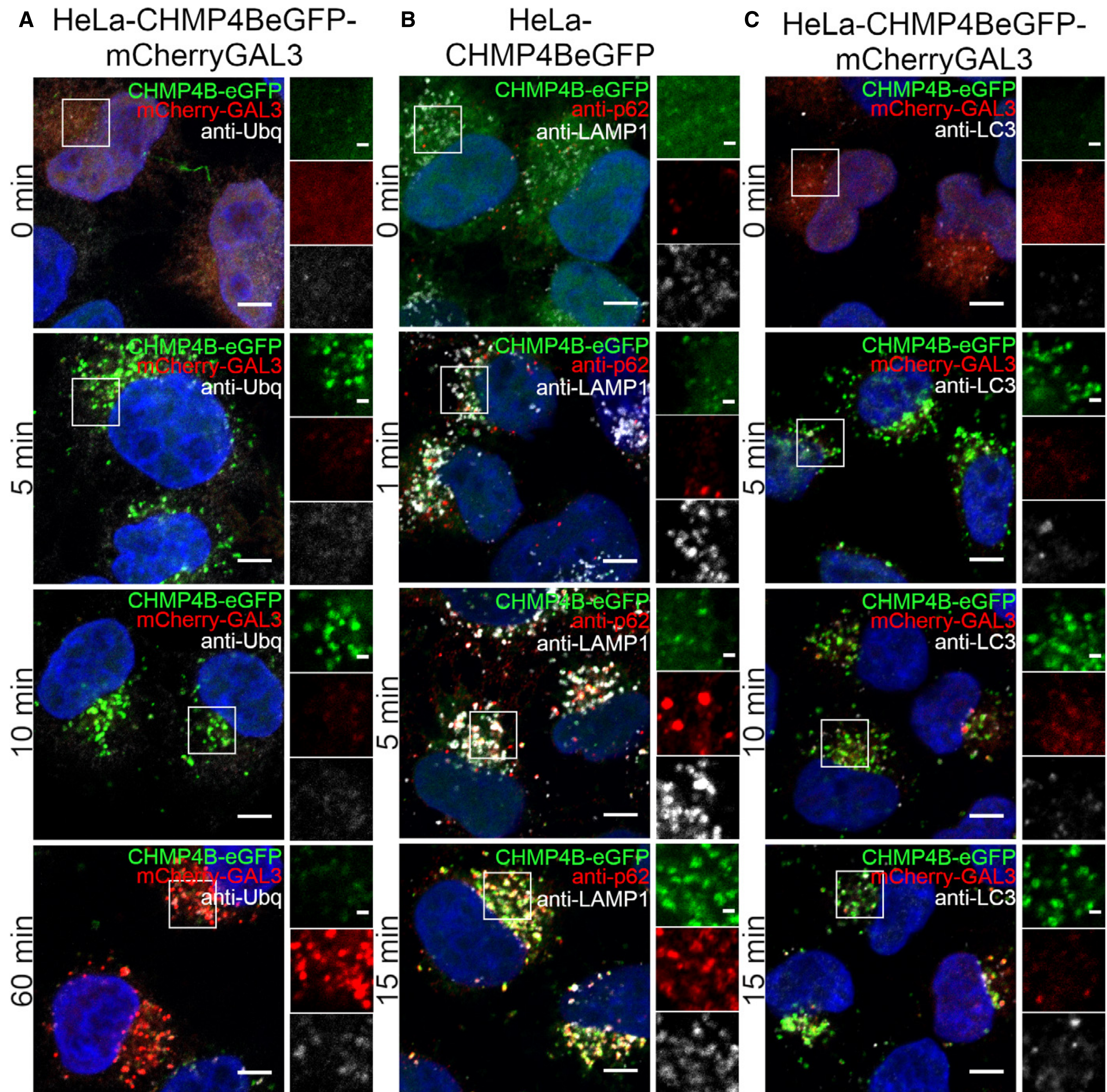

Figure EV4. Representative confocal images corresponding to Fig 6.

A Recruitment of CHMP4B as early as 5 min following LLOMe treatment whereas ubiquitin appears to be recruited later.

B, C Recruitment of CHMP4B to damaged endolysosomal membranes precedes p62 and LC3. In addition, p62 is recruited before LC3.

Data information: Scale bars: 5 and 1 μm (inset).

