

Supplemental material

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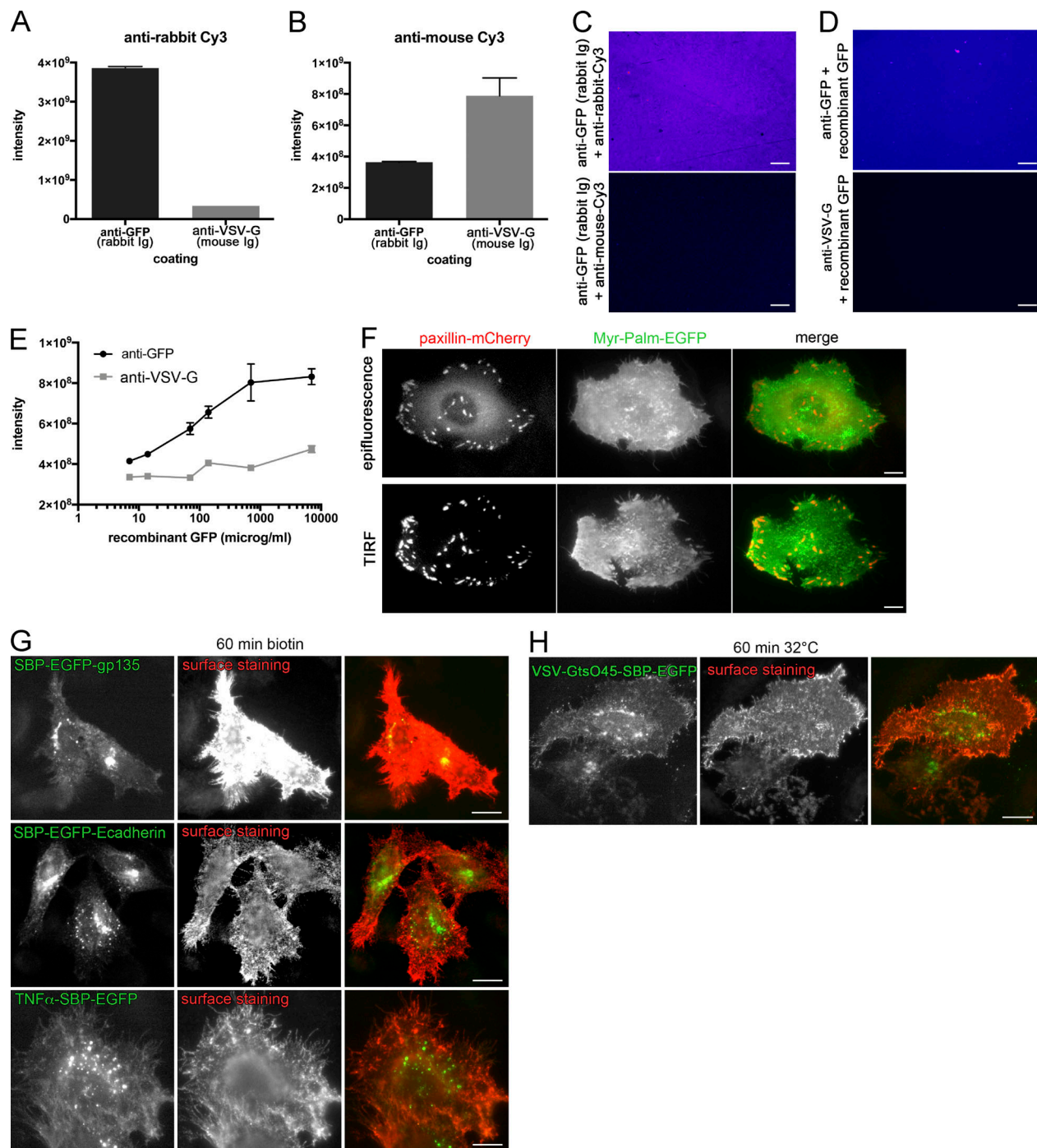


