Electronic Supplementary Material (ESI) for Biomaterials Science.

Supplementary Information

Illuminating endosomal escape of polymorphic lipid nanoparticles that boost mRNA delivery

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Evaluation of LNP cellular uptake using flow cytometry

293T/17 cells were seeded at 100,000 cell per well in 6-well plates, followed by overnight incubation for cell adhesion. The cells were treated with the LNPs encapsulating Cy5-labelled EGFP mRNA at 500 ng mRNA per well for 3 hr. Then, the cells were processed to single cell suspensions using TrypLE Express (Thermo Fisher), followed by staining with Fixable Viability Dye eFluor™ 780 and fixing with 1% paraformaldehyde. The samples were diluted with sterile PBS and analyzed in BD Fortessa with 670/30 and 780/60 filters. The data were processed in FlowJo software v10.7 (FlowJo LLC, Ashland, OR) to gate populations and to calculate mean fluorescence intensity of Cy5.

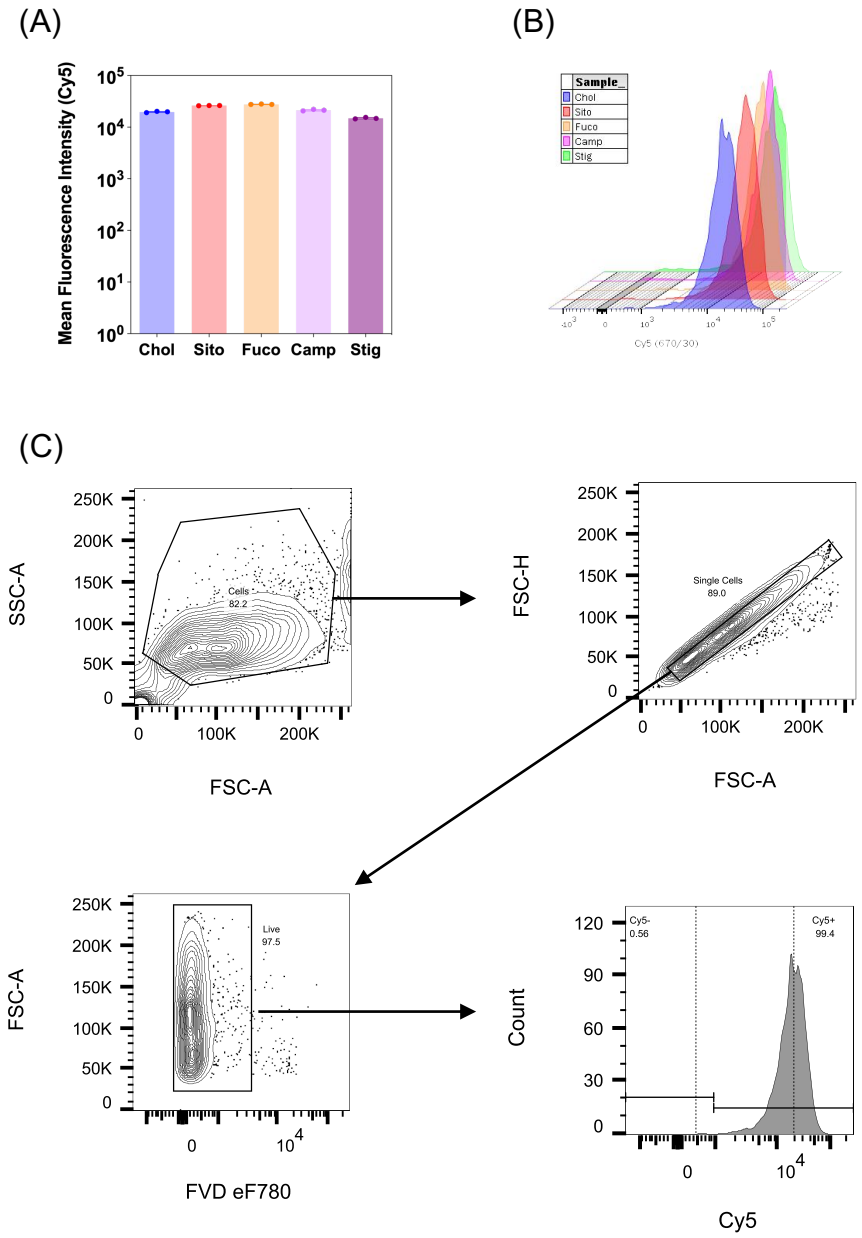

Fig. S1. (A) Cellular uptake of LNPs containing various sterol analogs (n = 3). **(B)** Multigraph overlay of Cy5 histograms of the samples treated with LNPs containing various sterol analogs: Chol (blue), Sito (red), Fuco (orange), Camp (pink), and Stig (green). **(C)** Gating strategy for Cy5-positive cell populations.

