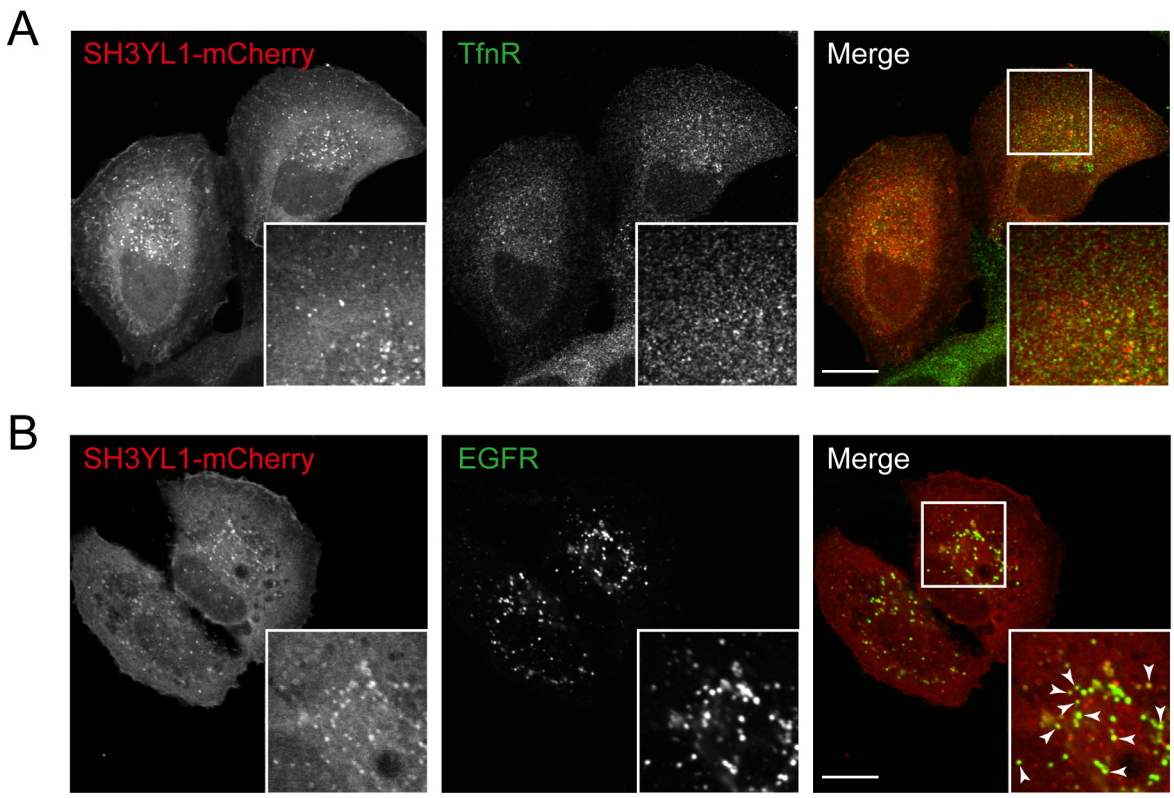


Fig. S1. Localization of SH3YL1 with endosomal markers. (A) HeLa cells stably expressing SH3YL1-mCherry (red) were immunostained with anti-transferrin receptor (TfnR) antibody (green). (B) SH3YL1-mCherry (red) stable HeLa cells were stimulated with 50 ng/ml EGF for 30 min and fixed. The localization of EGFR receptor was assessed by immunostaining with anti-EGFR antibody (green). The boxed region is magnified in inset. Arrowheads indicate colocalizations. Images are representative of three independent experiments. Scale bars, 10 µm.