Figure S1. Validation of the SPI assay and visualization of the whole ventral plasma membrane by TIRF. Arrival at the plasma membrane after the induction of secretion without SPI. (A–C) Glass coverslips were coated with either anti-GFP antibodies (rabbit Ig) or anti-VSV-G antibodies (mouse Ig). Secondary anti-rabbit or anti-mouse antibodies conjugated to Cy3 were used to detect the primary antibodies coated. (C) Fields of view of coverslips coated with anti-GFP and revealed as indicated. The same parameters of acquisition were used for the two fields of view. Fire lookup table was used (generated with Fiji). Error bars represent mean \pm SEM. (D and E) Glass coverslips were coated with either anti-GFP antibodies or anti-VSV-G antibodies as control. Serial dilutions of recombinant GFP were incubated onto these coated coverslips. GFP fluorescence signal was quantitated (arbitrary units). (E) Fields of view of coverslips coated with anti-GFP (top) or anti-VSV-G (bottom) antibodies and incubated with recombinant GFP at 700 μ g/ml. Fire lookup table was used (generated with Fiji). (F) The same cell overexpressing paxillin-mCherry to visualize FAs and myristoylated and palmitoylated EGFP to visualize the plasma membrane was imaged using a microscope in either epifluorescence or TIRF mode. (G) Induction of secretion using the RUSH system. HeLa cells stably expressing Str-KDEL were transfected with TNF α -SBP-EGFP, SBP-EGFP-Ecadherin, or SBP-EGFP-gp135. Induction of trafficking by addition of biotin was performed at time 0. Surface staining was performed using an anti-GFP antibody (red) on nonpermeabilized cells. (H) Trafficking induction of the thermo-sensitive mutant VSV-G. HeLa cells were transfected with VSV-G tsO45-EGFP. Induction of trafficking by a temperature switch from 40°C to 32°C was performed at time 0. Surface staining was performed using an anti-VSV-G antibody (red) on nonpermeabilized cells. Scale bars, 10 μ m.

