Fig. S1. A. The amount of Rab4b at the protein level was quantified in 4 independent experiments using anti Rab4b antibodies in HeLa cells (wt) and HeLa cells stably expressing HA-Rab4b treated with anti Rab4b siRNA or control siRNA. A representative western blot is shown in Fig. 2A (left panel). The right panel shows a comparison of the expression level of Rab4b in HeLa cells stably expression GFP-Rab4b or HA-Rab4b using anti Rab4 antibodies. The western blot was re-probed with anti tubulin antibodies as a loading control. **B**. HeLa cells stably expressing GFP-Rab4b were treated for indirect immunofluorescence with anti Rabip4 antibodies. A confocal image corresponding to GFP-Rab4b (green), endogenous Rabip4 (red), and the merged image were shown. **C**. HeLa cells stably expressing GFP-Rab4b were transiently transfected with pDsRes-Rabip4. Cells were treated for indirect immunofluorescence with anti EEA1 antibodies. A confocal image corresponding to GFP-Rab4b (green), DsRed-Rabip4 (red), the merged image of the green and red labeling, and EEA1were shown. The bar corresponds to 10 μm.

Fig. S2. A. HeLa cells (wt) were transfected for 48 h with control or anti Rab4b siRNA. Cells were prepared for glycerol gradient centrifugation as described in the Material and Methods section. The amount of TfR was analyzed in each fraction by western blot. A representative experiment and the quantification of 3 independent fractionations are shown. B. HeLa cells, wt or stably expressing GFP-Rab4b, were subjected to glycerol gradient centrifugation and TfR amount was determined as above. A representative experiment is shown.

Fig. S3. A. HeLa cells (wild type, wt) or expressing GFP-Rab4b were incubated for 1 h with AlexaFluor 594-coupled Tf at 20°C. Cells were rapidly washed and incubated for increasing periods of time at 37°C in presence of an excess of Holo-Tf. At the end of the incubation cells were washed and treated for confocal analysis. 20 fields (around 20 cells/field) in two independent experiments were quantified as described in Material and Methods. Amount of intracellular Tf was normalized by the cell number in each field and the results are expressed relative to one field in the time 0 of wt cells taken arbitrary to 100. **B.** The same experiment as in A was performed on wt HeLa cells transfected with control or anti Rab4b siRNA. * indicates that the difference is significant relative to the time 0 of the same experimental condition; # points to significant differences between wt and GFP-Rab4b expressing cells, or cells treated with control or anti Rab4b siRNA ($p<0.02$, Mann and Whitney test).

Fig. S4. A. HeLa cells stably expressing GFP-Rab4b were incubated for 90 min with Texas-Red coupled Tf. At the end of the incubation, cells were treated for immunofluorescence to detect EEA1 and were analyzed by confocal microscopy. The figure shows representative labeling for GFP-Rab4b (green), internalized Tf (iTf in red) and EEA1 (blue). A merge image of the three labeling is shown as well as the colocalized pixels between GFP-Rab4b and internalized Tf or between GFP-Rab4b and EEA1. **B.** HeLa cells stably expressing GFP-Rab4b were incubated 1 h at 4°C with Texas-Red coupled Tf. Cells were then incubated at 37°C for the indicated period of time and were treated for immunofluorescence to detect EEA1 and were analyzed by confocal microscopy as in A. The colocalization of internalized transferrin with EEA1 (open circles) or GFP-Rab4b (black circles) was determined by using the JACoP Plugin of ImageJ in order to determine the fraction of iTf overlapping either with EEA1 or GFP-Rab4b (Manders' coefficient). 100 cells were analyzed in 3 independent experiments. **C.** Wild type HeLa cells were transfected with control or anti Rab4b siRNA. 48 h later they were incubated for 90 min with Texas-Red coupled Tf. Cells were then fixed and analyzed by confocal microscopy.

Fig. S5. HeLa cells stably expressing GFP-Rab4b were transiently transfected with pIRES-Hygro-mCherryRab11a. One day later, they were treated for confocal analysis. The figure shows images corresponding to GFP-Rab4b, mCherryRab11a, and endogenous AP1γ and the indicated merge images. The delineated regions are shown enlarged in the right. Bar corresponds to 10 μm.

