

Expanded View Figures

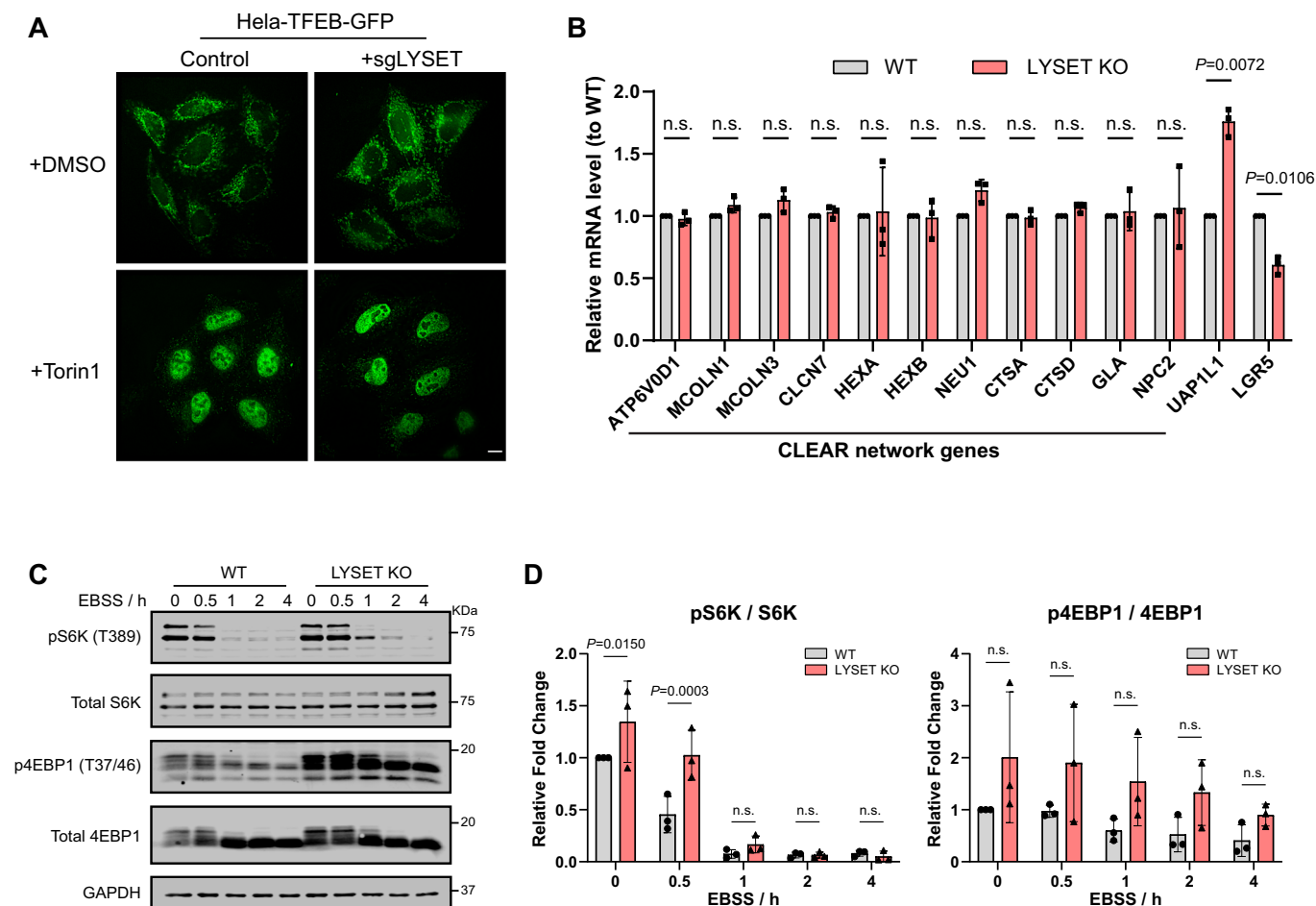


Figure EV1. Additional evidence for mTORC1- and TFEB/TFE3-independent lysosome accumulation in *LYSET* KO cells. Related to Figure 1.

(A) Fluorescence images of WT and *LYSET* KO HeLa cells stably expressing TFEB-GFP. Cells were treated with either dimethyl sulfoxide (DMSO) or 200 nM Torin1 (an mTORC1 inhibitor) for 1 hr. Scale bar: 10 μ m. (B) Comparison of CLEAR network gene transcription levels in *LYSET* KO and WT HEK293T cells. Data were presented as mean $\pm$ s.d.; $n = 3$ biological replicates. P values were calculated using multiple unpaired t-tests with Welch's correction. (C) Western blot analysis of S6K and 4EBP1 phosphorylation in WT and *LYSET* KO HEK293T cells under EBSS treatment. The experiments were repeated three times with similar results. (D) Quantification of (C). Data were presented as mean $\pm$ s.d.; $n = 3$ biological replicates; P values were calculated by two-way ANOVA with multiple comparisons. Source data are available online for this figure.