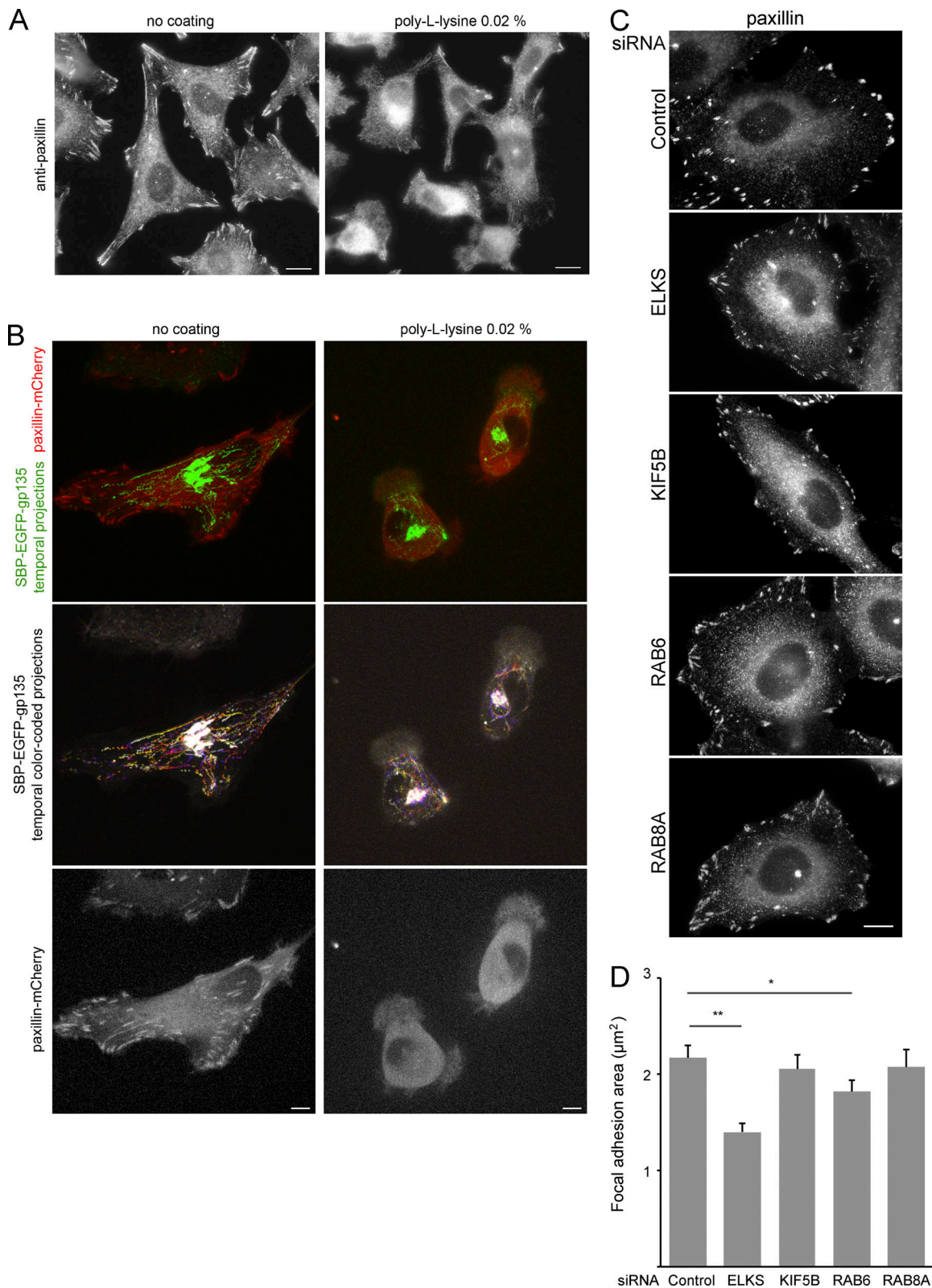


Figure S2. **Synchronized transport of SBP-EGFP-gp135 in cells coated on different substrates; effect of control, ELKS, KIF5B, RAB6, and RAB8A siRNAs on the size of FAs.** (A) HeLa cells seeded on glass coverslips either noncoated or coated with poly-L-lysine (0.02%). Focal adhesions were immunolabeled using an anti-paxillin antibody. (B) HeLa cells expressing paxillin-mCherry and Str-KDEL-SBP-EGFP-gp135 were seeded on glass coverslips either noncoated or coated with poly-L-lysine (0.02%). The transport of post-Golgi carriers containing SBP-EGFP-gp135 was monitored ~20 min after biotin addition. Temporal projections were obtained using the Fiji plugin Temporal-Color Code. (C) HeLa cells treated with control, ELKS, KIF5B, RAB6, or RAB8A siRNAs for 72 h were fixed and stained for paxillin. Representative images of cells treated with the different siRNAs and stained with an anti-paxillin antibody. (D) Quantification of the FA area (in μm^2) of cells treated as described in A (mean \pm SEM, $n = 3$). *, $P < 0.05$; **, $P < 10^{-4}$ (Student's t test). Scale bars, 10 μm .