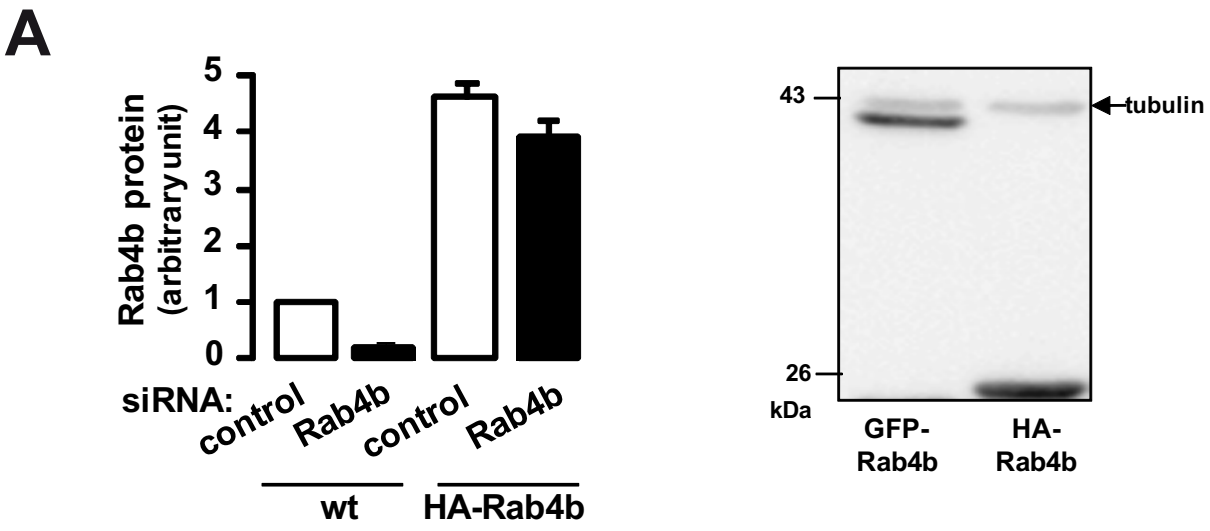
Fig. S6. Endogenous Vamp3 is partially colocalized with TfR but not EEA1. HeLa cells (wt) were treated for indirect immunofluorescence of TfR (green) and Vamp3 (Red) in panel **A** or EEA1 (green) and Vamp3 (red) in panel **B**. Cells were then analyzed by confocal microscopy and the figure shows representative images of the green and red labeling as well as the merge image and the colocalized pixels. Bars correspond to 10 μm.

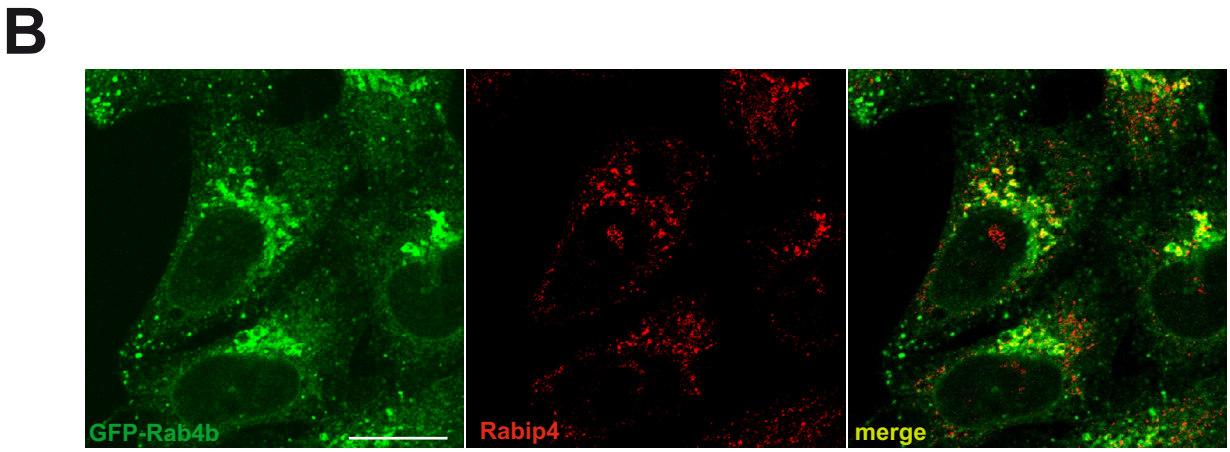
Fig. S7. Rab4b interaction with adaptins. A. Yeasts were co-transformed with the bait Rab4b Q67L and the 3 adaptins AP1γ, AP2α, and AP3δ as the prey. The expression of the expressed proteins is controlled with the indicated antibodies and the result of an interaction assay detecting β-galactisidase activity is shown. **B.** 293 cells were transfected either with a mock vector or pcDNA3-HA-mRab4b. 36 h later lysates were prepared and subjected to immunoprecipitation with anti HA antibodies. Total lysates and proteins associated with the immune pellet were analyzed by Western blot to detect AP1γ, AP2α, AP3δ, and HA-mRab4b as described in Fig.4. **C-D**. HeLa cells stably expression GFP-Rab4b (green) were treated for indirect immunofluorescence with anti AP2α (α adaptin, red)) in C and anti AP3δ (δ adaptin, red) in D. A confocal section of each labeling and the merge image are shown as well as an enlarged view of peripheral GFP-Rab4b positive structures.

