Supplemental figure 1

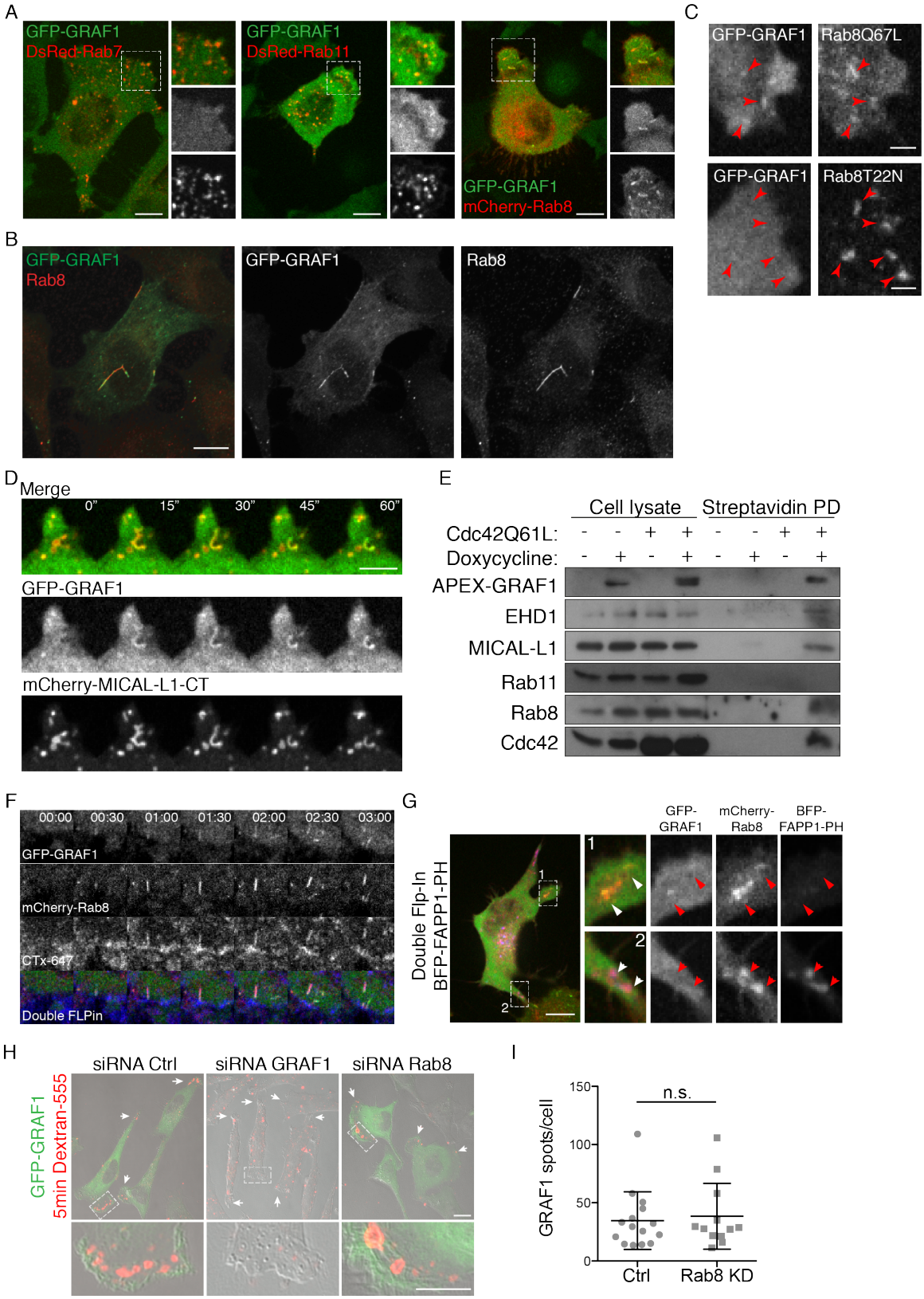


Figure S1.