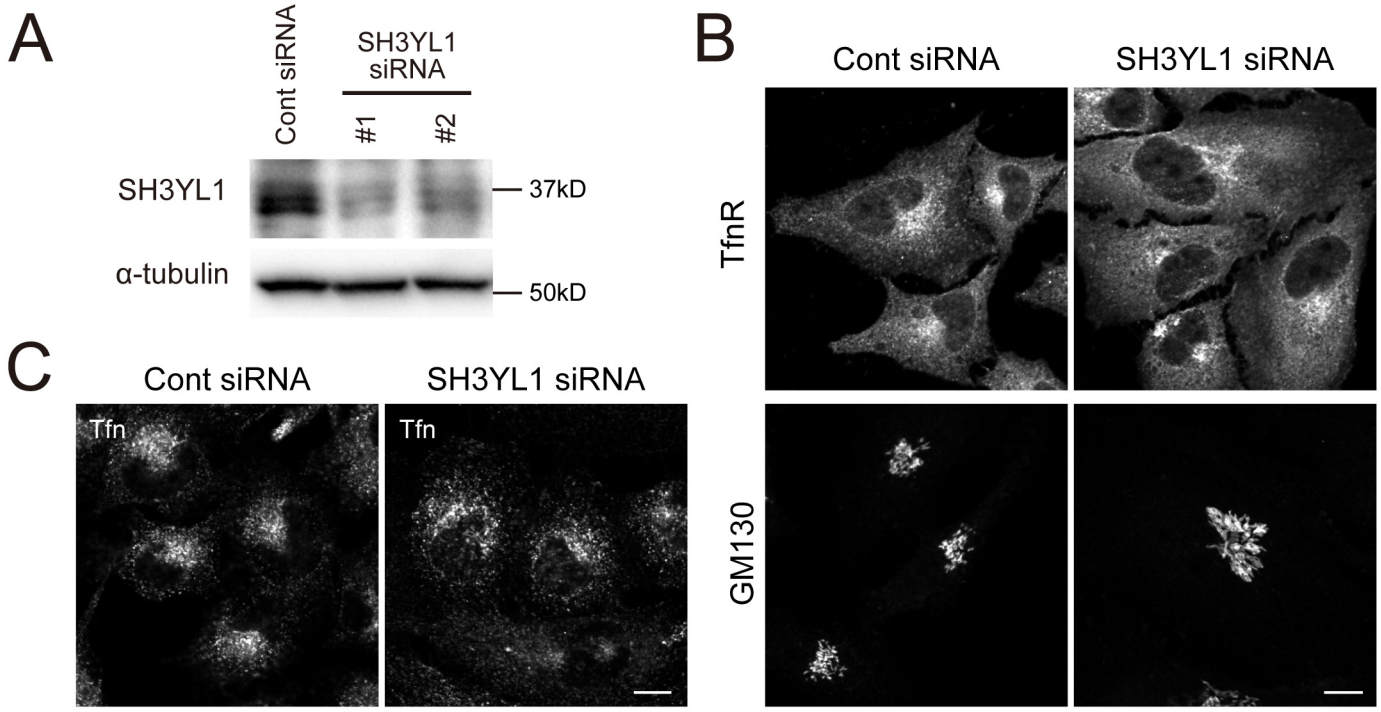


Fig. S2. Depletion of SH3YL1 does not affect transferrin uptake. (A) HeLa cells were treated with control or SH3YL1-specific siRNAs for 72 h, and cell lysates were detected by immunoblotting. (B) SH3YL1 knockdown cells (control or SH3YL1#2) were stained with anti-transferrin receptor (TfnR) or anti-GM130 antibodies. Images are representative of three independent experiments. Scale bars, 10 µm. (C) HeLa cells treated with siRNAs (control or SH3YL1#2) were incubated with 1 µg/ml Alexa Fluor 568-conjugated transferrin (Tfn) for 5 min at 37ºC and then chased in serum-free medium without ligand for 10 min. Images are representative of three independent experiments. Scale bars, 10 µm.