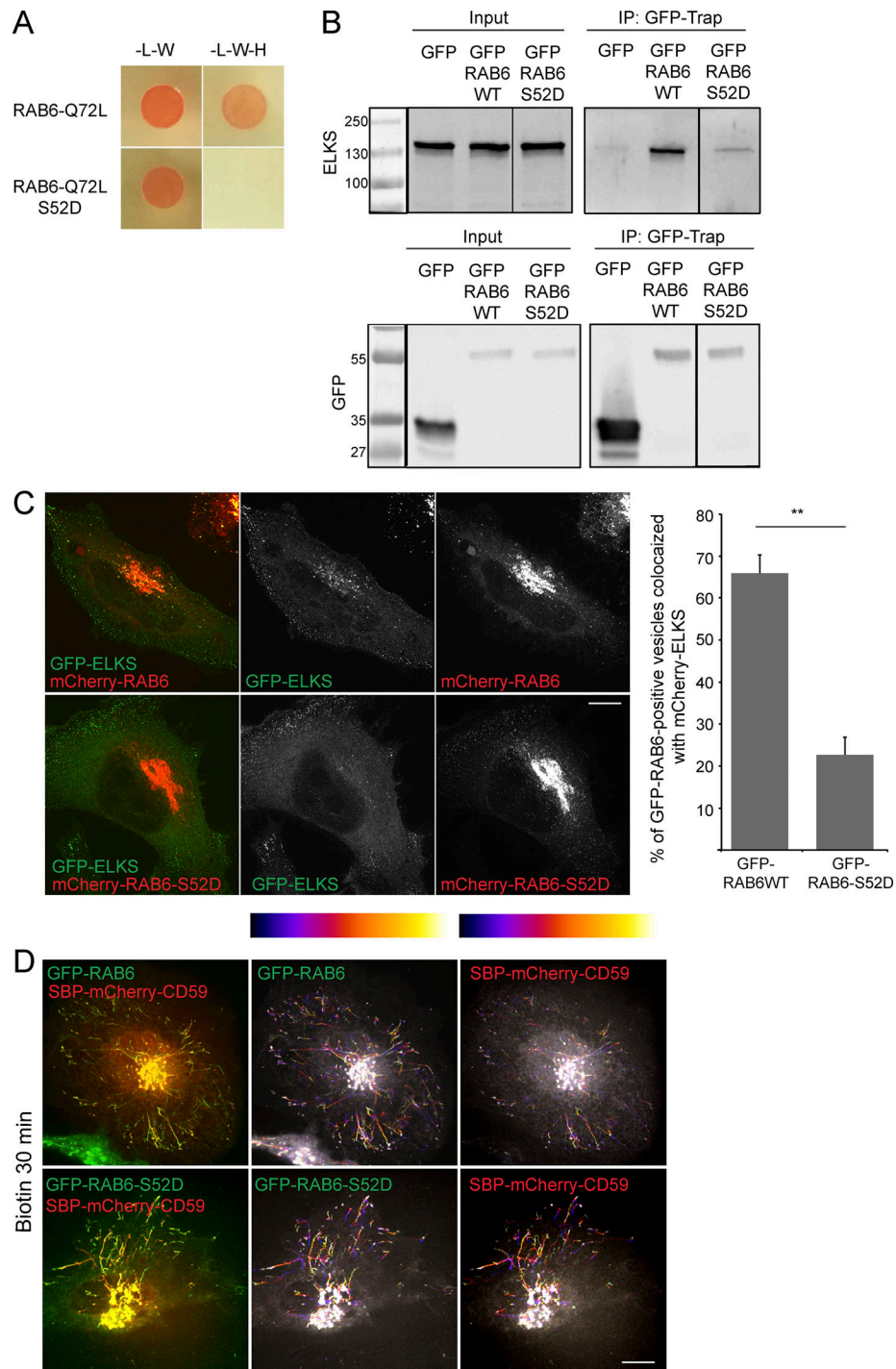


Figure S3. Characterization of the RAB6A-S52D mutant. (A) Yeast two-hybrid interactions between the RAB6-binding domain of ELKS (Monier et al., 2002) and RAB6A-Q72L WT or S52D. The *S. cerevisiae* reporter strain L40 was cotransformed with a plasmid encoding fusion proteins to detect interactions between RAB6-binding domain of ELKS and RAB6A-Q72L or RAB6A-Q72L-S52D. Growth on medium lacking histidine (-W-L-H) indicates an interaction between the encoded proteins. (B) Endogenous ELKS is pulled down by GFP-RAB6A and not by GFP-RAB6A-S52D. GFP-RAB6A, GFP-RAB6A-S52D, or GFP-transfected HeLa cell extracts were immunoprecipitated using the GFP-trap system. ELKS bound to GFP-RAB6 was revealed by Western blot analysis using anti-ELKS antibody. GFP, GFP-RAB6A, and GFP-RAB6A-S52D were revealed by anti-GFP antibody. Input represents a 5% load of the total cell extracts used in all conditions. (C) Left: HeLa cells coexpressing GFP-ELKS with mCherry-RAB6 or mCherry-RAB6-S52D were fixed and imaged. Right: Quantification of the number of GFP-RAB6- or GFP-RAB6-S52D-positive vesicles colocalizing with mCherry-ELKS (mean \pm SEM, $n = 3$). **, $P < 0.005$ (Student's *t* test). (D) HeLa cells were co-transfected with mCherry-CD59 and GFP-RAB6 or GFP-RAB6-S52D. Cells were observed by time-lapse imaging using a spinning disk microscope. Biotin was added at time 0. After a 30-min incubation with biotin, mCherry-CD59 localizes in the Golgi apparatus, and fast acquisition imaging was performed. A temporal projection of mCherry-CD59 and GFP-RAB6A WT or S52D signal over a 60-s video was performed using Fiji software and is represented with a temporal color code (top of each image). GFP-RAB6 and GFP-RAB6-S52D were colocalized on post-Golgi tracks exiting the Golgi complex en route to the plasma membrane. Scale bars, 10 μ m.