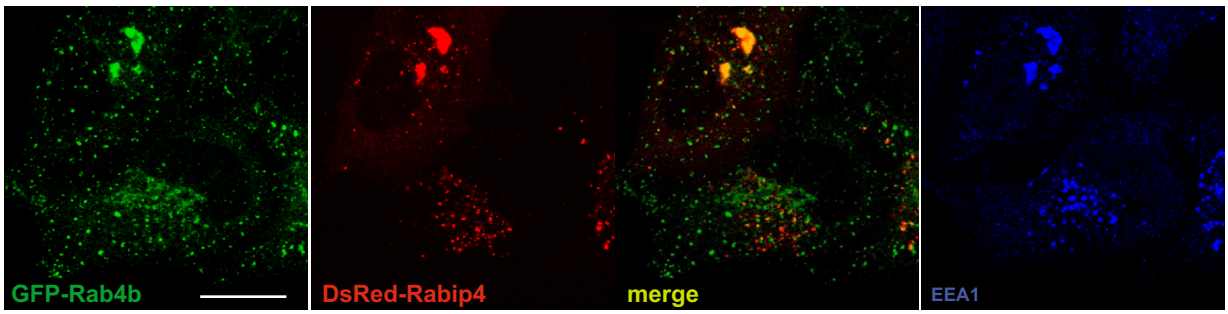
Fig. S8. GFP-Rab4b is colocalized with clathrin heavy and light chain. A-B. HeLa cells stably expressing GFP-Rab4b were treated for indirect immunofluorescence to detect clathrin heavy chain (CHC) and EEA1. Figure A shows confocal images corresponding to GFP-Rab4b (green), CHC (red), the merge of these two images, and the colocalized pixels. Enlarged views of the delineated squares were shown on the right. **B.** The corresponding confocal stacks were treated with Volocity 5.0 software to obtain a representation of the colocalized voxels (3D colocalized), and the merge 3D image of the three labeling (GFP-Rab4b in green, CHC in red, and EEA1 in purple). An enlarged view of the delineated squares is shown for GFP-Rab4b and CHC. Arrows point to structures that contain GFP-Rab4b, CHC, but not EEA1 whereas open arrows point to structures positive for the three proteins. **C.** HeLa cells stably expressing HA-Rab4b were transiently transfected with a vector expressing a clathrin light chain (LCa) in fusion with GFP. Cells were treated for indirect immunofluorescence to detect HA-tag. The figure shows confocal images of GFP-LCa (green), HA-Rab4b (red), the merge of these two images, and the colocalized pixels. Enlarged views of the delineated squares are shown on the right.

Fig. S9. Rab4b is required for AP1γ association with light density vesicles. Wt HeLa cells were transfected with control or anti Rab4b siRNA. 48 later post nuclear supernatants were prepared and subjected to a glycerol density gradient and 13 fractions were collected at the end of the centrifugation. Proteins from an equal volume of each fraction were separated by SDS-PAGE and AP1γ is detected by Western blot. **A.** A representative Western blot is shown. A coloration of all the proteins by ponceau red shows that the amount of proteins is equivalent in the two experimental conditions. **B.** The graph represents the quantification ± s.e.m. of three independent experiments. The amount of AP1γ is expressed as the percentage of the sum of each fraction. * indicates significant difference with $p<0.05$ (paired Student t test).

