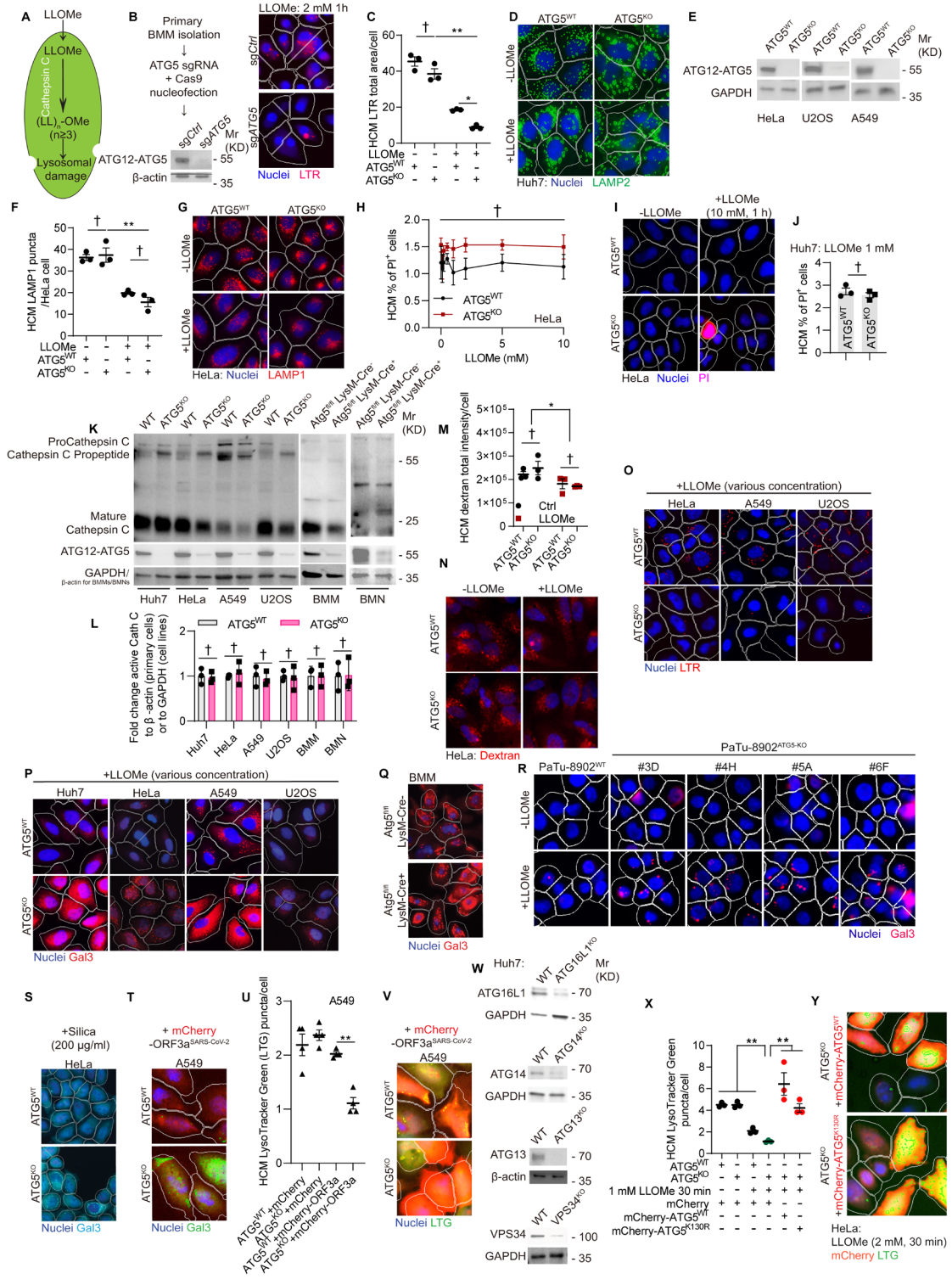
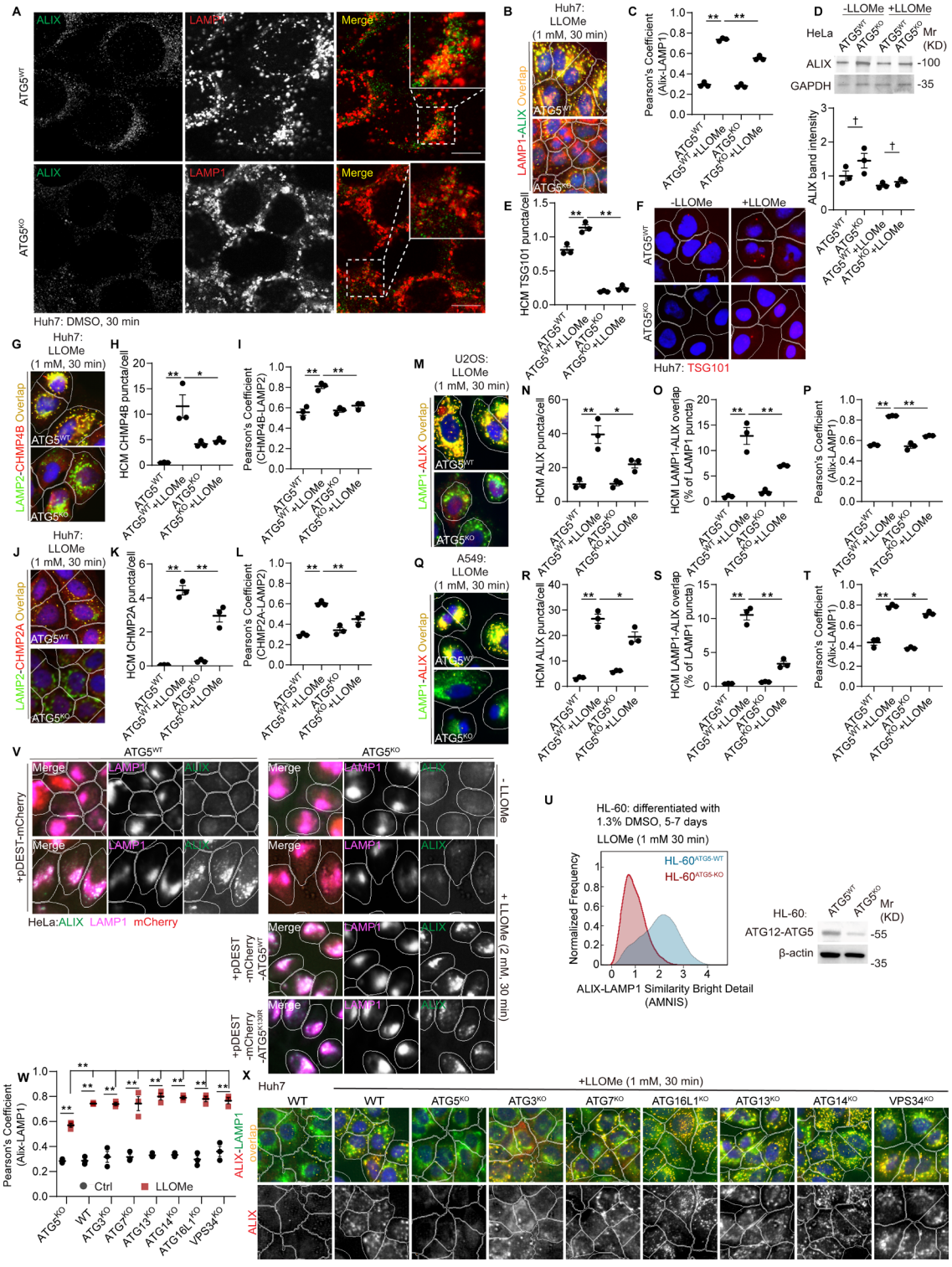


Supplementary Figure 1. Loss of ATG5 but not of other ATGs renders lysosomes excessively susceptible to damage continued (related to Figure 1)

A, Schematic for LLOMe mechanism. **B,** Schematic and immunoblot of CRISPR knockout of ATG5 in primary BMMs, and representative images of LTR puncta following 2 mM LLOMe incubation for 1 hour. **C,** HCM quantification of LTR area in Huh7$^{ATG5\text{-}WT}$ and Huh7$^{ATG5\text{-}KO}$ cells treated with or without 1 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **D,**

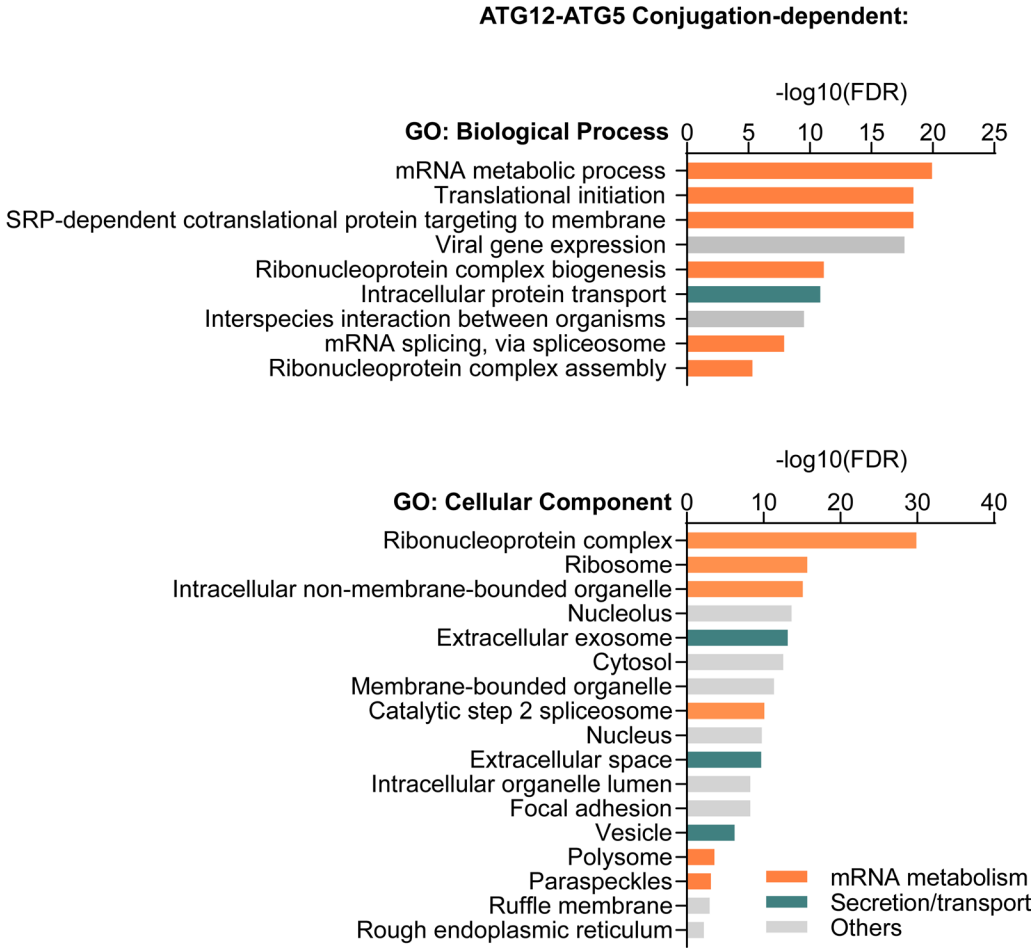
Representative image of LAMP2 puncta in Huh7$^{ATG5\text{-}WT}$ and Huh7$^{ATG5\text{-}KO}$ cells treated with or without 1 mM LLOMe for 30 min. **E,** Immunoblot of ATG5 knockout in U2OS, HeLa, and A549 cells. **F,** HCM quantification and **G,** representative images of LAMP1 puncta in HeLa$^{ATG5\text{-}WT}$ and HeLa$^{ATG5\text{-}KO}$ cells treated with or without 2 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **H,** HCM quantification and **I,** representative images % PI^{+} cells in HeLa$^{ATG5\text{-}WT}$ and HeLa$^{ATG5\text{-}KO}$ cells treated with indicated concentration of LLOMe for 1 h. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **J,** HCM quantification of % PI^{+} cells in Huh7$^{ATG5\text{-}WT}$ and Huh7$^{ATG5\text{-}KO}$ cells treated with 1 mM LLOMe for 1 h. Data, means ± SE (n=3); unpaired *t*-test. **K,** Immunoblot and quantification (**L**) of Cathepsin C expression in ATG5^{WT} and ATG5^{KO} cells from indicated cell types. **M**, HCM quantification and representative images (**N**) of dextran TexasRed 3K intensity in HeLa$^{ATG5\text{-}WT}$ and HeLa$^{ATG5\text{-}KO}$ cells treated with or without 2 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **O,** Representative images of LTR puncta in ATG5^{WT} and ATG5^{KO} cells of indicated cell types treated with LLOMe for 30 min. **P,** Representative images of Gal3 puncta in ATG5^{WT} and ATG5^{KO} cells of indicated cell types treated with LLOMe for 30 min. **Q,** Representative images of Gal3 puncta in Atg5$^{fl/fl}$ LysM-Cre^{-} and Atg5$^{fl/fl}$ LysM-Cre^{+} BMMs treated with LLOMe for 30 min. **R,** Representative images of Gal3 puncta in PaTu-8902^{WT} and PaTu-8902$^{ATG5\text{-}KO}$ cells treated with or without 1.5 mM LLOMe for 30 min. **S,** Representative images of Gal3 puncta in HeLa$^{ATG5\text{-}WT}$ and HeLa$^{ATG5\text{-}KO}$ cells treated with 200 µg/mL silica crystals. **T,** Representative images of Gal3 puncta in A549$^{ATG5\text{-}WT}$ and A549$^{ATG5\text{-}KO}$ cells transfected with mCherry-ORF3a$^{SARS\text{-}CoV\text{-}2}$. **U,** HCM quantification and representative images (**V**) of Gal3 puncta in A549$^{ATG5\text{-}WT}$ and A549$^{ATG5\text{-}KO}$ cells transfected with mCherry-ORF3a$^{SARS\text{-}CoV\text{-}2}$. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **W,** Immunoblot of CRISPR knockout of ATG16L1, ATG14, ATG13, and VPS34 in Huh7 cells. **X,** HCM quantification and **Y,** representative images of LysoTracker Green (LTG) puncta in HeLa$^{ATG5\text{-}WT}$ and HeLa$^{ATG5\text{-}KO}$ cells overexpressing or not overexpressing mCherry-ATG5^{WT} or mCherry-ATG5^{K130R} treated with or without 2 mM LLOMe for 30 min. Data, means ± SE (n=3); unpaired *t*-test. In all HCM graphs, each individual data point represents independent biological replicate, with values based on 49-64 fields/well, >500 primary objects (cells) per well, 5 wells per sample (used only for sampling error) with bars denoting means for biological replicates and standard errors of the mean. Statistical significance symbols in all panels, †p≥0.05, *p<0.05, **p<0.01.



Supplementary Figure 2. ATG5 is required for recruitment of ESCRTs to repair damaged lysosomes continued (related to Figure 2)

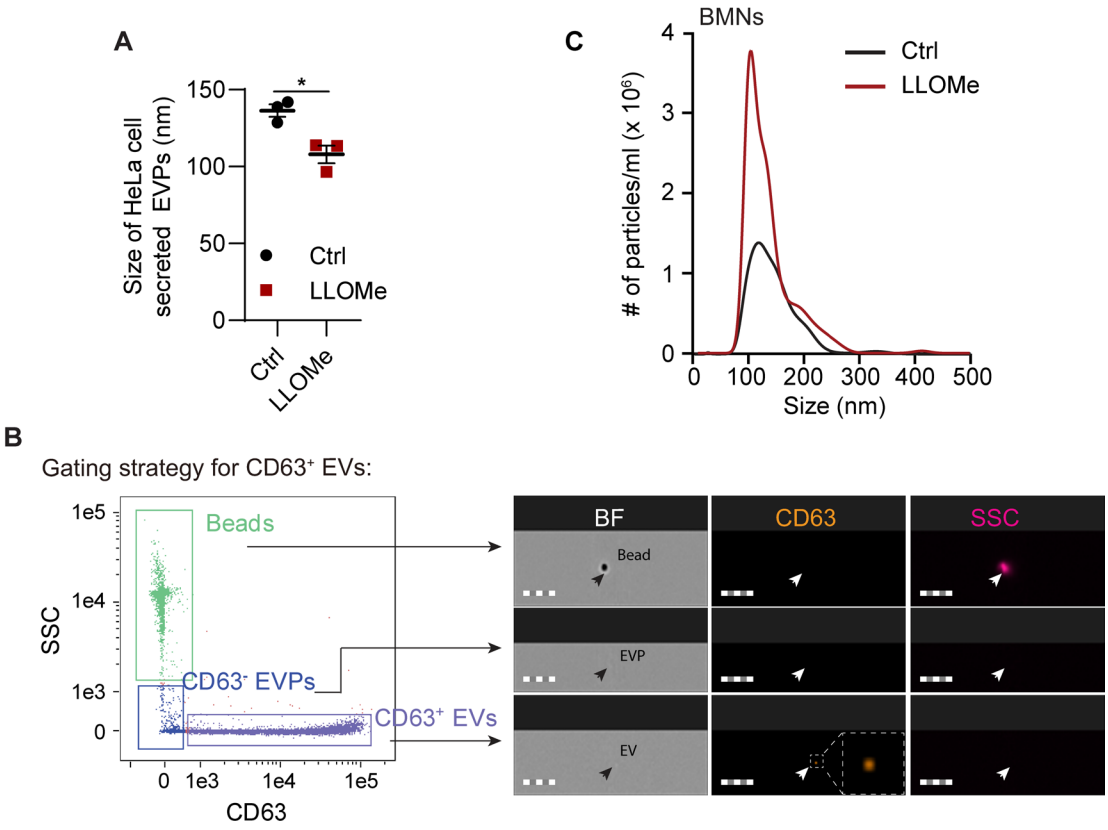
A, Representative confocal images of ALIX and LAMP1 in Huh7$^{ATG5\text{-}WT}$ and Huh7$^{ATG5\text{-}KO}$ cells treated with DMSO for 30 min. Scale bars, 10 μm. **B,** Representative HCM images of ALIX-LAMP1 overlap in Huh7$^{ATG5\text{-}WT}$ and Huh7$^{ATG5\text{-}KO}$ cells treated with 1 mM LLOMe for 30 min. **C,** HCM quantification of Pearson's coefficients of ALIX-LAMP1 in Huh7$^{ATG5\text{-}WT}$ and Huh7$^{ATG5\text{-}KO}$ cells treated with or without 1 mM LLOMe for