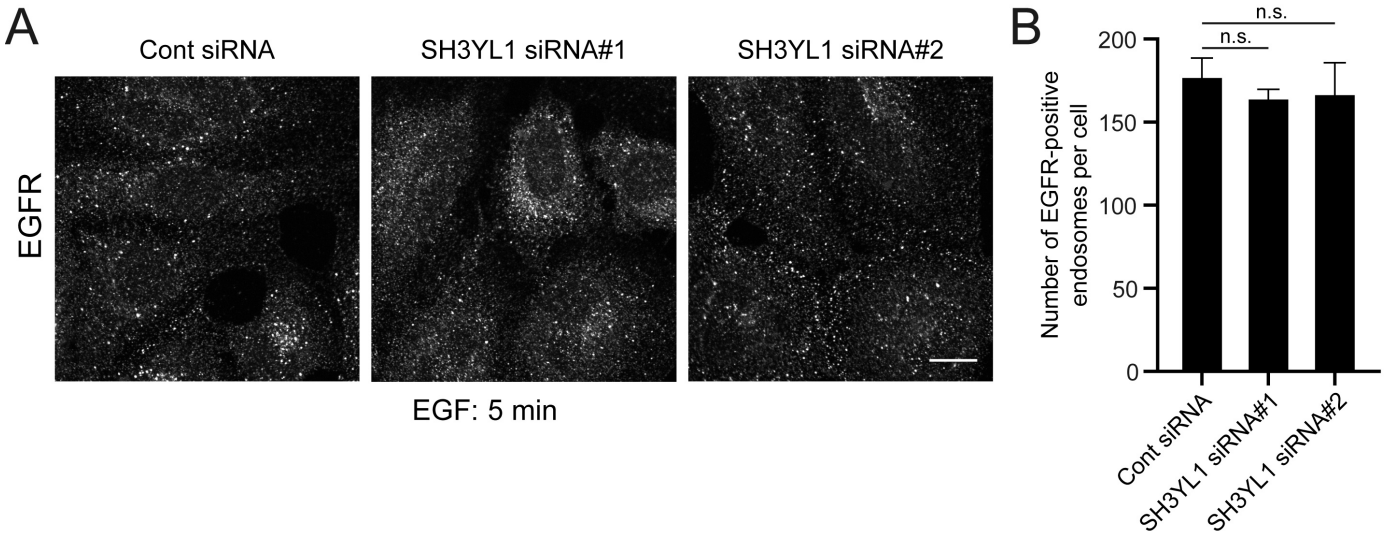
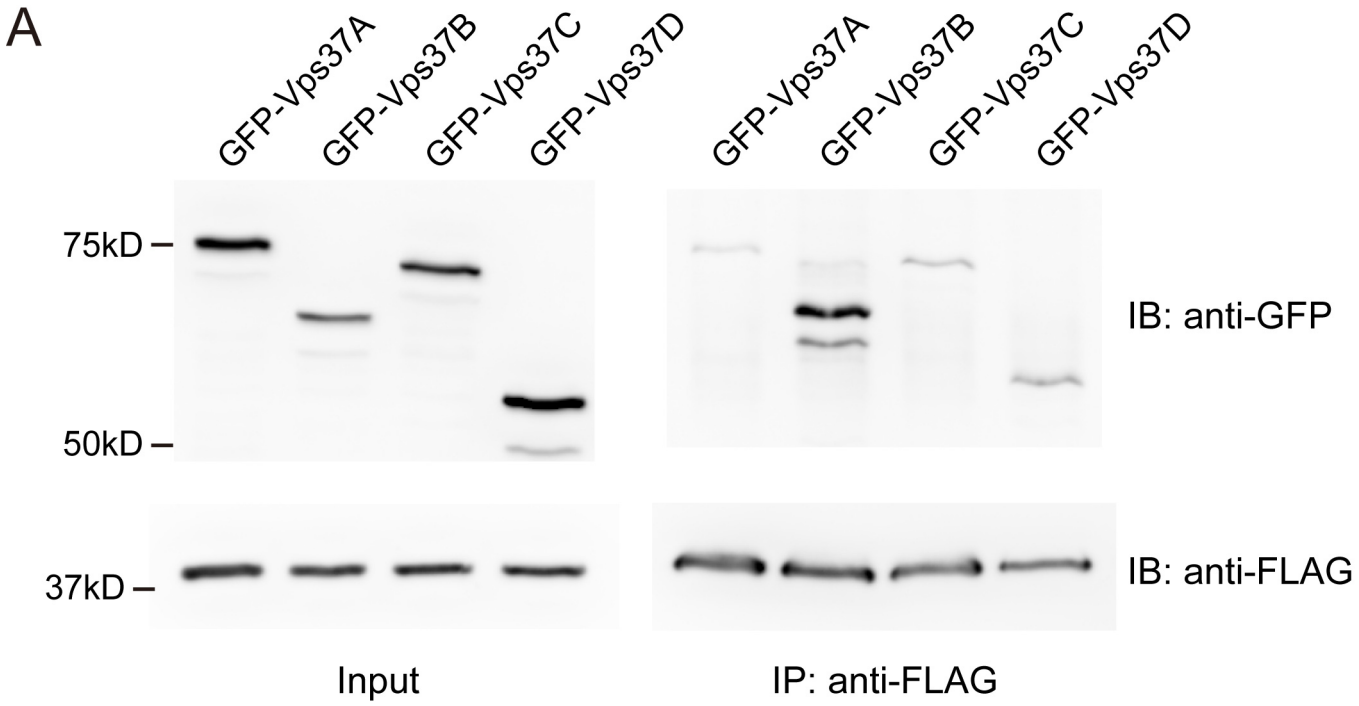