A) Micrographs showing GFP-GRAF1 expressing Flp-In T-REx HeLa cells transfected with different Rab proteins as indicated. B) Micographs showing fixed GFP-GRAF1 Flp-In T-REx HeLa cells stained with antibodies to detect endogenous Rab8. C) Fluorescent image from a time-lapse of double Flp-In T-REx HeLa cells expressing GFP-GRAF1 and mCherry-Rab8Q67L (upper panel) or GFP-GRAF1 Flp-In T-REx HeLa cells transfected with mCherry-Rab8T22N (lower panel). Red arrows indicate colocalisation between GFP-GRAF1 and mCherry-Rab8Q67L or absence of colocalisation between GFP-GRAF1 and mCherry-Rab8T22N. Scale bar = 2μm. D) Time lapse images of GFP-GRAF1 Flp-In T-REx HeLa cells transfected with the mCherry-MICAL-L1-CT probe, as a marker of active Rab8. E) Immunoblot analysis of biotinylated proteins pulled-down from Cdc42Q61L transfected or non-transfected induced and non-induced Flp-In T-REx-GRAF1-APEX2 cell lysates. Cells were labeled with Biotin-Phenol for 30 minutes followed by 10 minutes incubation with H_2O_2, proteins in close proximity of GRAF1-APEX2 were biotinylated and pulled-down with neutravidin beads. Displayed blots are not from the same blot due to that some of the proteins have almost the same running size. F) Micrograph sequence from live cell imaging of double Flp-In T-REx HeLa cells during uptake of fluorescently labeled cholera toxin. Time is indicated in minutes and seconds. G) Fluorescent micrographs of double Flp-In T-REx HeLa cells transfected with the PH domain of FAPP1 (BFP-FAPP1-PH), which marks Golgi-derived secretory vesicles. H) Fluorescent micrographs showing GRAF1 expressing Flp-In T-REx HeLa cells transfected with control siRNA (Ctrl) or siRNA against GRAF1 or Rab8 and incubated with dextran for 5 minutes, washed and fixed. White arrows indicate the presence or absence of dextran accumulation at cell protrusions. I) Quantification of the number of GRAF1 structures (fluorescent spots determined by the Imaris software) at protrusions per cell in GFP-GRAF Flp-In T-REx HeLa cells transfected with control siRNA (Ctrl) or siRNA against Rab8. 15 cells per transfection from three independent experiments were analyzed. (n.s. not significant. t-test). Scale bar= 10μm.