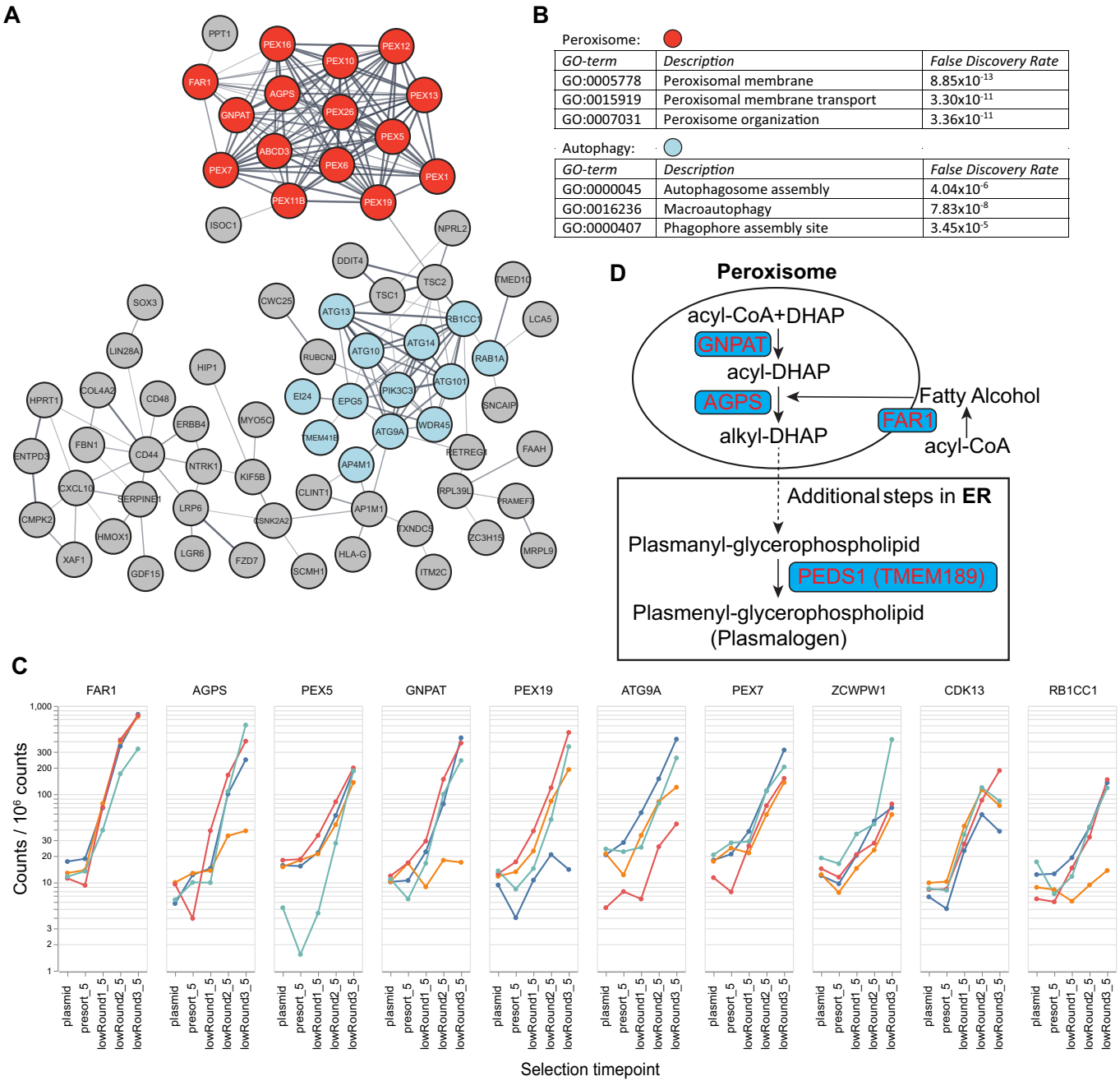
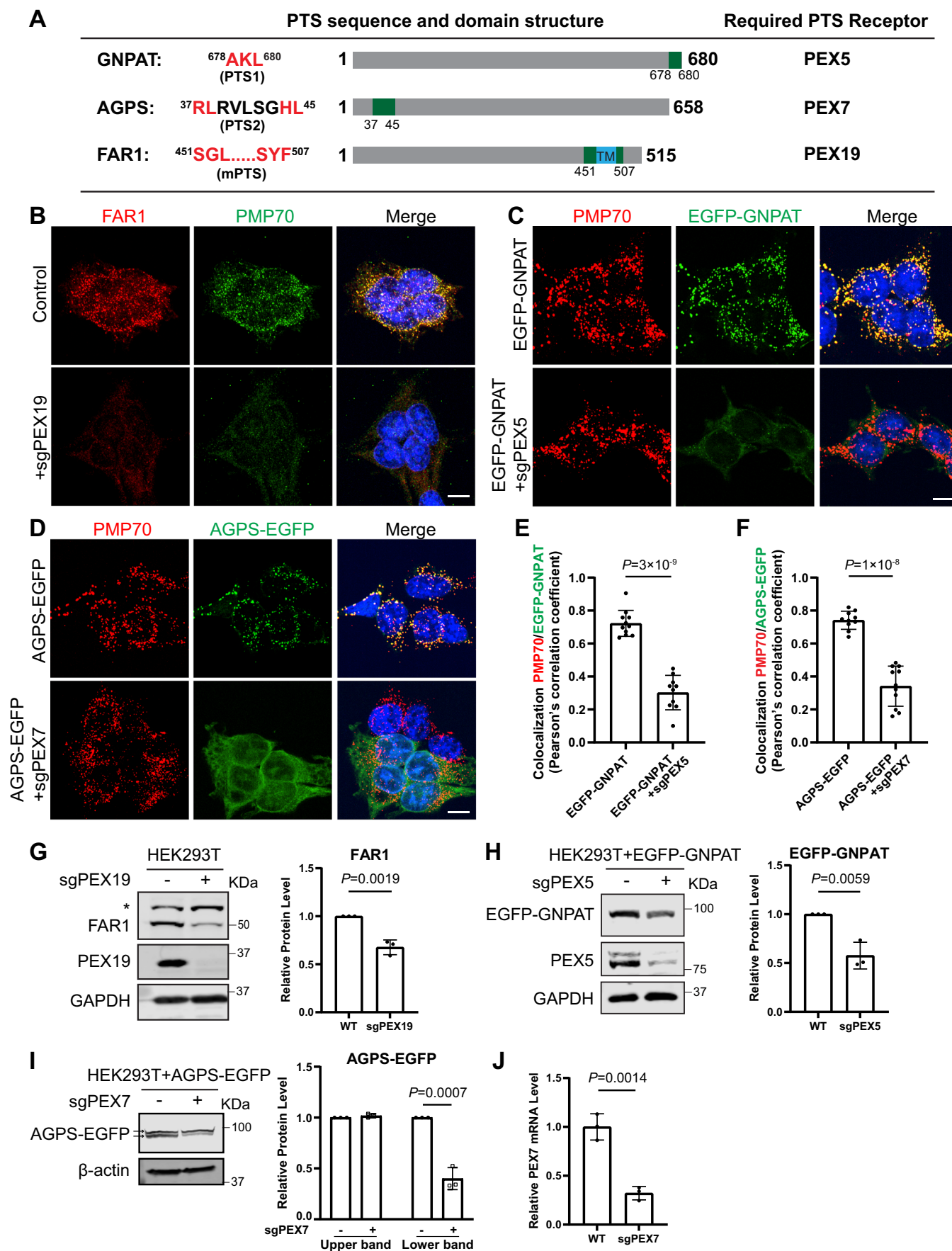