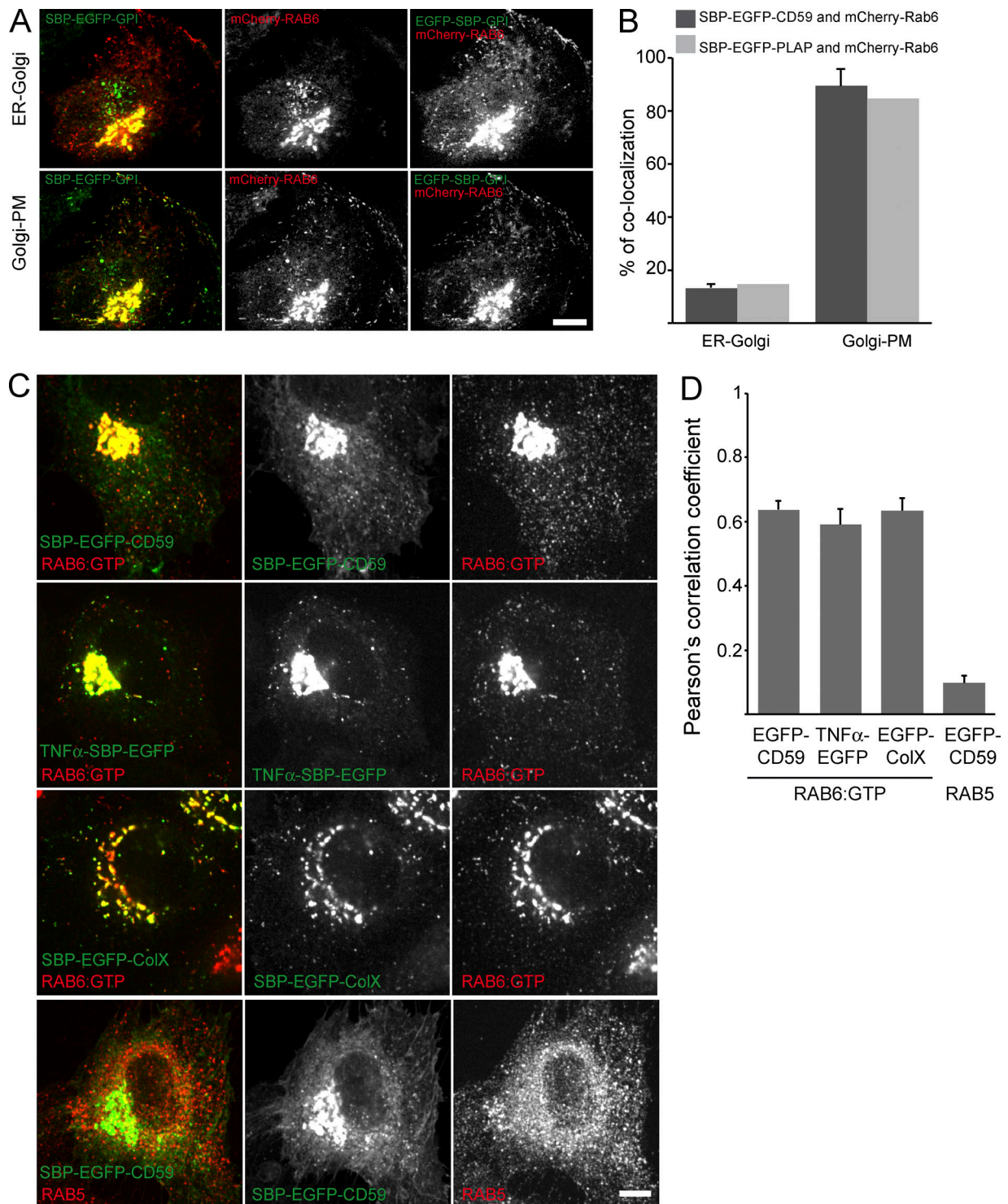


Figure S4. **Colocalization of RAB6 with the GPI-APs between ER-Golgi and Golgi to plasma membrane; colocalization of the cargos with endogenous RAB6:GTP.** (A) RPE-1 cells coexpressing mCherry-RAB6 with SBP-EGFP-CD59 or SBP-EGFP-PLAP were incubated for 10 min (to monitor ER to Golgi transport) or 30 min (to monitor Golgi to plasma membrane transport) with biotin and imaged using time-lapse video-microscopy. Representative images taken from videos of cells expressing SBP-EGFP-GPI are displayed. (B) Quantification of the percentage of colocalization between mCherry-RAB6 and SBP-EGFP-CD59 or SBP-EGFP-PLAP at 10 and 30 min after biotin addition (mean \pm SEM, $n = 2-12$ cells). Scale bar: 10 μ m. (C) Staining for endogenous RAB6:GTP of HeLa or RPE-1 cells expressing SBP-EGFP-CD59, TNF α -SBP-EGFP, or SBP-EGFP-CoIX incubated with biotin for 30 min. Staining for endogenous RAB5 of HeLa cells expressing SBP-EGFP-CD59 and incubated with biotin for 30 min. Scale bar: 10 μ m. (D) Colocalization (Pearson's coefficient) between RAB6, RAB5, and the cargos (mean \pm SEM, $n = 35-39$ regions from 12 cells).