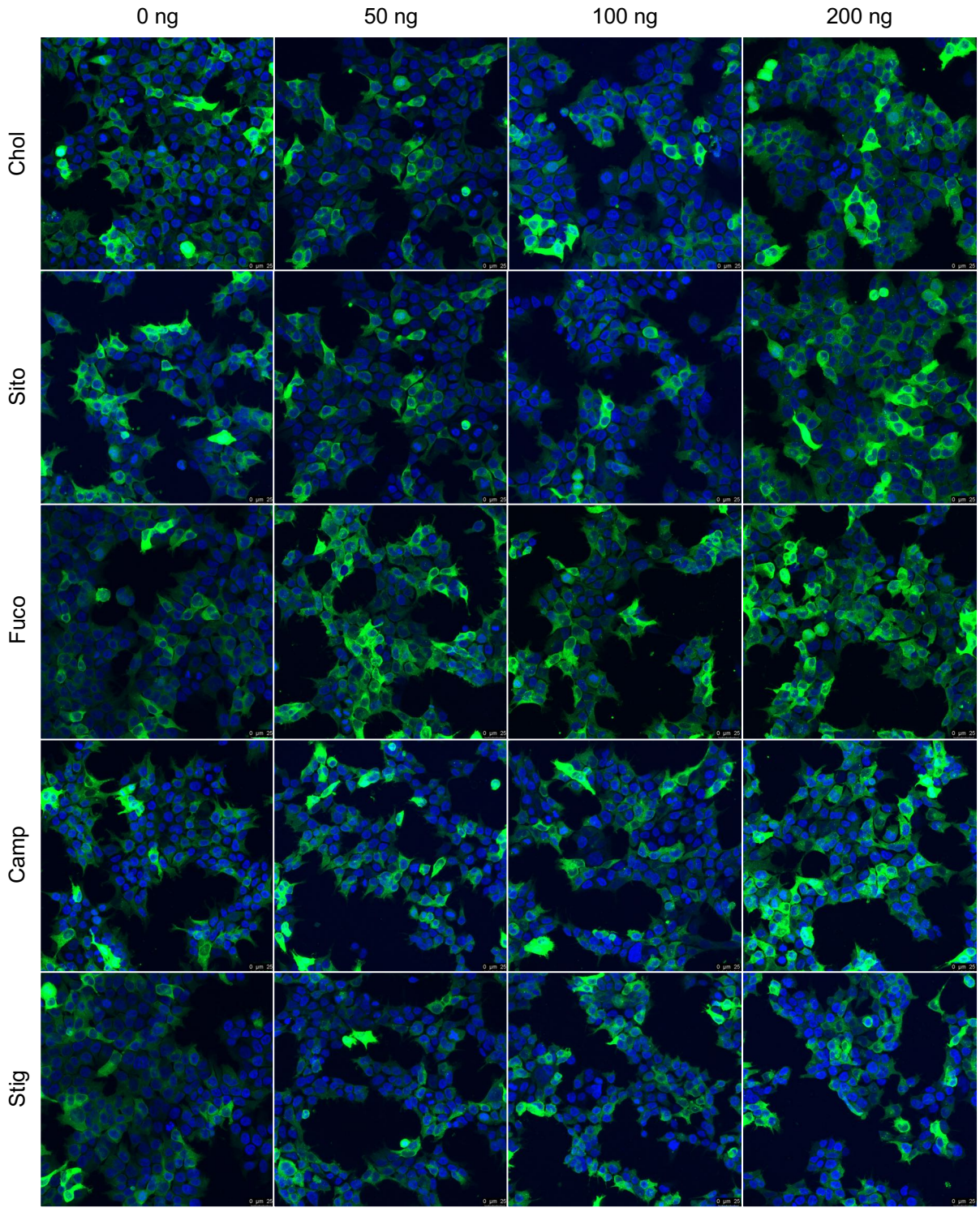

Fig. S2. Representative images of the Gal8-GFP reporter cells after treatment of the LNPs containing various sterol analogs at various mRNA doses for 3 hr. Presented in

maximum intensity projection. Gal8-GFP (green) and nucleus (blue). Scale bars show 25 μm.