30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **D,** Representative immunoblot images and quantification of ALIX in HeLa^{WT} and HeLa$^{ATG5\text{-}KO}$ cells treated with or without 2 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **E,** HCM quantification and **F,** representative images of TSG101 puncta in Huh7$^{ATG5\text{-}WT}$ and Huh7$^{ATG5\text{-}KO}$ cells treated with or without 1 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **G,** Representative images and HCM quantification of CHMP4B puncta **(H)** and Pearson's coefficients of LAMP2-CHMP4B **(I)** in Huh7$^{ATG5\text{-}WT}$ and Huh7$^{ATG5\text{-}KO}$ cells treated with or without 1 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **J,** Representative images and HCM quantification of CHMP2A puncta **(K)** and Pearson's coefficients of LAMP2-CHMP2A **(L)** in Huh7$^{ATG5\text{-}WT}$ and Huh7$^{ATG5\text{-}KO}$ cells treated with or without 1 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **M-P,** Representative images (**M**) and HCM quantification of ALIX puncta (**N**), LAMP1-ALIX overlap (**O**), and Pearson's coefficients of LAMP1-ALIX (**P**) in U2OS$^{ATG5\text{-}WT}$ and U2OS$^{ATG5\text{-}KO}$ cells treated with or without 1 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **Q,** Representative images and HCM quantification of ALIX puncta (**R**), LAMP1-ALIX overlap (**S**), and Pearson's