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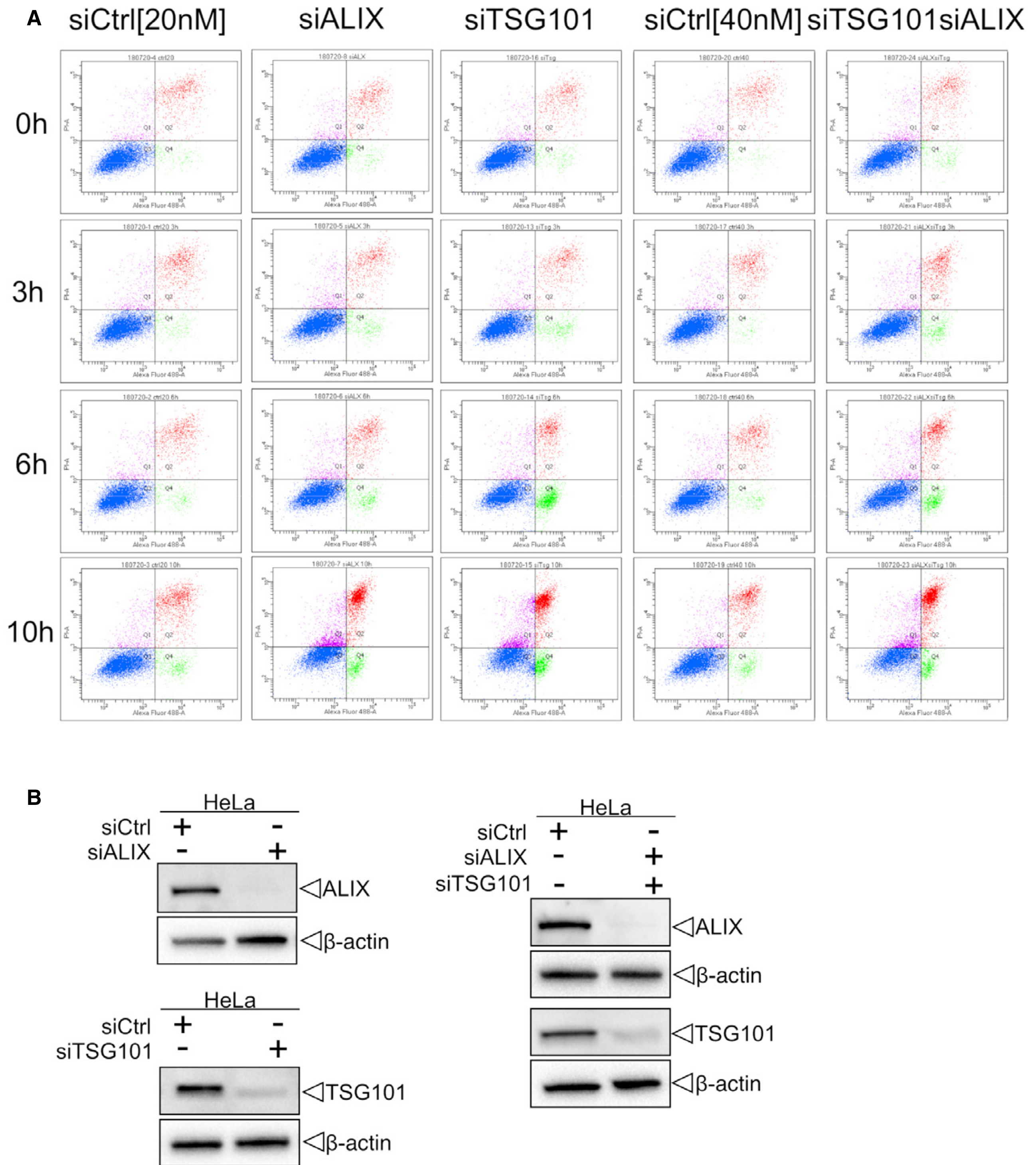

Figure EV5. ESCRTs are essential for cell viability after endolysosomal damage.

A Cells were transfected with control (siCtrl), TSG101, ALIX, or both siRNAs. Forty-eight hours after transfection, cells were harvested and profiled with flow cytometry. Dot plots and gating strategy of one out of three experiments are shown.

B HeLa cells were transfected with control (siCtrl), ALIX, TSG101, or both siRNA. Efficient depletion was confirmed by Western blot analysis. β-actin was used as loading control.

Source data are available online for this figure.

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