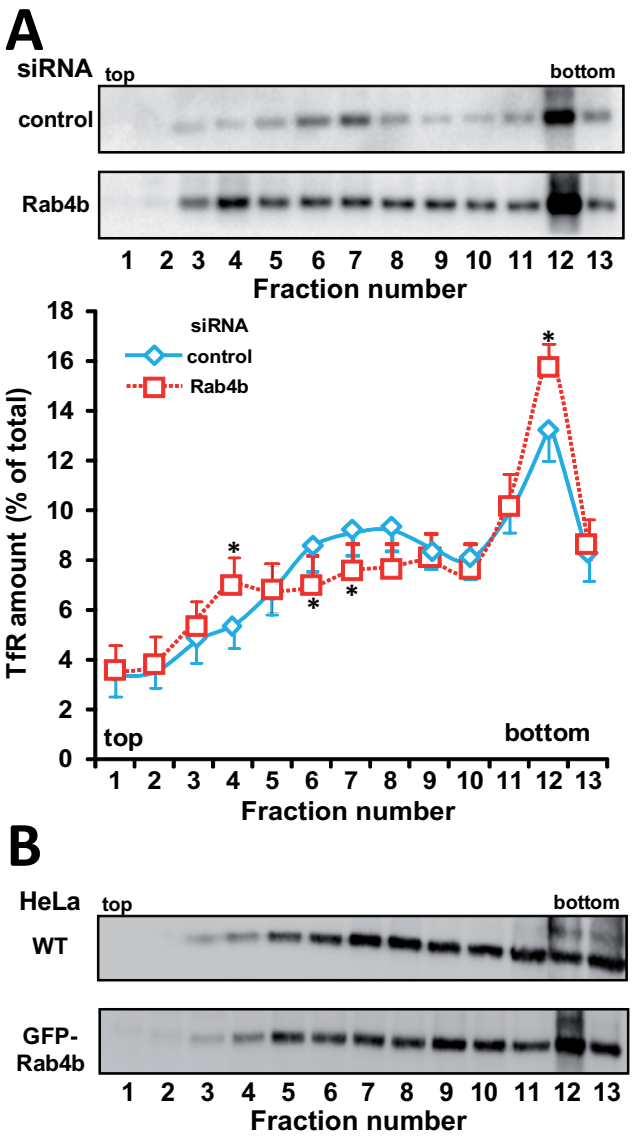
Fig. S10. Comparison between GFP-Rab4b and GFP-Rab4a stably expression cells. A-D HeLa cells expressing GFP-Rab4b and two clones of HeLa cells expressing different levels of GFP-Rab4a were analyzed for the localization of the overexpressed GFP-Rab4 (green), AP1γ (red), and EEA (blue) (A-C). The confocal images in A and B were acquired without changing the setting parameters. **D**. The comparison of the levels of expression between the different clones (cln) was made by Western blot by using anti GFP antibodies. **E**. The graph represents the number of GFP-Rab4 containing structures with a circularity comprised between 0.5 and 1 and with a size between 5 and 40 (pixel units) in 10 confocal images for each conditions as those shown in A, B, and C. The obtained numbers are normalized by the average intensity of EEA1 labeling in each field as an index of cell number (ImageJ, analyze particles). **F**. Steady state HRP-Tf binding was determined in HeLa cells, wt or stably expressing GFP-Rab4b or 3 different levels of GFP-Rab4a, as described in Fig.2. The expression levels of the GFP-Rab4 and of HSP90 as a loading control are shown. **G**. Schematic representation of the consequences of changes Rab4 expression in HeLa cells. Arrows in red were for Rab4 (a or b) overexpression. Arrows in blue was for Rab4b down regulation. TGN: *trans* Golgi network; EE: early endosomes; RE: recycling endosomes.







Supplemental figure 1



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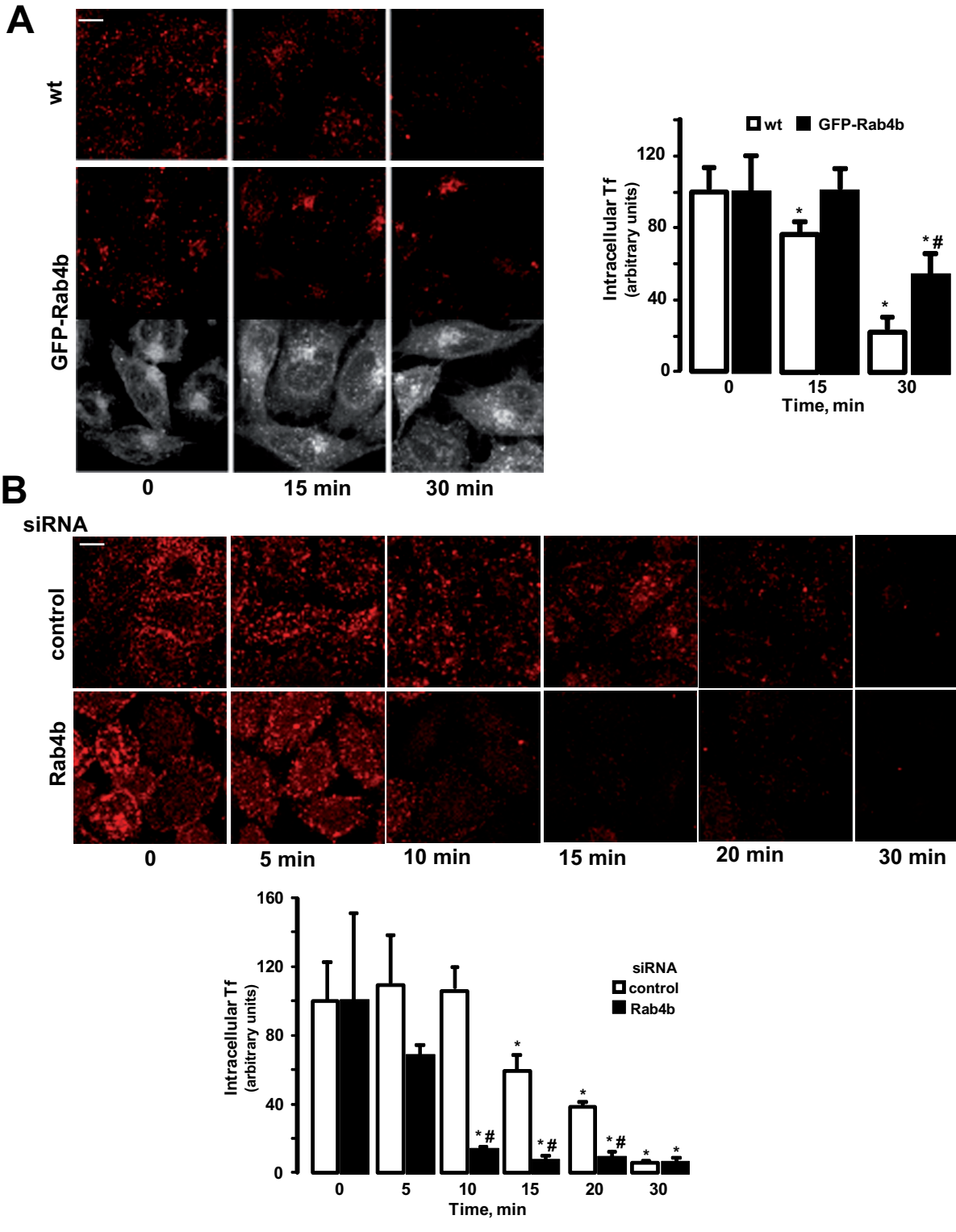