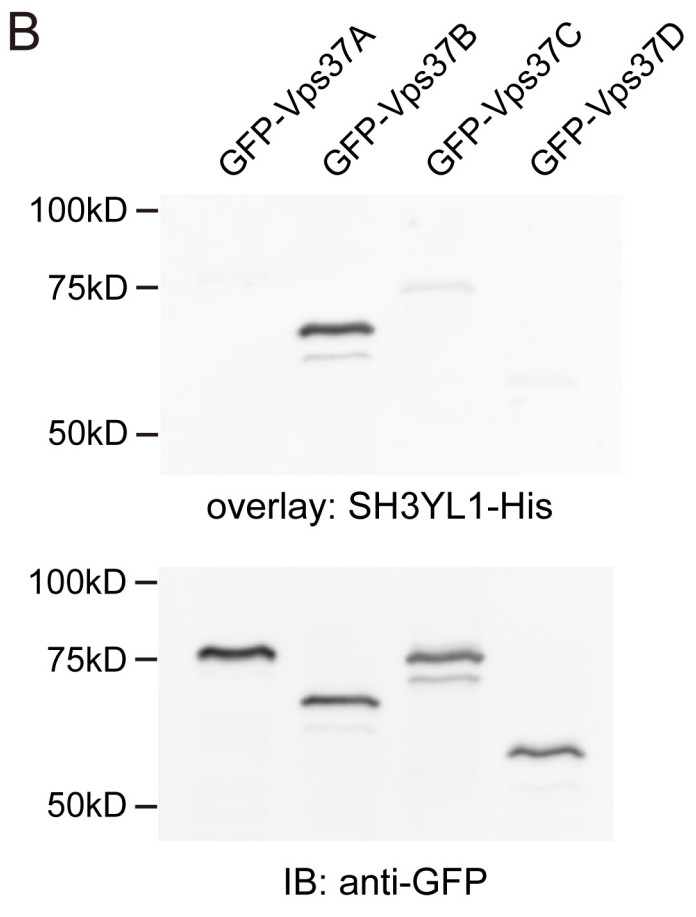