Supplemental figure 2

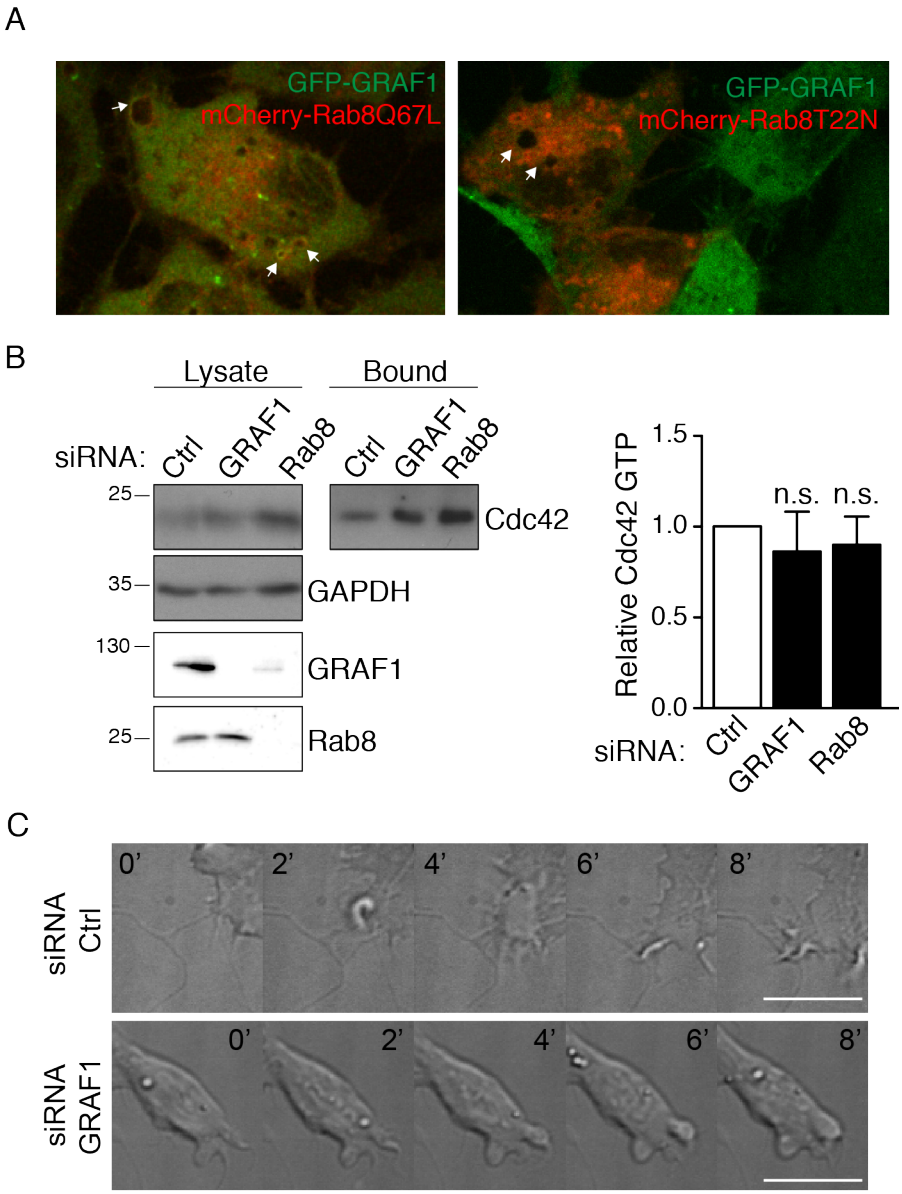


Figure S2.

A) Representative fluorescent micrographs of double Flp-In T-REx HeLa cells expressing GFP-GRAF1 and mCherry-Rab8Q67L and GFP-GRAF1 expressing cells transfected with mCherry-Rab8T22N during recovery from hypotonic treatment. White arrows indicate *vacuole like dilatations* (VLDs) that formed during hypotonic treatment. B) Left panel, immunoblot analysis of total lysates and the bound fractions from a Cdc42 activity pull down using PAK-PBD beads were used to pull down Cdc42 from GFP-GRAF1 expressing Flp-In T-REx HeLa cells transfected with the control siRNA (Ctrl) or

siRNA against GRAF1 or Rab8. Immunoblot analysis of total lysates of GRAF1 and Rab8 show the knock down efficiencies and GAPDH reference protein loading. Right panel, quantification of three independent Cdc42 pull down experiments performed as described in the material and methods section (n.s= not significant, Kruskall-Wallis test). C) DIC time-lapse images of GFP-GRAF1 Flp-In T-REx HeLa cells transfected with control siRNA (Ctrl) or siRNA against GRAF1. Scale bar = 2 µm