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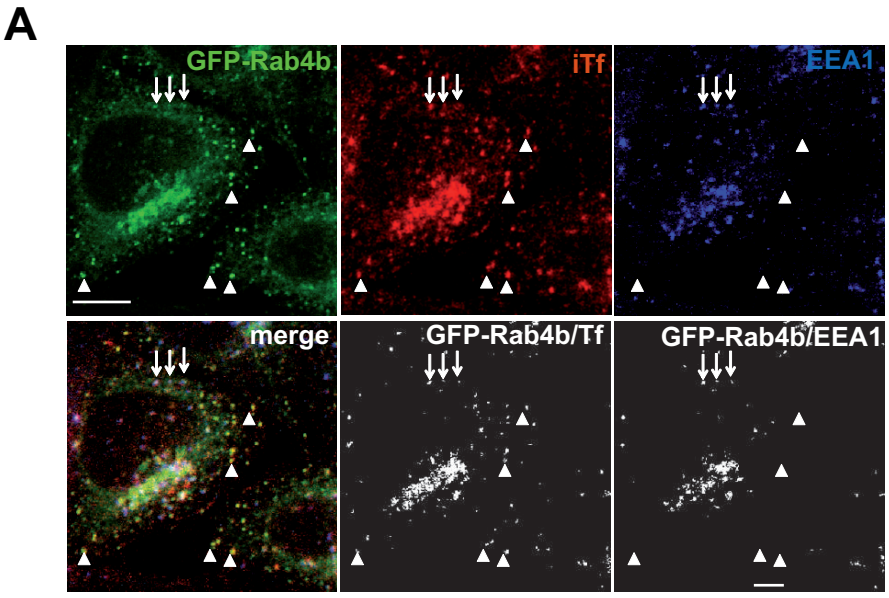

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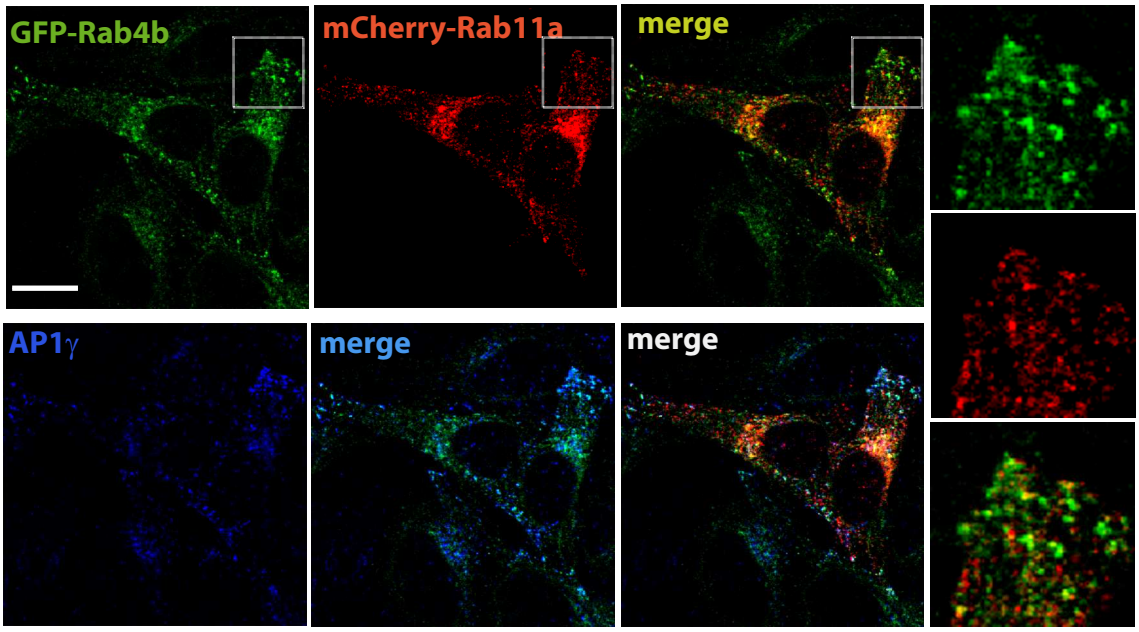