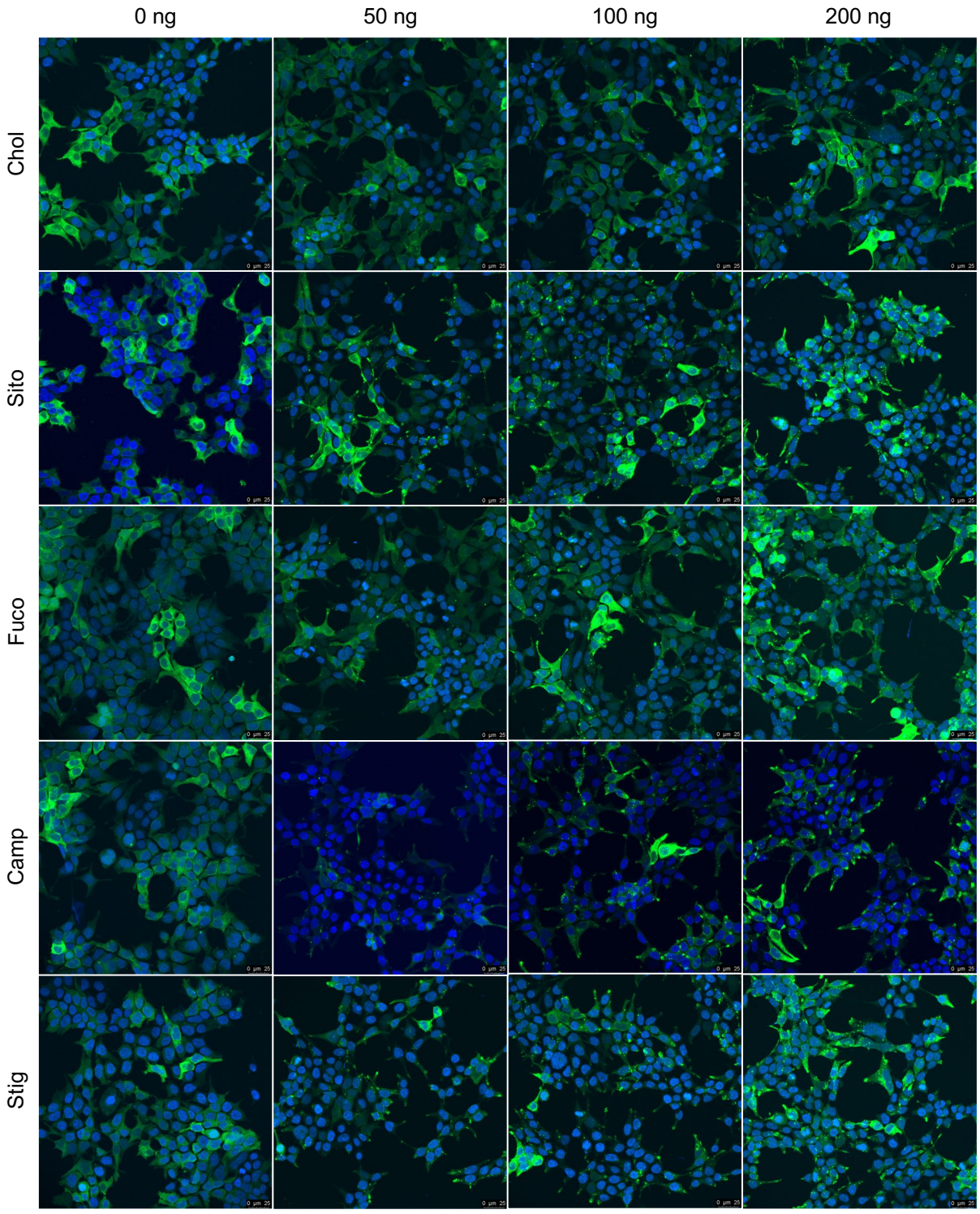

Fig. S3. Representative images of the Gal8-GFP reporter cells after treatment of the LNPs containing various sterol analogs at various mRNA doses for 24 hr. Presented in

maximum intensity projection. Gal8-GFP (green) and nucleus (blue). Scale bars show 25 μm.