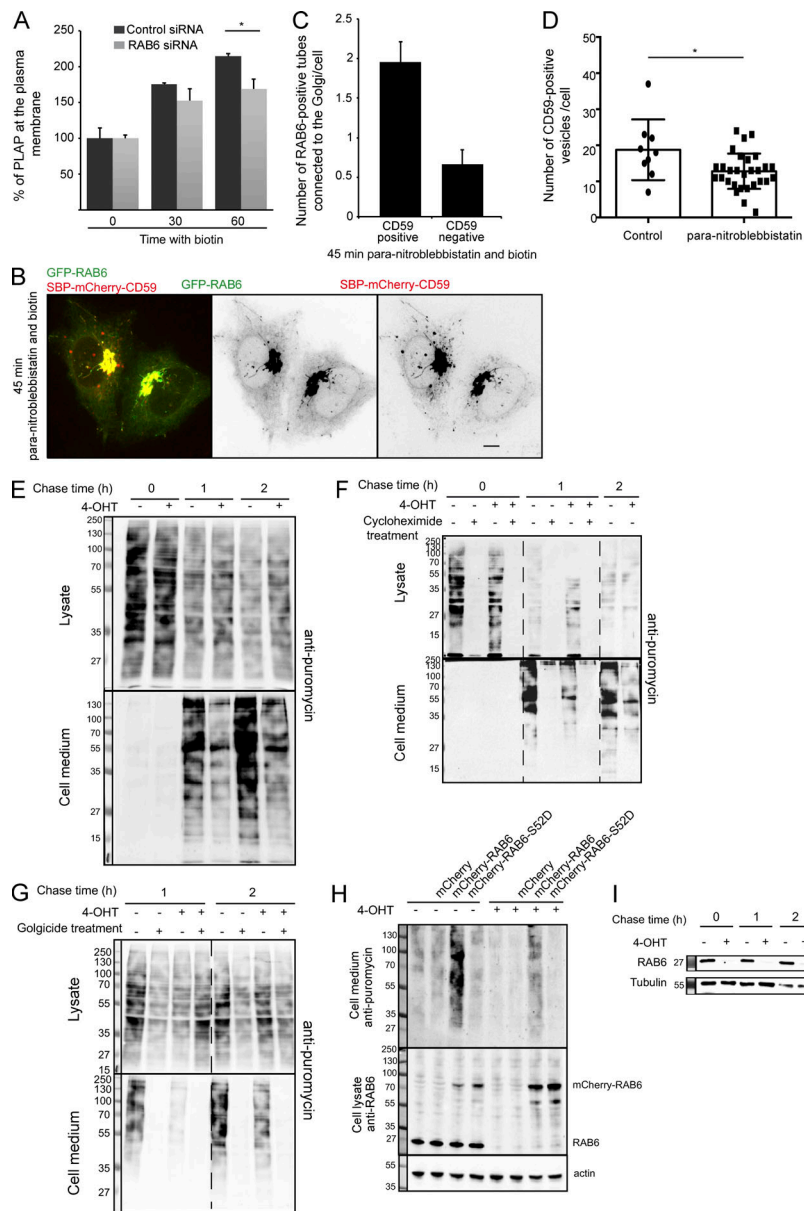


Figure S5. **RAB6 depletion affects PLAP arrival at the plasma membrane; myosin II-dependent fission of EGFP-CD59-positive vesicles from Golgi membranes; SUNSET assay for quantification of protein secretion in MEF RAB6^{lox/lox} cells; and effect of mCherry-RAB6wt and mCherry-RAB6-S52D on protein secretion.** (A) Quantification of SBP-EGFP-PLAP at the cell surface by FACS in cells transfected with control or RAB6 siRNA and incubated for 30 or 60 min with biotin (mean ± SEM, n = 3). P < 0.05 (Student's t test). After a 60-min incubation with biotin, we observed a 50% decrease in the arrival of PLAP at the plasma membrane. *, P < 0.05 (Student's t test). (B) HeLa cells coexpressing GFP-RAB6 and SBP-mCherry-CD59 were treated for 45 min with para-nitroblebbistatin. After 15 min, biotin was added in the medium to allow cargo release from the ER, and cells were imaged using time-lapse video-microscopy. Representative images taken from videos. Higher magnification on the right. Scale bar, 10 μm. (C) Quantification of the number of tubes connected to the Golgi complex containing RAB6 with or without CD59 in cells treated as indicated in B (mean ± SEM, n = 10 cells). (D) Quantification of the number of transport carriers in cells treated as indicated in B (mean ± SEM, n = 9–29 cells). *, P < 0.05 (Student's t test). (E) MEFs prepared from RAB6^{loxP/KO} Rosa26CreERT2-TG embryos (described in Bardin et al. [2015]; named after MEF RAB6^{lox/lox}) were treated with ethanol or 4-OHT for 96 h to induce RAB6 depletion. Cells were then incubated with puromycin and chased in puromycin-free medium