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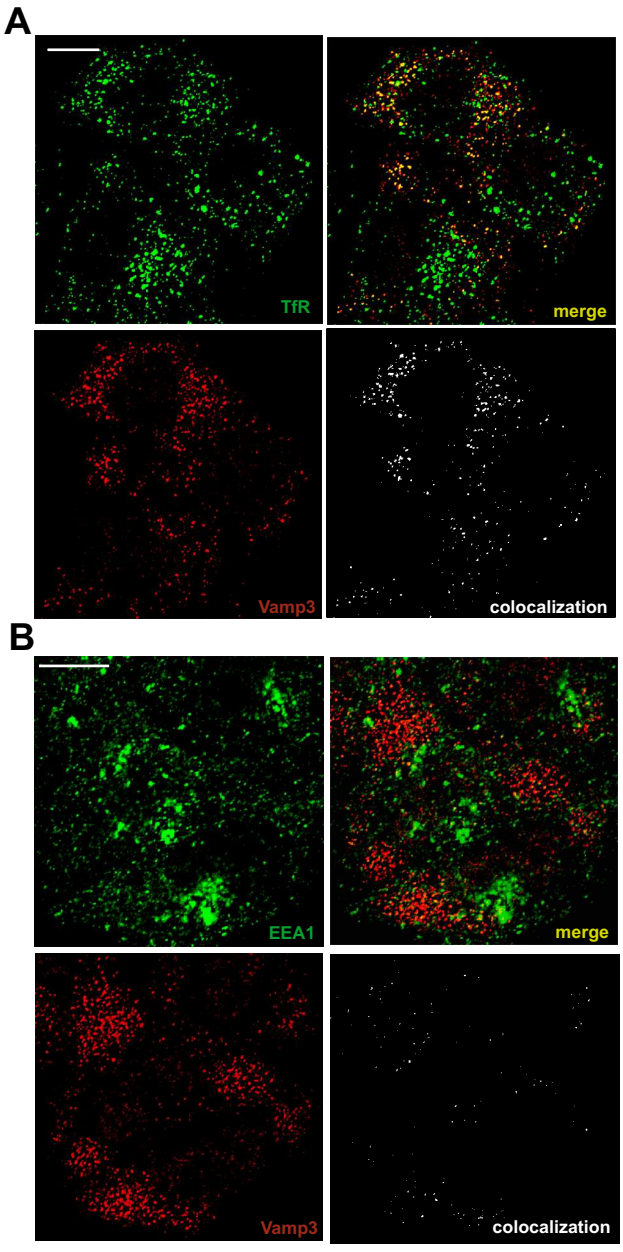

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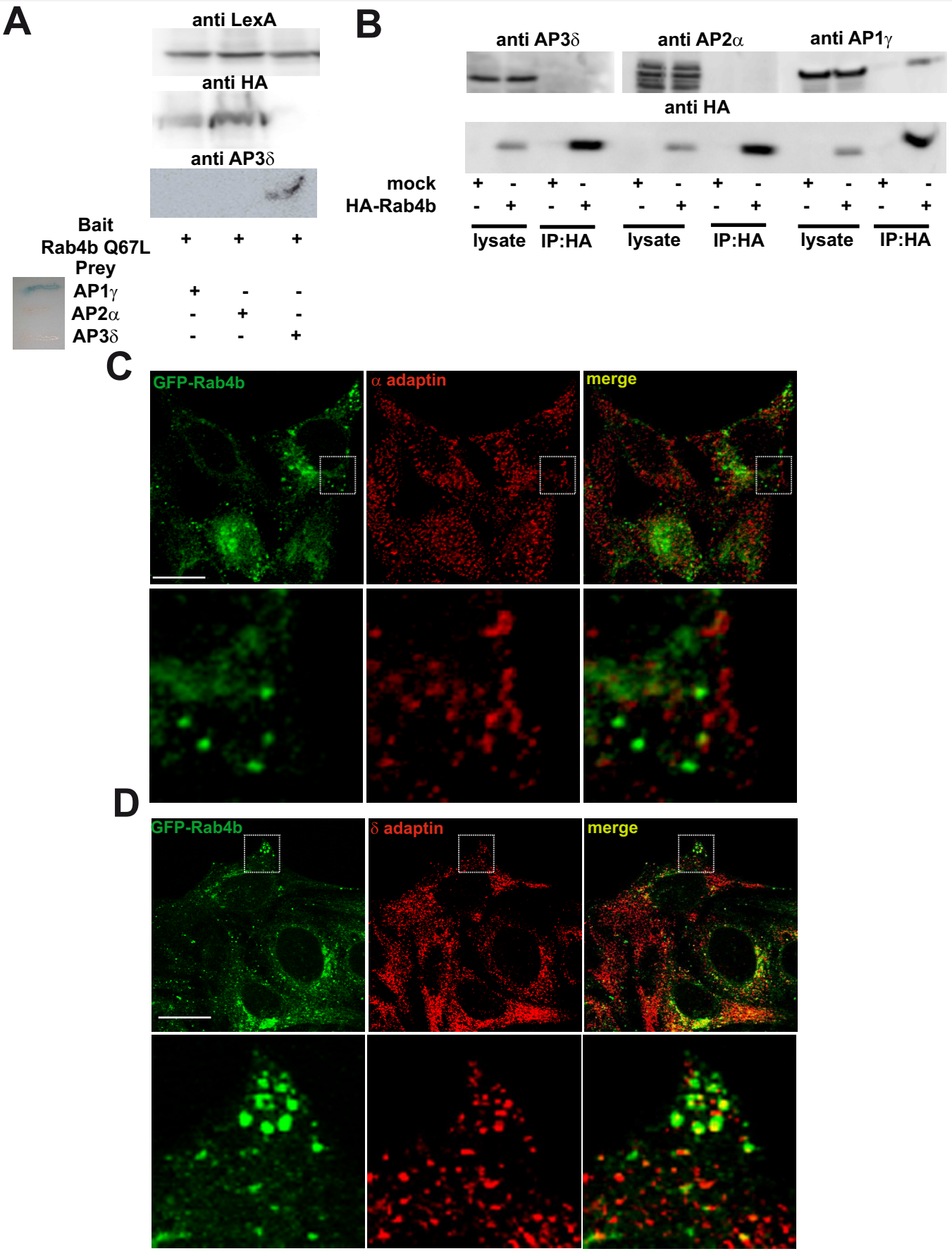