Fig. S3. Depletion of SH3YL1 does not affect EGF uptake. (A) HeLa cells were stimulated with 50 ng/ml EGF for 5 min, fixed, and then immunostained with anti-EGFR antibody. Images are representative of three independent experiments. Scale bars, 10 µm. (B) EGFR-positive endosomes were counted and presented with the mean (±SEM) of three independent experiments with at least 10 cells per experiment. n.s., not significant (Tukey's multiple comparison test).





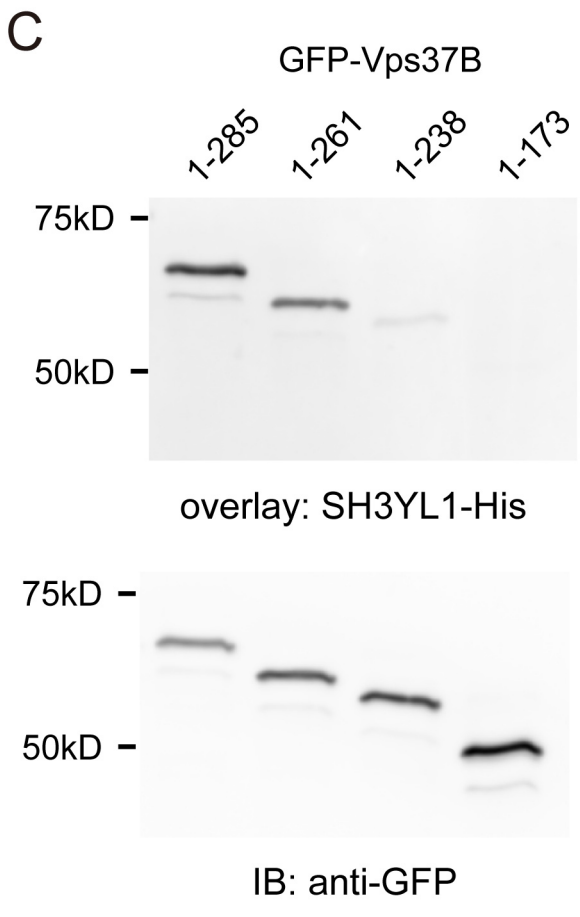


Fig. S4. SH3YL1 binds almost exclusively to Vps37B. (A) Indicated constructs were expressed in 293FT cells together with FLAG-SH3YL1 for 48 h, and cell lysates were prepared. SH3YL1 proteins were immunoprecipitated with anti-FLAG antibody and then analyzed by immunoblotting with anti-GFP antibody. Input; cell lysate. Data are representative of three independent experiments. (B) Indicated constructs of GFP-Vps37 proteins were expressed in 293FT cells and immunoprecipitated with anti-GFP antibody. Immunoprecipitates were transferred onto PVDF membrane after SDS-PAGE and then overlaid by SH3YL1-His recombinant protein (upper). GFP-Vps37 proteins were visualized by western blotting with anti-GFP antibody (lower). Data are representative of three independent experiments. (C) SH3YL1-Vps37B interaction requires “PxLPxR” motifs in Vps37B. Indicated constructs of GFP-Vps37B were subjected to an overlay assay as in (B). Data are representative of three independent experiments.

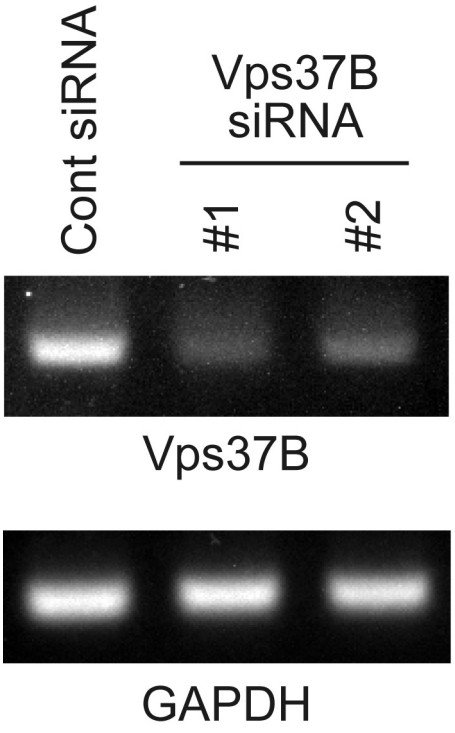


Fig. S5. Knockdown of Vps37B in HeLa cells. HeLa cells were treated with control or Vps37B-specific siRNAs for 72 h. Knockdown levels were assessed by RT-PCR by specific primer sets for human Vps37B (upper) or GAPDH (lower). Data are representative of three independent experiments.