Figure EV2. Further analysis of CRISPR-Cas9 KO screen results. Related to Figure 2.

(A) STRING network analysis of enriched genes ($\log_2(\text{fold change}) > 1$) from the screen. Peroxisome genes are highlighted in red, and autophagy genes in blue. (B) GO terms, descriptions, and FDR values of enriched pathways identified by STRING analysis in (A). (C) Consistent enrichment across individual sgRNAs. For each top-hit gene, per-sgRNA frequencies (counts per million) are plotted across selection time points. "Plasmid" denotes the starting plasmid library, and "presort" denotes the transduced cell population prior to sorting. Each color represents an independent guide targeting a given gene. (D) The peroxisomal steps of the plasmalogen synthesis pathway. The final ER step, in which PEDS1 converts plasmanyl to plasmenyl phospholipids, is also highlighted.



◀ **Figure EV3. Peroxisomal targeting of GNPAT, AGPS, and FAR1 depends on PEX5, PEX7, and PEX19, respectively. Related to Figure 2.**

(A) Schematic representations of GNPAT, AGPS, and FAR1 proteins. Peroxisomal targeting sequences and their positions within each protein are highlighted in red and green. The corresponding peroxisomal-targeting receptors are also indicated. TM: transmembrane domain. (B) Immunofluorescence images of endogenous FAR1 and PMP70 in WT and *PEX19* KO HEK293T cells. Scale bar: 10 μ m. (C) Localization of EGFP-GNPAT and endogenous PMP70 in WT and *PEX5* KO HEK293T cells. Scale bar: 10 μ m. (D) Localization of AGPS-EGFP and endogenous PMP70 in WT and *PEX7* KO HEK293T cells. Scale bar: 10 μ m. (E, F) Pearson's coefficient analysis of co-localization between EGFP-GNPAT and PMP70 in (C), and between AGPS-EGFP and PMP70 in (D). Data were presented as mean $\pm$ s.d.; $n = 10$ ROIs from three biological replicates. *P* values were calculated by a two-tailed unpaired t-test. (G) Western blot analysis for endogenous FAR1 in WT and *PEX19* KO HEK293T cells, with quantification. * Indicates a non-specific band. Data were presented as mean $\pm$ s.d.; $n = 3$ biological replicates. *P* values were calculated by a two-tailed unpaired t-test. (H) Western blot analysis of EGFP-GNPAT in WT and *PEX5* KO HEK293T cells, probed with a GFP antibody. Data were presented as mean $\pm$ s.d.; $n = 3$ biological replicates. *P* values were calculated using a two-tailed unpaired t-test. (I) Western blot analysis of AGPS-EGFP in WT and *PEX7* KO HEK293T cells, probed with a GFP antibody. The upper and lower bands of AGPS-EGFP are indicated by arrows. Data were presented as mean $\pm$ s.d.; $n = 3$ biological replicates. *P* values were calculated using a two-tailed unpaired t-test. (J) RT-qPCR analysis confirms the reduction of *PEX7* mRNA in *PEX7* KO cells. Data were presented as mean $\pm$ s.d.; $n = 3$ biological replicates; *P* values were calculated by a two-tailed unpaired t-test. For (B–D), these experiments were repeated three times with similar results. Source data are available online for this figure.