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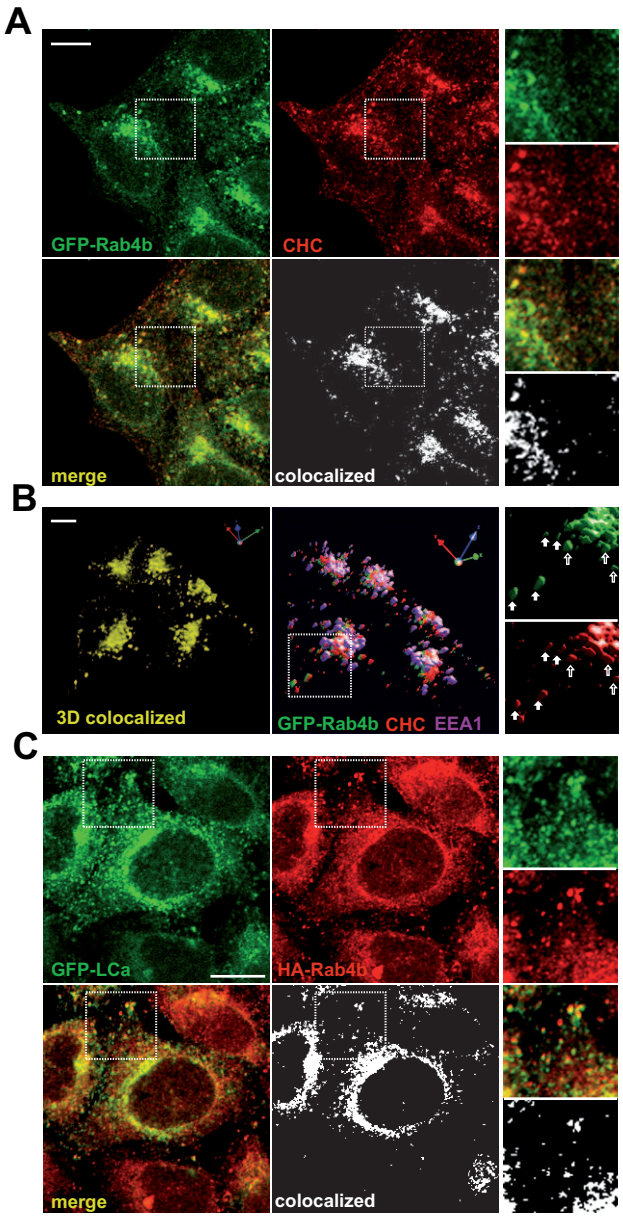