coefficients of LAMP1-ALIX (**T**) in A549$^{ATG5\text{-}WT}$ and A549$^{ATG5\text{-}KO}$ cells treated with or without 1 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **U,** Representative images of ALIX-LAMP1 similarity bright detail (top) and immunoblot of ATG5 expression (bottom) in HL-60$^{ATG5\text{-}WT}$ and HL-60$^{ATG5\text{-}KO}$ cells incubated with 1 mM LLOMe for 30 min. **V,** Representative images of ALIX puncta and ALIX-LAMP1 overlap in HeLa$^{ATG5\text{-}WT}$ and HeLa$^{ATG5\text{-}KO}$ cells transfected with mCherry, mCherry-ATG5^{WT}, or mCherry-ATG5^{K130R} followed by incubation with or without 2 mM LLOMe for 30 min. **W,** Pearson's coefficients of LAMP1-ALIX and representative images (**X**) of ALIX puncta and ALIX-LAMP1 overlap in Huh7$^{ATG5\text{-}WT}$, Huh7$^{ATG5\text{-}KO}$, Huh7$^{ATG3\text{-}KO}$, Huh7$^{ATG7\text{-}KO}$, Huh7$^{ATG13\text{-}KO}$, Huh7$^{ATG14\text{-}KO}$, Huh7$^{ATG16L1\text{-}KO}$ and Huh7$^{VPS34\text{-}KO}$ cells incubation with 1 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. In all HCM graphs, each individual data point represents an independent biological replicate, with values based on 49-64 fields/well, >500 primary objects (cells) per well, 5 wells per sample (used only for sampling error) with bars denoting means for biological replicates and standard errors of the mean. Statistical significance symbols in all panels, †$p \geq 0.05$, *$p < 0.05$, **$p < 0.01$.