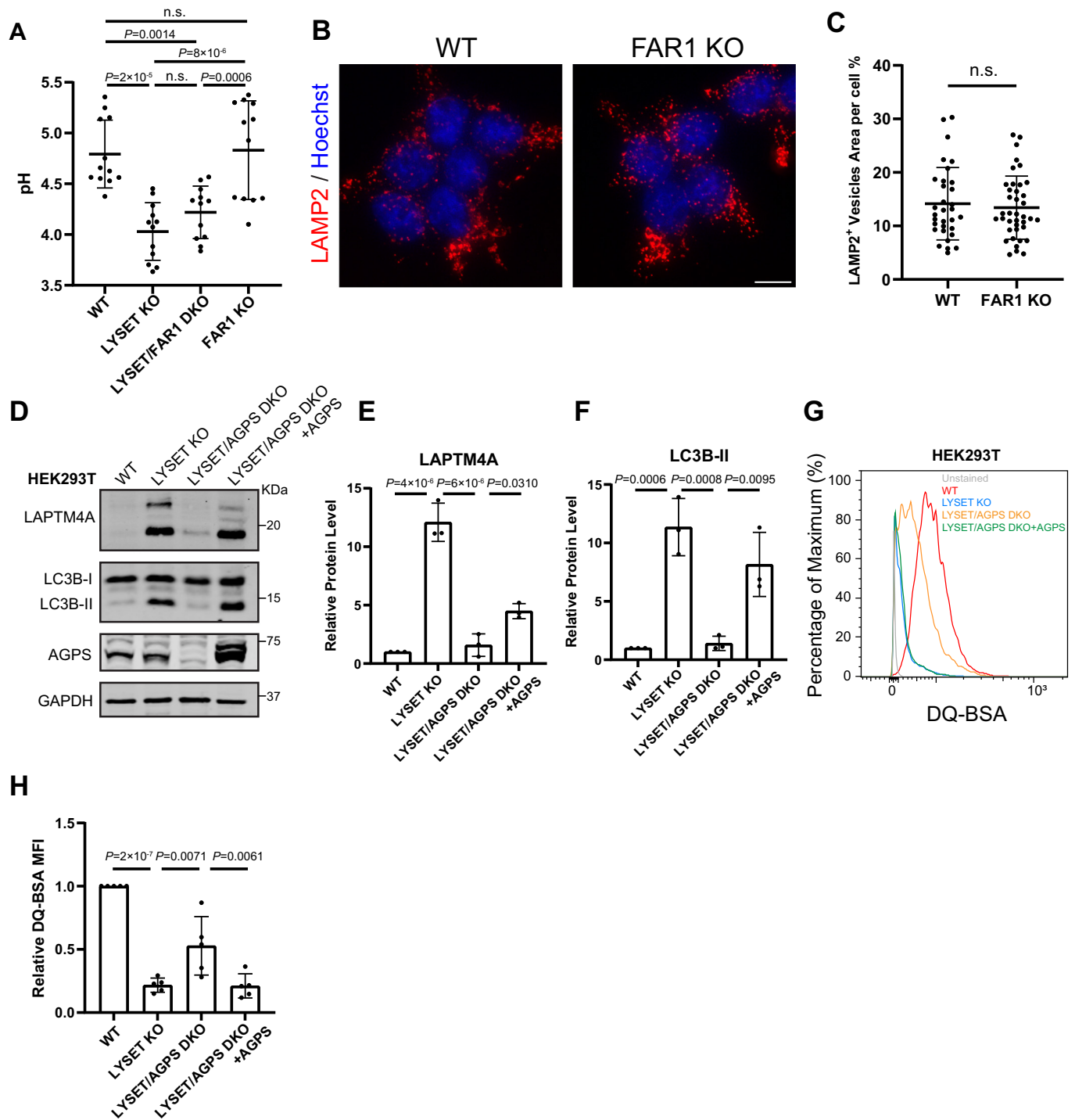


Figure EV4. Effects of ether lipid deficiency on lysosomal pH, number, and protein degradation. Related to Figure 3.

(A) Lysosomal pH in WT, LYSET KO, LYSET/FAR1 DKO, and FAR1 KO HEK293T cells. Cells are stably transfected with the FIRE-pHly (a ratiometric pH biosensor), and the signal intensities of mTFP and mCherry were measured by flow cytometry. Lysosomal pH was calculated from the ratio of mTFP to mCherry signals. Data were presented as mean $\pm$ s.d.; $n = 12$ from three biological replicates; P values were calculated by one-way ANOVA with multiple comparisons. (B, C) LAMP2 immunostaining (B) and quantification (C) in WT and FAR1 KO HEK293T cells. Data were presented as mean $\pm$ s.d.; $n = 32$ cells from three biological replicates; P values were calculated by two-tailed unpaired t-test. Scale bar: 10 μ m. (D) Steady-state levels of LAPT4A and LC3B-II in WT, LYSET KO, LYSET/AGPS DKO, and LYSET/AGPS DKO cells complemented with AGPS overexpression. (E, F) Quantification of full-length LAPT4A and LC3B-II levels in (D). Data were presented as mean $\pm$ s.d.; $n = 3$ biological replicates; P values were calculated by one-way ANOVA with multiple comparisons. (G) DQ-BSA assay in WT, LYSET KO, LYSET/AGPS DKO cells, and LYSET/AGPS DKO cells complemented with AGPS overexpression. (H) Normalized MFI in (G). Data were presented as mean $\pm$ s.d.; $n = 5$ biological replicates; P values were calculated by one-way ANOVA with multiple comparisons. Source data are available online for this figure.