Cell viability and mRNA transfection assay

293T/17 or 293T/17 Gal8-GFP cells were seeded in clear-bottom, 96 well plates. After overnight incubation, cells were treated with LNPs encapsulating *Fluc* mRNA at various doses. For cell viability assay, 293T/17 Gal8-GFP cells were treated with alamarBlue™ cell viability reagent, followed by measuring absorbances at 570 nm and 600 nm. Cell viability was calculated according to the manufacturer's instruction. For mRNA transfection assay, 293T/17 cells were treated with ONE-Glo™ + Tox luciferase reporter and cell viability kit (Promega, Madison, WI).

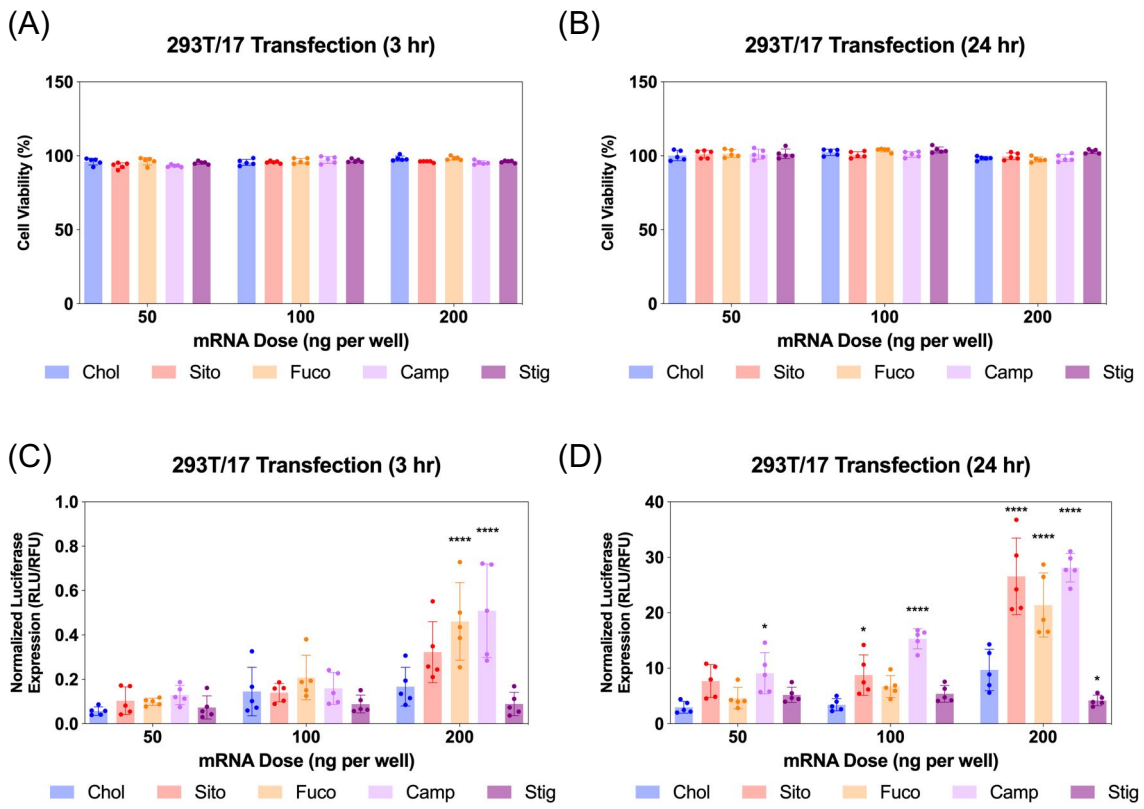

Fig. S4. mRNA transfection assay in 293T/17 cells treated with *Fluc* mRNA-loaded LNPs containing various sterol analogs: Chol (blue), Sito (red), Fuco (orange), Camp (lavender), Stig (purple) **(A, B)** Cell viability of 293T/17 cells treated with the LNPs containing various sterol analogs for **(A)** 3 hr and **(B)** 24 hr. **(C, D)** Luciferase expression in the transfected 293T/17 cells using the LNPs containing various sterol analogs for **(C)** 3 hr and **(D)** 24 hr. Statistical analyses were performed against LNP-Chol at each dose using Tukey's multiple comparisons test (n = 5); *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