for 0, 1, 2, 4, and 5.5 h. Total proteins in cell lysates and supernatants (medium) were labeled with an anti-puromycin antibody. Representative immunoblots are displayed. (F) MEF RAB6^{lox/lox} were treated with ethanol or with 4-OHT for 96 h to induce RAB6 depletion. Cells were treated with cycloheximide for 15 min, then incubated for 30 min with puromycin and chased in a medium without puromycin for 0, 1, and 2 h. Total proteins in cell lysates and supernatants were labeled with an anti-puromycin antibody. Representative immunoblots are displayed. (G) MEFs RAB6^{lox/lox} were treated with ethanol or 4-OHT for 96 h. Cells were treated with Golgicide A for 30 min, then incubated for 30 min with puromycin and chased in a medium without puromycin for 0, 1, and 2 h. Total proteins in cell lysates and supernatants were labeled with an anti-puromycin antibody. Representative immunoblots are displayed. (H) MEF RAB6^{lox/lox} cells were transfected with mCherry, mCherry-RAB6, or mCherry-RAB6-S52D and treated with ethanol or 4-OHT for 96 h. Cells were then incubated with puromycin and chased in puromycin-free medium for 1 h. Total proteins in cell supernatants (medium) were labeled with anti-puromycin. Cell lysates were incubated with anti-RAB6 antibody. Actin was used as a loading control. Representative immunoblots are displayed. (I) MEF cells treated as indicated in A were subjected to Western blot analysis. RAB6 was revealed using anti-RAB6 antibody. Tubulin signal was used as a loading control.

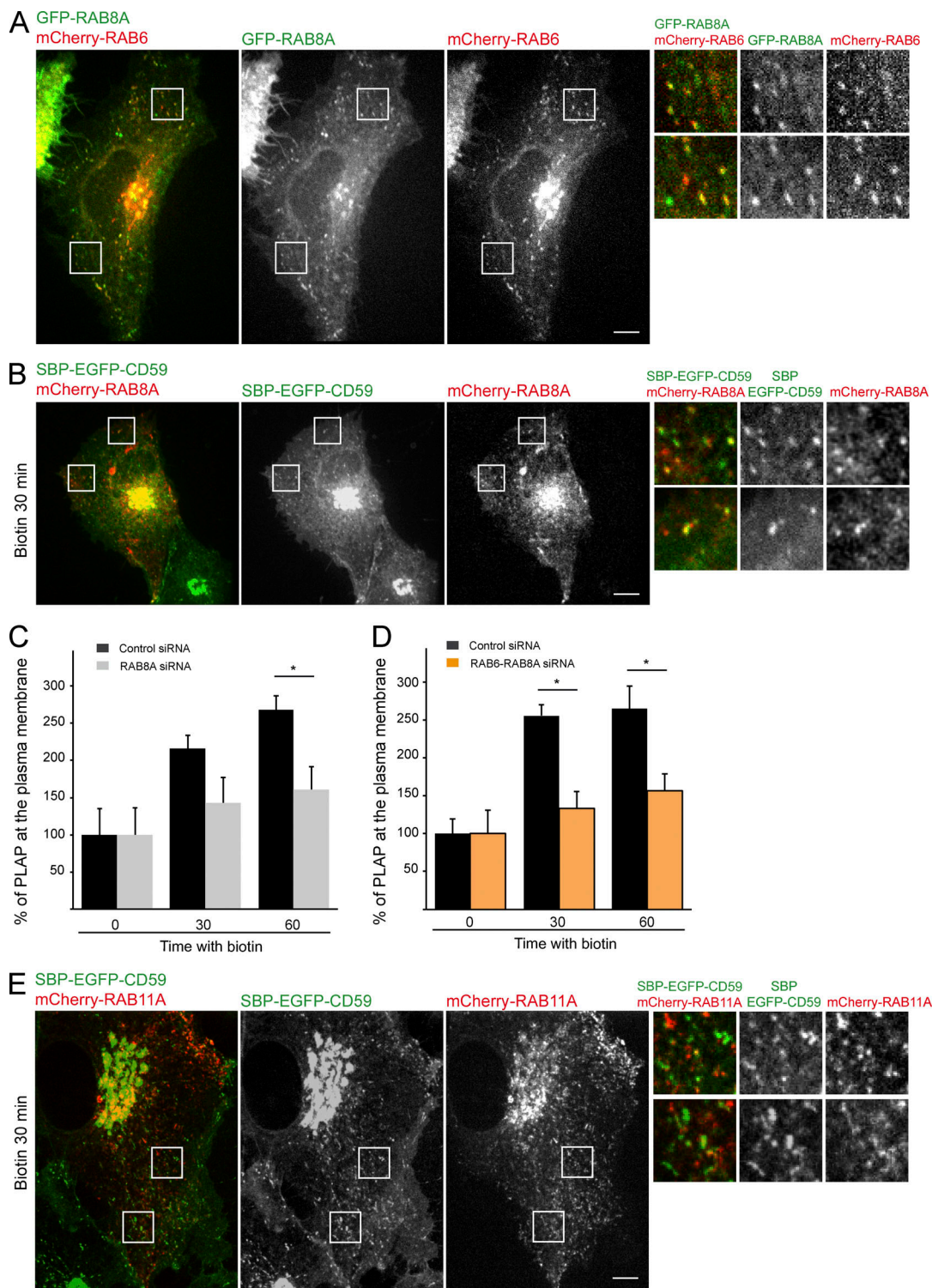
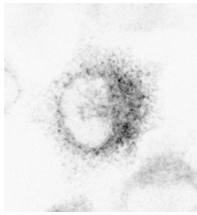
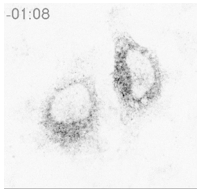