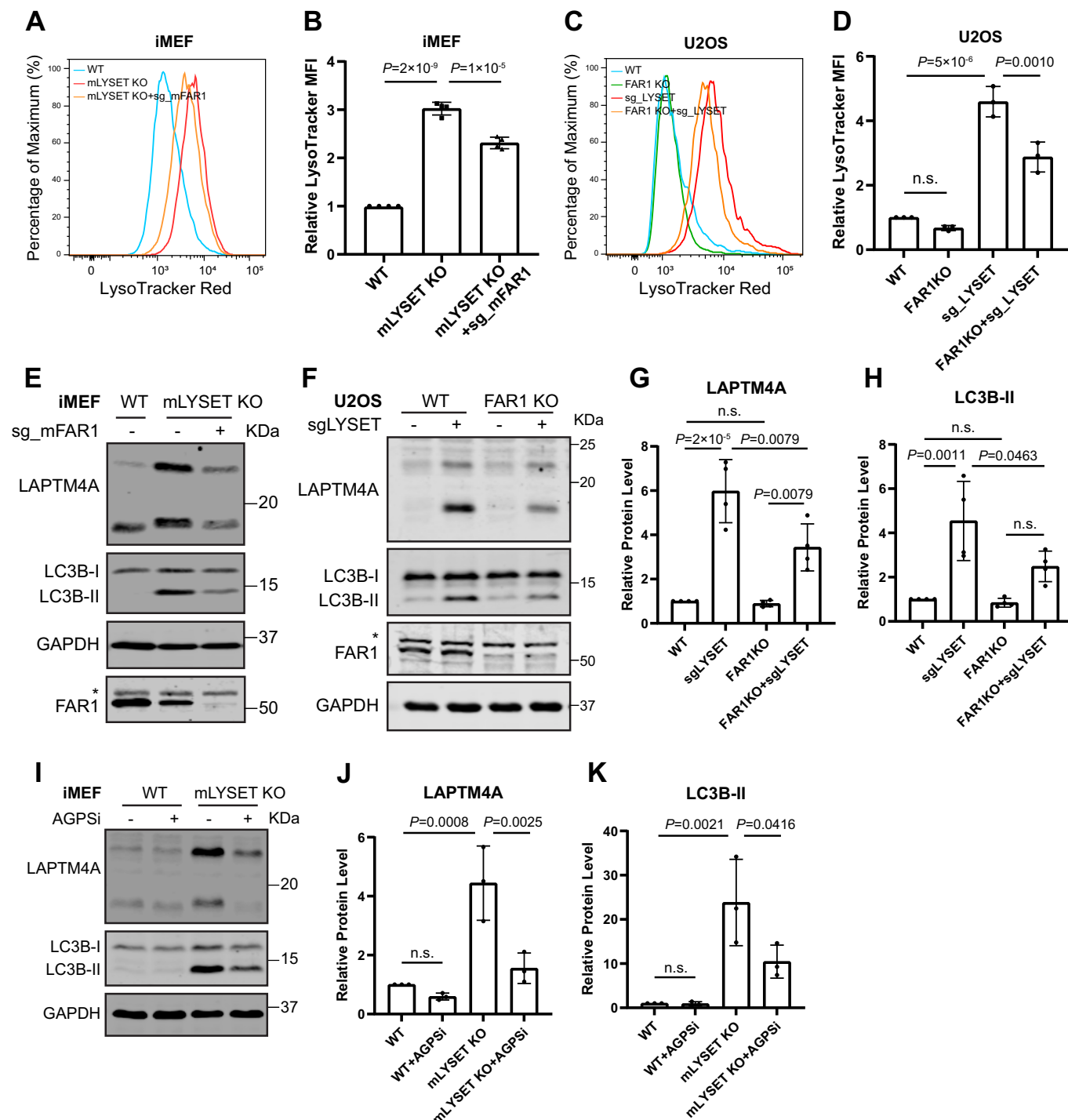