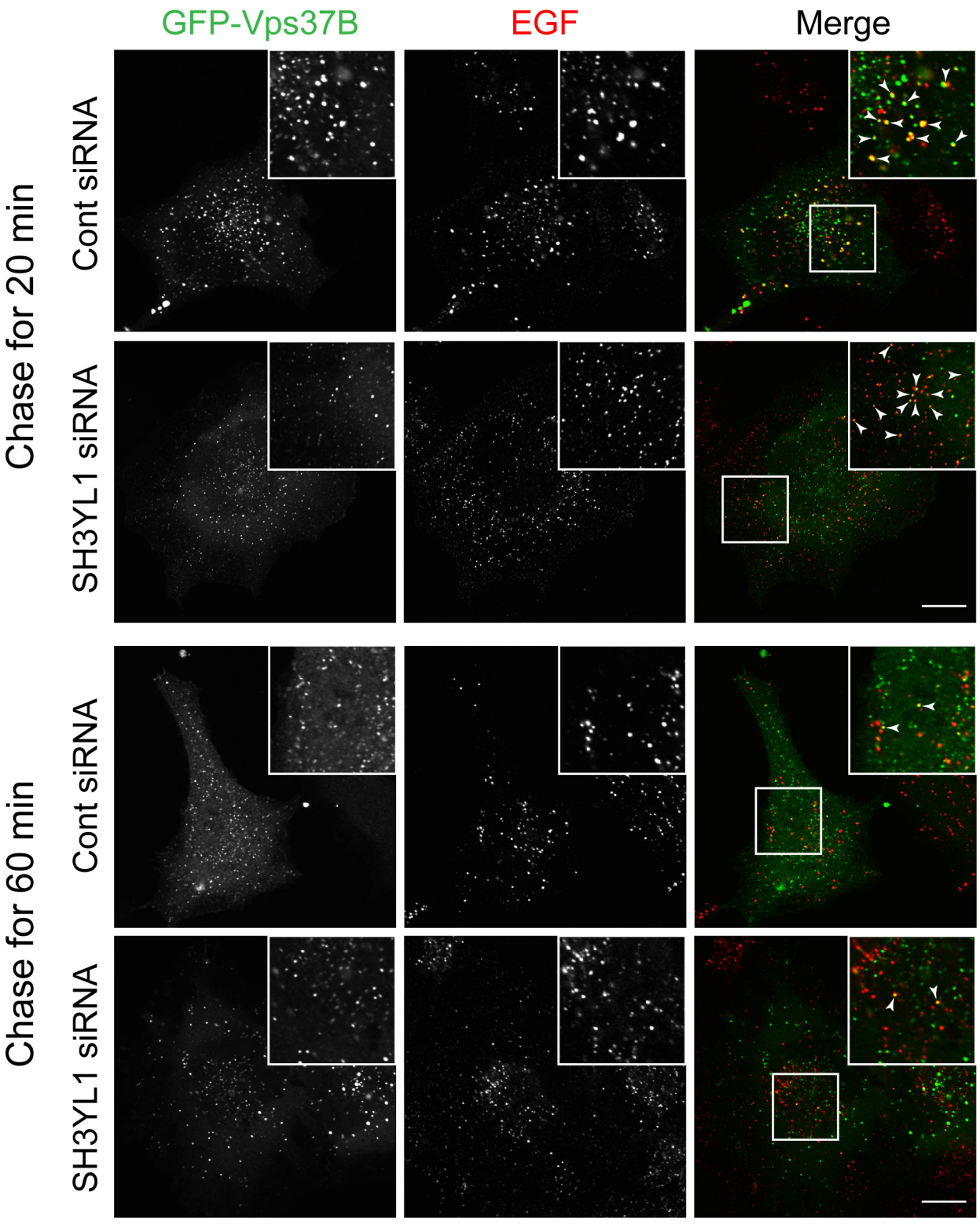

GFP-Vps37B
EGF
Merge
Chase for 20 min
Cont siRNA
SH3YL1 siRNA
Chase for 60 min
Cont siRNA
SH3YL1 siRNA