Figure S6. **RAB6 and RAB8 are found colocalized on post-Golgi carriers; RAB6 and RAB8 act in the same post-Golgi trafficking pathway.** (A) HeLa cells coexpressing GFP-RAB8A and mCherry-RAB6 were imaged using a time-lapse spinning-disk confocal microscope. Representative images taken from videos are displayed. (B) RPE1-SBP-EGFP-CD59 coexpressing mCherry-RAB8A were incubated for 30 min with biotin to allow cargo release from the ER. Cells were imaged using a time-lapse spinning-disk confocal microscope. Representative images taken from videos are displayed. (C) Quantification of SBP-EGFP-PLAP at the cell surface by FACS in cells transfected with control or RAB8A siRNA and incubated for 30 or 60 min with biotin (mean \pm SEM, $n = 3$). *, $P < 0.05$ (Student's t test). After a 60-min incubation with biotin, we observed a 50% decrease in the arrival of PLAP at the plasma membrane. (D) Quantification of SBP-EGFP-PLAP at the cell surface by FACS in cells transfected with control or RAB6 and RAB8A siRNA and incubated for 30 or 60 min with biotin (mean \pm SEM, $n = 3$). *, $P < 0.05$ (Student's t test). After a 30- or 60-min incubation with biotin, we observed a 50% decrease in the arrival of PLAP at the plasma membrane. (E) RPE1-SBP-EGFP-CD59 coexpressing mCherry-RAB11A were incubated for 30 min with biotin to allow cargo release from the ER. Cells were imaged using a time-lapse spinning-disk confocal microscope. Representative images taken from videos are displayed. Scale bars, 10 μ m.



Video 1. **Synchronized secretion of SBP-EGFP-ColX.** HeLa cells stably expressing SBP-EGFP-ColX were observed in real time using a spinning disk microscope. A projection of several Z-slices is shown. Acquisition was performed every 40 s for 36 min, and biotin was added at frame 2. Corresponds to [Fig. 1 A](#).



Video 2. **Synchronized secretion of SBP-EGFP-ColX using the SPI assay.** HeLa cells stably expressing SBP-EGFP-ColX and seeded on anti-GFP-coated coverslips (SPI assay) were observed in real time using a spinning disk microscope. A projection of several Z-slices is shown. Time is indicated in minutes:seconds. Biotin was added at frame 2 (00:00). Corresponds to [Fig. 1 C](#).