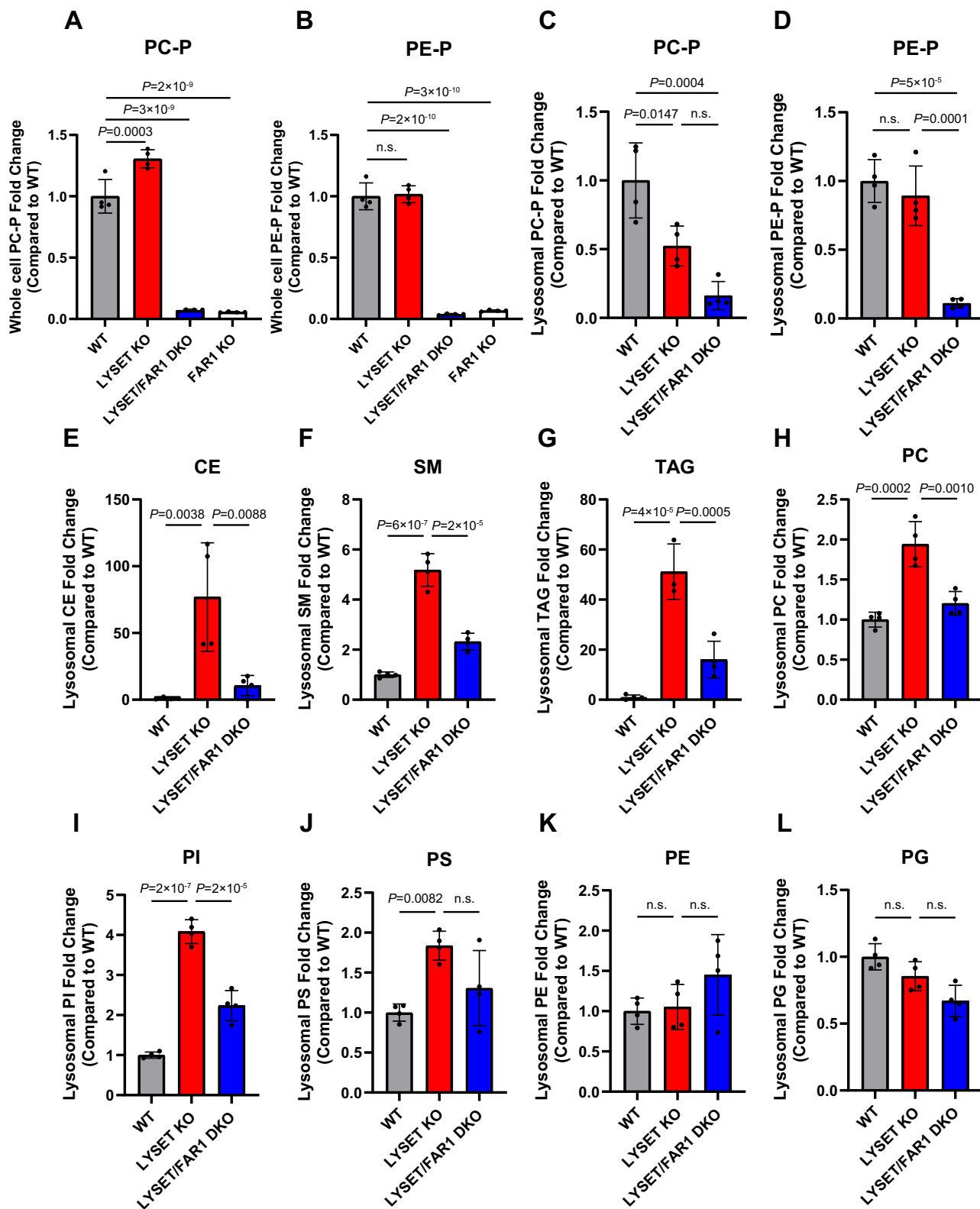