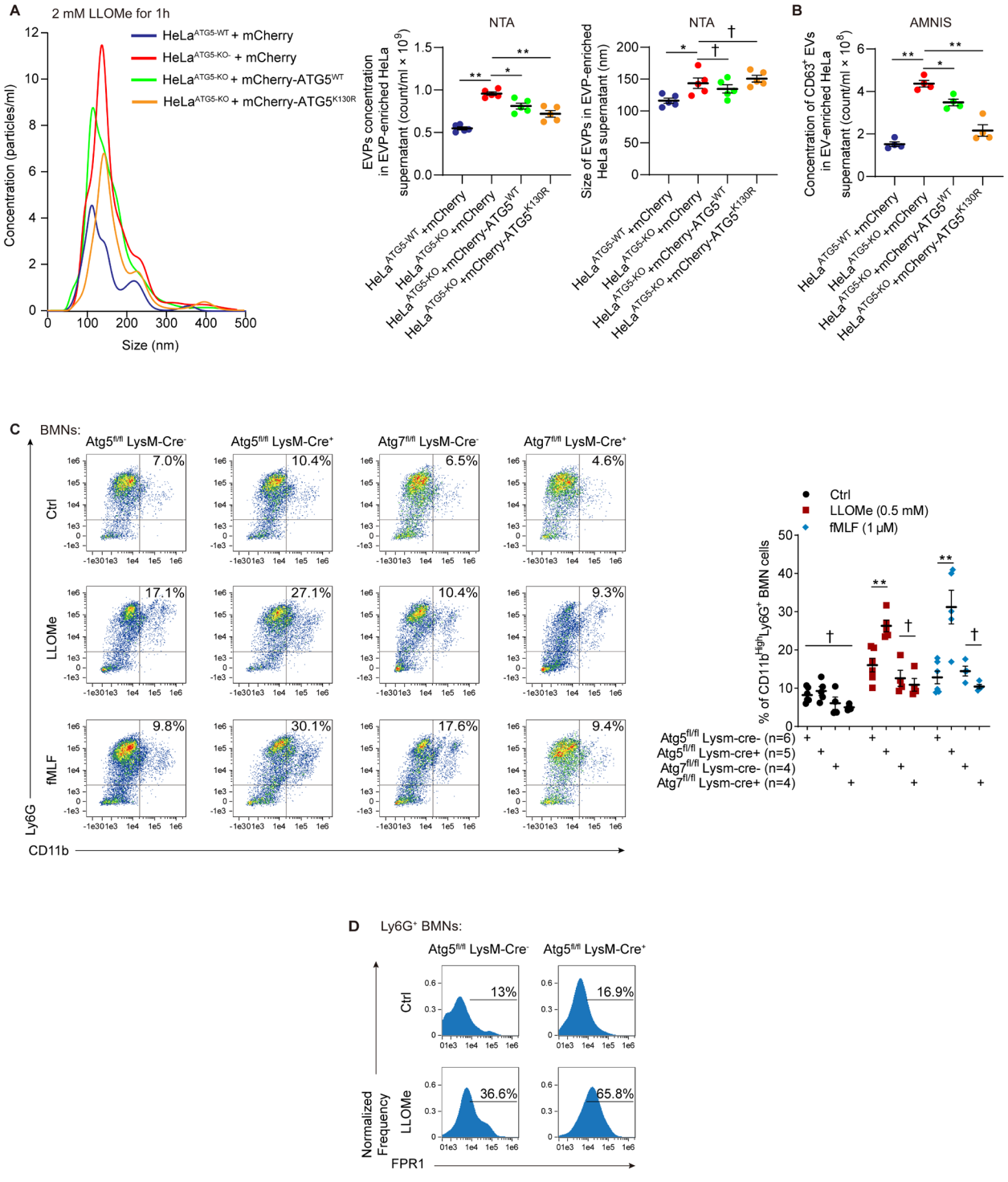
Supplementary Figure 3. Proximity biotinylation proteomics of ATG5 interactors continued (related to Figure 3)

Pathway enrichment analysis (cellular components and biological processes) of conjugation-dependent proteins.