Fig. S6. Loss of SH3YL1 does not affect EGF trafficking via Vps37B-positive endosomes. (A) HeLa cells co-expressing GFP-Vps37B and mCherry-Tsg101 were treated with siRNAs (control or SH3YL1#2) for 72 h, incubated with 0.1 µg/ml Alexa647-EGF for 5 min at 37ºC and then chased in serum-free medium without EGF for 20 and 60 min. Boxed regions are magnified in insets. Arrowheads indicate colocalization of Alexa647-EGF (red) with GFP-Vps37B (green). Images are representative of three independent experiments. Scale bars, 10 µm. Arrowheads indicate colocalizations with Pearson correlation coefficients of 0.44±0.05 (cont siRNA, 20 min), 0.13±0.08 (cont siRNA, 60 min), 0.29±0.04 (SH3YL1 siRNA#2, 20 min), and 0.13±0.02 (SH3YL1 siRNA#2, 60 min) (mean±SD of 10 cells).

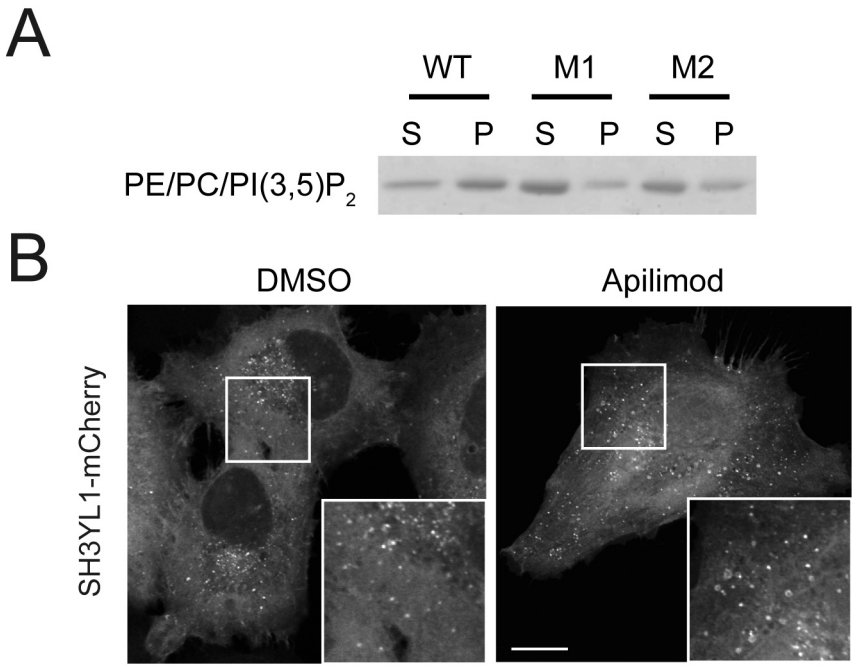


Fig. S7. Vesicular localization of SH3YL1 is not dependent on PI(3,5)P_2. (A) Liposome co-sedimentation assay using PE/PC-based liposomes supplemented with 10% of PI(3,5)P_2 (See Materials and methods in detailed). SH3YL1 recombinant proteins wild-type (WT), amino acid substitutions of $Lys^{14}Lys^{15}$ to $Ala^{14}Ala^{15}$ (M1), and amino acid substitutions of $Lys^{18}Arg^{21}$ to $Ala^{18}Ala^{21}$ (M2) are used in this assay. Data are representative of three independent experiments. **(B)** HeLa cells stably expressing SH3YL1-mCherry were treated by DMSO or 1 μM apilimod (a PIKfyve inhibitor) for 1 h. SH3YL1 is also localized at enlarged endosomes caused by the inhibition of PI(3,5)P_2 synthesis by PIKfyve, indicating its membrane localization is independent of PI(3,5)P_2. Images are representative of three independent experiments.