Figure EV5. Conserved effects of the ether lipid synthesis pathway across species and cell lines. Related to Figure 3.

(A) LysoTracker Red intensity in WT, *LYSET* KO, and *LYSET*/*FAR1* DKO iMEF cells. (B) Normalized MFI in (A). Data were presented as mean $\pm$ s.d.; $n = 4$ biological replicates; P values were calculated by one-way ANOVA with multiple comparisons. (C) LysoTracker Red intensity in WT, *FAR1* KO, *LYSET* KO, and *LYSET*/*FAR1* DKO U2OS cells. (D) Normalized MFI in (C). Data were presented as mean $\pm$ s.d.; $n = 3$ biological replicates; P values were calculated by one-way ANOVA with multiple comparisons. (E) Steady-state levels of LAPT4A and LC3B-II in WT, *LYSET* KO, and *LYSET* KO iMEF cells transfected with *FAR1* guide RNA. * Indicates a non-specific band. The experiments were repeated three times with similar results. (F) Steady-state levels of LAPT4A and LC3B-II in WT and *FAR1* KO U2OS cells transfected with or without *LYSET* guide RNA. * Indicates a non-specific band. (G, H) Quantification of total LAPT4A (G) and LC3B-II (H) levels in (F). Data were presented as mean $\pm$ s.d.; $n = 4$ biological replicates; P values were calculated by one-way ANOVA with multiple comparisons. (I) Steady-state levels of LAPT4A and LC3B-II in WT, *LYSET* KO iMEF cells treated with or without 250 μ M AGPS inhibitor for 48 h. (J, K) Quantification of total LAPT4A (J) and LC3B-II (K) levels in (I). Data were presented as mean $\pm$ s.d.; $n = 3$ biological replicates; P values were calculated by one-way ANOVA with multiple comparisons. Source data are available online for this figure.



◀ **Figure EV6. Lysosomal lipid accumulation is reduced in *LYSET/FAR1* DKO cells. Related to Figure 5.**

(A, B) Whole-cell plasmalogen-phosphatidylcholine (PC-P) and plasmalogen-phosphatidylethanolamine (PE-P) levels in WT, *LYSET* KO, *LYSET/FAR1* DKO, and *FAR1* KO HEK293T cells. Fold changes were normalized to WT levels. Data were presented as mean $\pm$ s.d.; $n = 4$ biological replicates; P values were calculated by one-way ANOVA with multiple comparisons. (C–L) Relative levels of plasmalogen-phosphatidylcholine (PC-P), plasmalogen-phosphatidylethanolamine (PE-P), cholesteryl esters (CE), sphingomyelin (SM), triacylglycerols (TAG), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) in purified lysosomes from WT, *LYSET* KO, and *LYSET/FAR1* DKO HEK293T cells. Fold changes were normalized to WT levels. Data were presented as mean $\pm$ s.d.; $n = 4$ biological replicates; P values were calculated by one-way ANOVA with multiple comparisons. Source data are available online for this figure.