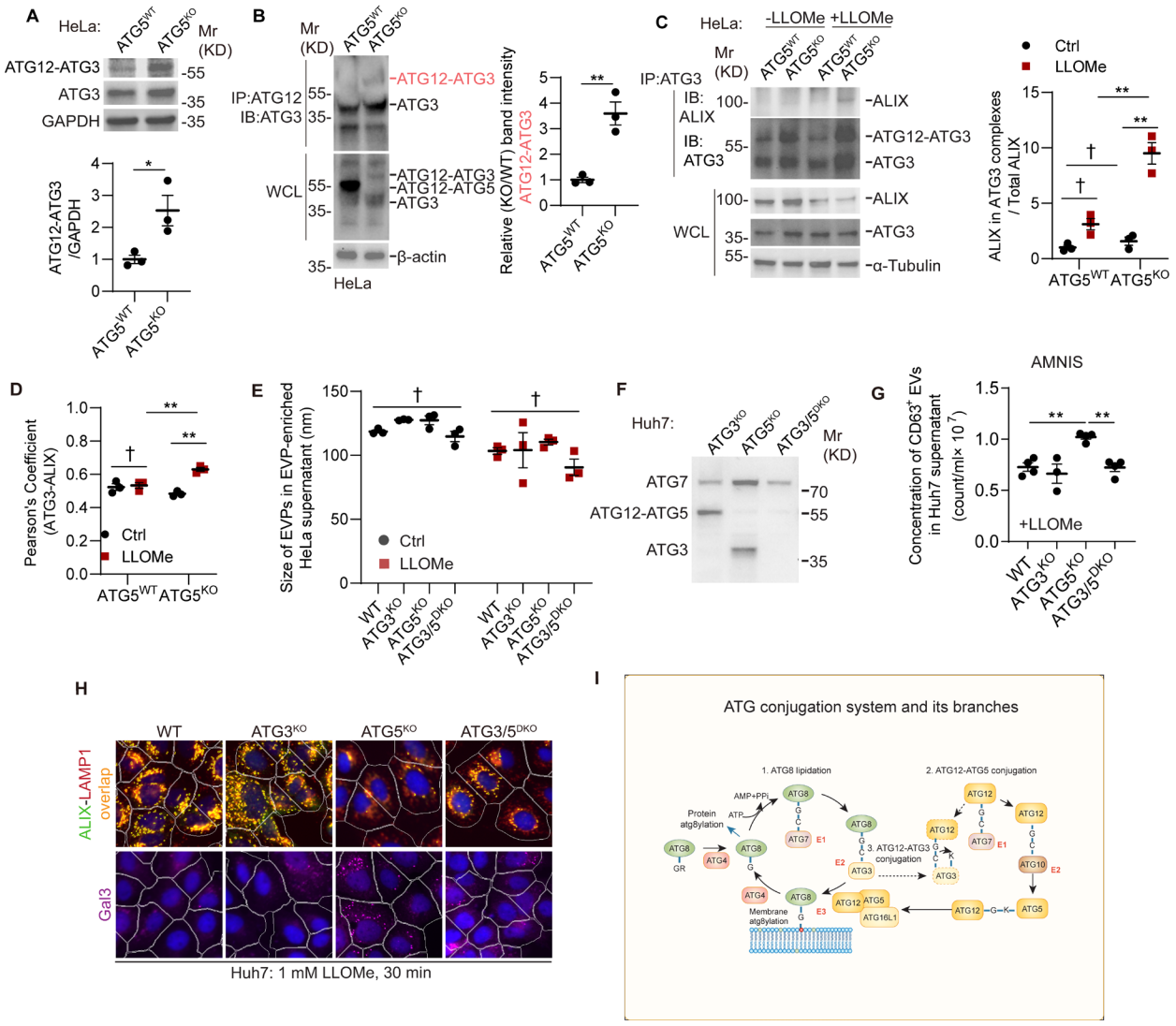
Supplementary Figure 4. Lysosomal damage induces extracellular vesicle release and neutrophil degranulation continued (related to Figure 4)

A, Quantification of the size of EVPs extracted from supernatant of HeLa cells treated with or without 2 mM LLOMe for 1 h using NTA. Data, means ± SE (n=3); unpaired *t*-test. Each individual data point represents independent biological replicate. **B,** Gating strategy for CD63⁺ EVs using AMNIS. Scale bars, 10 μm. **C,** Representative image of concentration and size distribution of EVPs extracted from supernatant of BMNs treated with 1 mM LLOMe for 1 h using NTA. Statistical significance symbols in all panels, †p≥0.05, *p<0.05, **p<0.01.



Supplementary Figure 5. ATG5 knockout enhances EV release in HeLa cells whereas in PMNs loss of ATG5 promotes degranulation in response to lysosomal damage (related to Figures 5 and 6).