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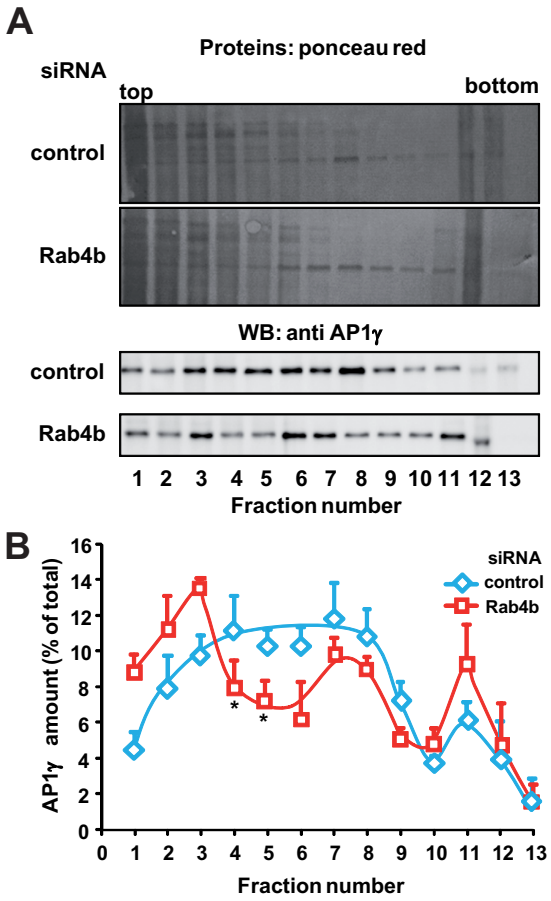

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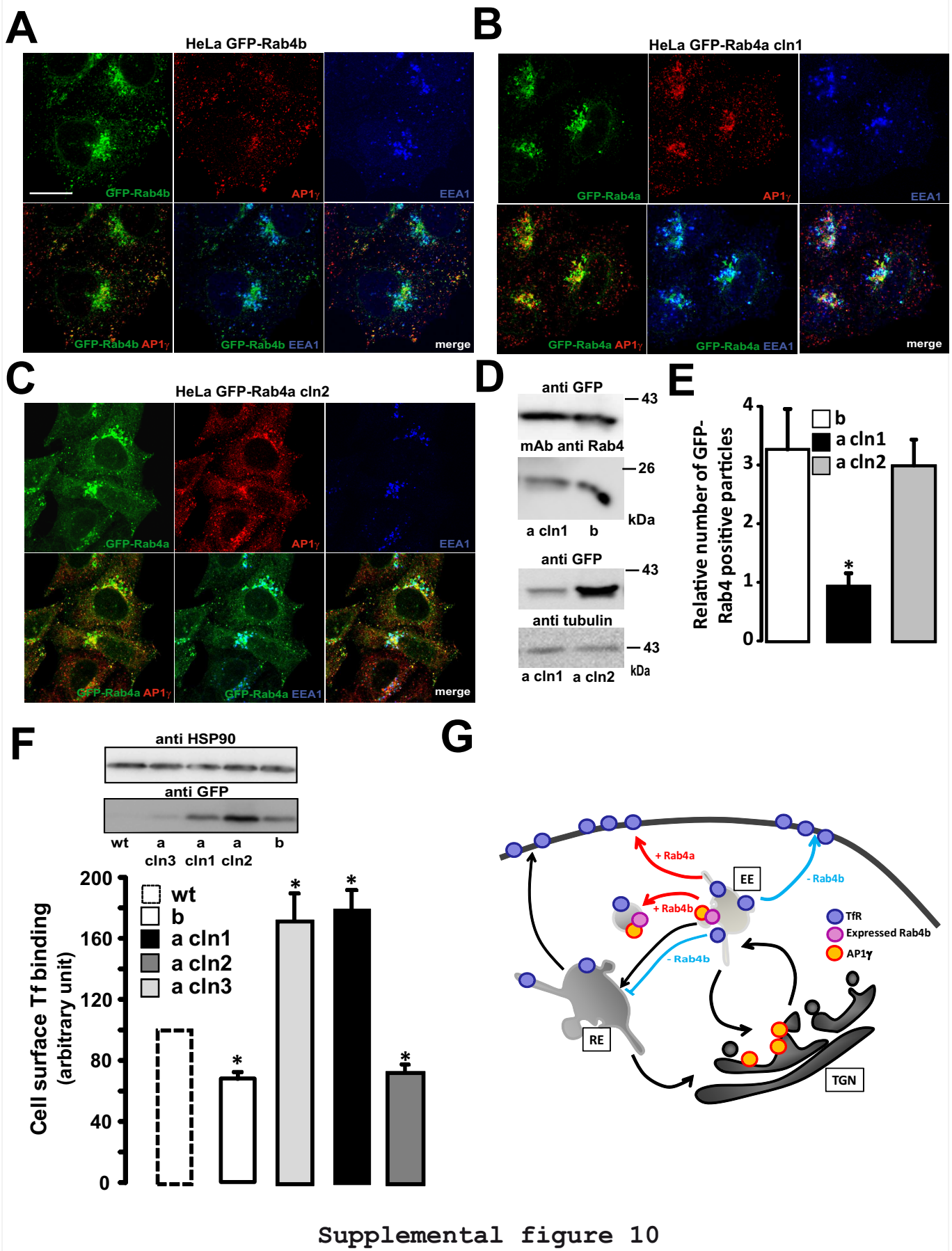

Supplemental figure 10