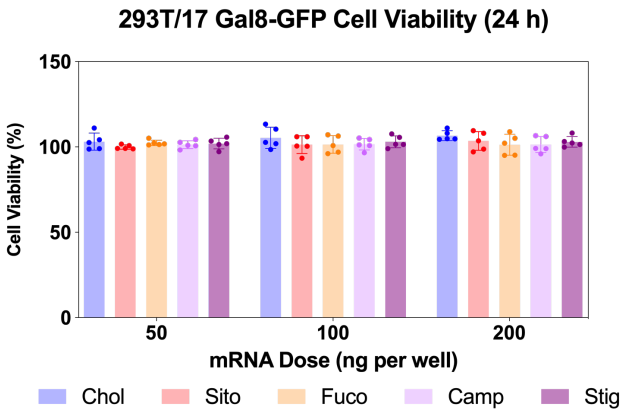

Fig. S5. Cell viability assay of 293T/17 Gal8-GFP reporter cells after treatment of *Fluc* mRNA-loaded LNPs containing various sterol analogs: Chol (blue), Sito (red), Fuco (orange), Camp (lavender), Stig (purple).

Colocalization analysis

Colocalization analysis was performed on maximum intensity Z-projections of confocal image stacks using Coloc2 plugin of ImageJ. In order to disregard the cytosolic GFP signal, we only selected the areas with puncta in GFP-Gal8 channel by applying a mask. To create the mask, we selected the maxima with prominence > 3000 and created the mask with maxima points within tolerance. Pearson's (before automatic bisection thresholding) and Mander's correlation coefficients (after automatic thresholding) were selected for the discussion.

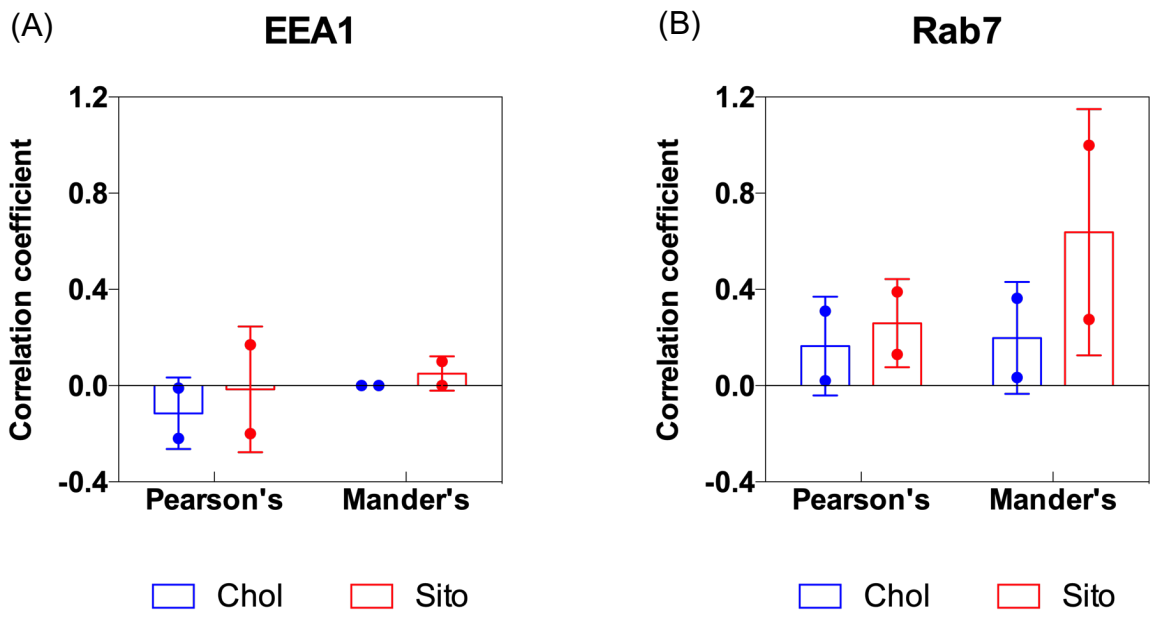

Fig. S6. Measuring colocalization of Gal8 puncta with endosomal markers, **(A)** EEA1 for early endosomes and **(B)** Rab7 for late endosomes. The Gal8-GFP reporter cells were treated with LNP-Chol (blue) or LNP-Sito (red) for 24 hr, followed by immunocytochemistry staining with EEA1 and Rab7 antibodies. The resulting images were processed to maximum intensity projection, followed by Pearson's and Mander's correlation analyses to calculate correlation coefficients in ImageJ (n = 2).