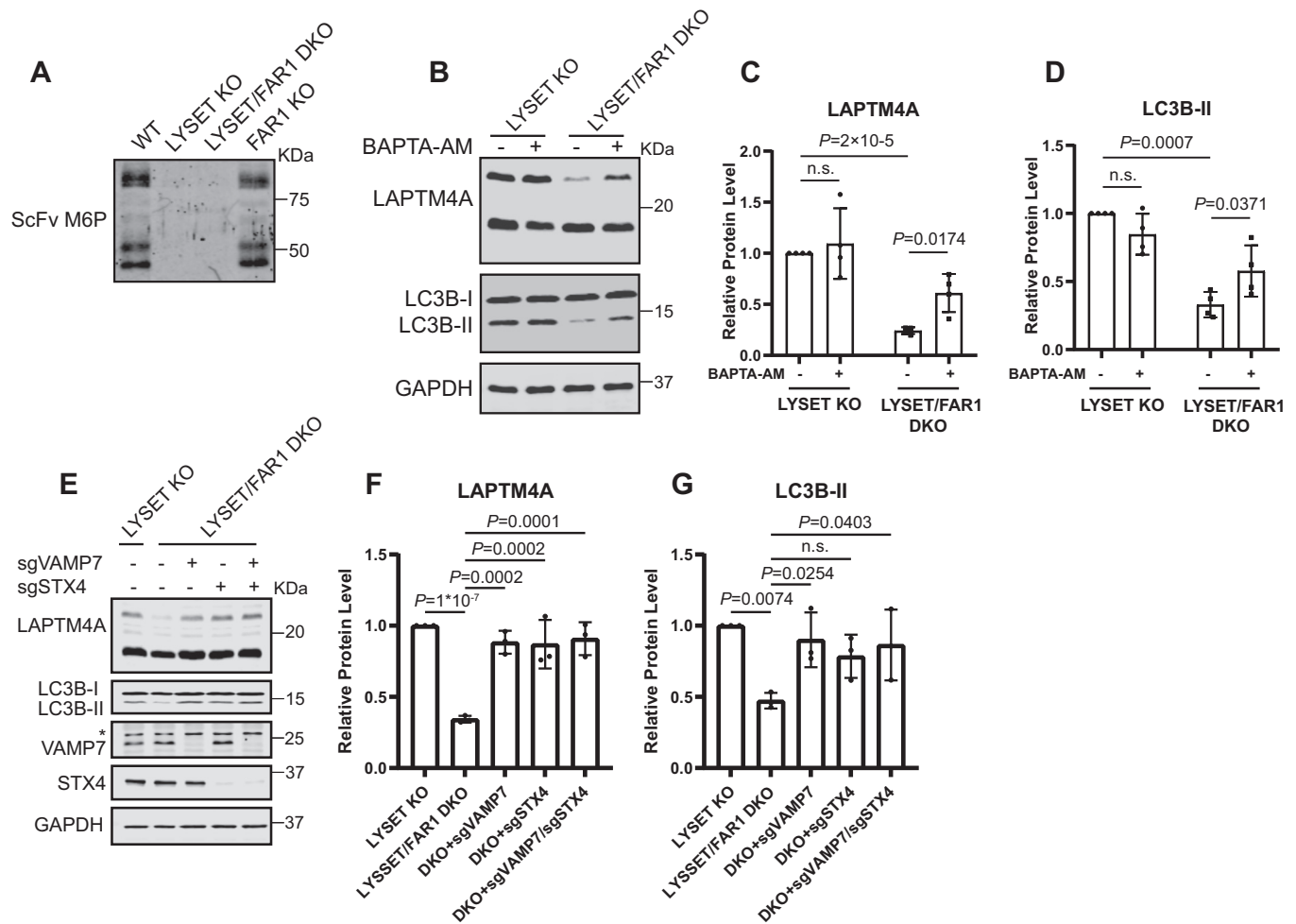


Figure EV7. Inhibition of lysosomal exocytosis restores LAPT4A and LC3B-II levels in LYSET/FAR1 DKO cells. Related to Figure 7.

(A) Western blot analysis of the M6P modifications in purified lysosomes from the indicated cell lines, detected by a single-chain antibody against M6P (scFv M6P). (B) Western blot analysis of LAPT4A and LC3B-II in LYSET KO and LYSET/FAR1 DKO HEK293T cells treated with or without 10 μ M BAPTA-AM for 48 h. (C, D) Normalized protein levels of full-length LAPT4A and LC3B-II in (B). Data were presented as mean $\pm$ s.d.; $n = 4$ biological replicates; P values were calculated by two-tailed paired t-test. (E) Western blot analysis of LAPT4A and LC3B-II in LYSET KO, LYSET/FAR1 DKO, LYSET/FAR1/VAMP7 TKO, LYSET/FAR1/STX4 TKO, and LYSET/FAR1/VAMP7/STX4 QKO HEK293T cells. * Indicates a non-specific band. (F, G) Normalized protein levels of full-length LAPT4A and LC3B-II in (E). Data were presented as mean $\pm$ s.d.; $n = 3$ biological replicates; P values were calculated by one-way ANOVA with multiple comparisons. Source data are available online for this figure.