A, Representative flow cytometry and quantifications of % CD11b^{High}Ly6G^{+} BMNs from Atg5$^{fl/fl}$ LysM-Cre^{-} and Atg5$^{fl/fl}$ LysM-Cre^{+}, Atg7$^{fl/fl}$ LysM-Cre^{-} and Atg7$^{fl/fl}$ LysM-Cre^{+} mice incubated with or without 0.5 mM LLOMe for 30 min. Data, means ± SE (n=4-6); two-way ANOVA with Tukey's multiple comparisons. **B,** Representative flow cytometry of FPR1 on Atg5$^{fl/fl}$ LysM-Cre^{-} and Atg5$^{fl/fl}$ LysM-Cre^{+} BMNs incubated with

or without 0.5 mM LLOMe for 30 min. In all panels, each data point represents an independent biological replicate. **C,** Representative graph of concentration, size distribution and quantification (NTA) of EVPs of $HeLa^{ATG5\text{-}WT}$ and $HeLa^{ATG5\text{-}KO}$ cells transfected with mCherry, mCherry-$ATG5^{WT}$, mCherry-$ATG5^{K130R}$ incubated with 2 mM LLOMe for 1 h. Data, means ± SE (n=5); one-way ANOVA with Tukey's multiple comparisons. **D,** Flow cytometry quantification of $CD63^{+}$ EVs extracted from supernatant of $HeLa^{ATG5\text{-}WT}$ and $HeLa^{ATG5\text{-}KO}$ cells transfected with mCherry, mCherry-$ATG5^{WT}$, mCherry-$ATG5^{K130R}$ and incubated with 2 mM LLOMe for 1 h. Data, means ± SE (n=5); one-way ANOVA with Tukey's multiple comparisons. In all panels, each data point represents an independent biological replicate. Statistical significance symbols in all panels, †$p \geq 0.05$, *$p < 0.05$, **$p < 0.01$.



Supplementary Figure 6. Alternative conjugation contributes to lysosomal vulnerability and exocytic processes continued (related to Figure 7)

A, Immunoblot and quantifications of ATG12-ATG3 conjugate in HeLa$^{ATG5\text{-}WT}$ and HeLa$^{ATG5\text{-}KO}$ cells. Data, means ± SE (n=3); unpaired *t*-test. **B,** Co-IP analysis of ATG12-ATG3 conjugate identity and quantification in HeLa$^{ATG5\text{-}WT}$ and HeLa$^{ATG5\text{-}KO}$ cells. Data, means ± SE (n=3); unpaired *t*-test. **C,** Co-IP analysis and quantifications of ALIX and ATG12-ATG3 interactions in HeLa$^{ATG5\text{-}WT}$ and HeLa$^{ATG5\text{-}KO}$ cells treated with or without 2 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **D,** Pearson's coefficients of ALIX-ATG3 in Huh7$^{ATG5\text{-}WT}$ and Huh7$^{ATG5\text{-}KO}$ cells treated with or without 1 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **E,** Quantification (NTA) of EV sizes extracted from supernatant of HeLa^{WT}, HeLa$^{ATG3\text{-}KO}$, HeLa$^{ATG5\text{-}KO}$, and HeLa$^{ATG3/5\text{-}DKO}$ cells treated with or without 2 mM LLOMe for 30 min. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. **F,** Immunoblot of Huh7$^{ATG3\text{-}KO}$, Huh7$^{ATG5\text{-}KO}$, and Huh7$^{ATG3/5\text{-}DKO}$ cells. **G,** Flow cytometry quantification of CD63^{+} EVs extracted from supernatant of Huh7^{WT}, Huh7$^{ATG3\text{-}KO}$, Huh7$^{ATG5\text{-}KO}$, and Huh7$^{ATG3/5\text{-}DKO}$ cells treated with 1 mM LLOMe for 1 h. Data, means ± SE (n≥3); one-way ANOVA with Tukey's multiple comparisons. **H,** Representative images of ALIX-LAMP1 overlap area (top panel) and Gal3 puncta (bottom panel) in Huh7^{WT}, Huh7$^{ATG3\text{-}KO}$, Huh7$^{ATG5\text{-}KO}$, and Huh7$^{ATG3/5\text{-}DKO}$ cells treated with 1 mM LLOMe for 30 min. **I,** The branches of ATG conjugation are modified from (Deretic and Lazarou, 2022). In all panels, each data point represents independent biological replicate. Statistical significance symbols in all panels, †p≥0.05, *p<0.05, **p<0.01.