Supplemental figure 3

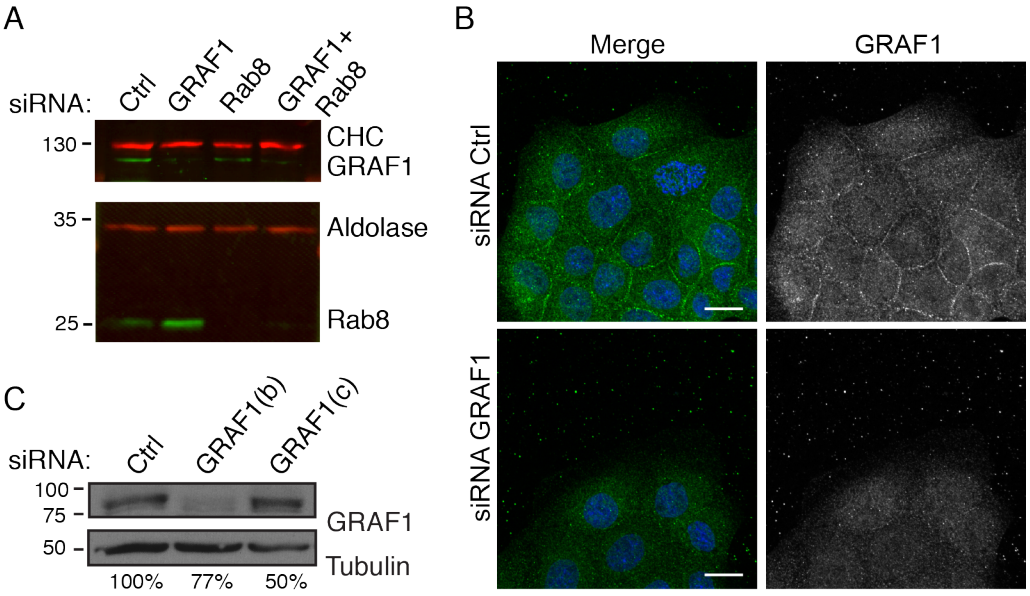
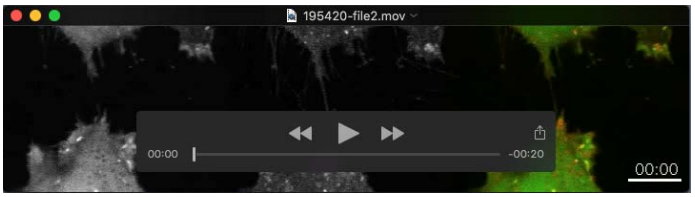


Figure S3.

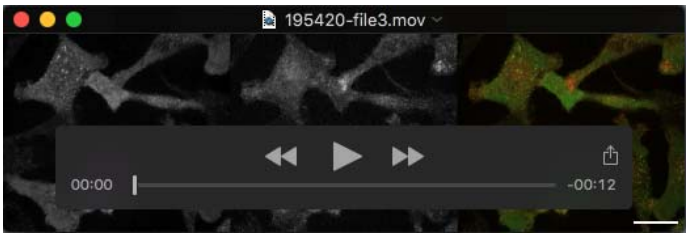
A) Immunoblot analysis of Flp-In T-REx GFP-GRAF1 cells treated with the indicated siRNAs. Aldolase and clathrin heavy chain were used as loading controls. B) Fluorescent micrographs showing MDCK type II cells grown on coverslips, fixed and stained for GRAF1 after transfection with control siRNA (Ctrl) or siRNA against GRAF1. Scale bar= 10µm. C) Immunoblot analysis of MDCK type II cells treated with two different siRNAs against GRAF1. Knock down efficiencies are indicated as %. Tubulin was used as a loading control.

Link to supplemental videos

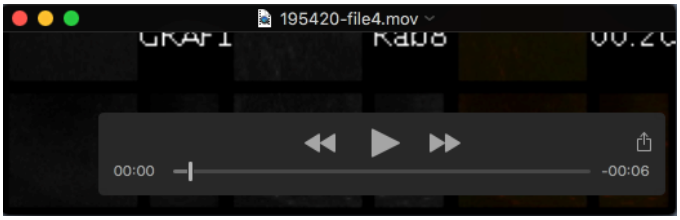
https://figshare.com/s/9c525522fbadf219fad2



Movie 1. Dynamics of GFP-GRAF1 and mCherry-Rab8 at cell protrusion. Double Flp-In T-REx HeLa cells expressing GFP-GRAF1 (green) and mCherry-Rab8 (red). White arrows and numbers highlight one example per category. Note that several events per category appear throughout the movie. Images were acquired with a spinning disk confocal microscope every 3 seconds for 10 minutes (Zeiss Cell Observer).



Movie 2. Effect of osmotic changes on the localization of GFP-GRAF1 and mCherry-Rab8 in double FLPin cells. Double Flp-In T-REx HeLa cells expressing GFP-GRAF1 (green) and mCherry-Rab8 (red) seeded on microfluidic plates. Normal medium was exchanged to hypotonic medium and replaced 10 minutes later with isotonic medium as indicated. Images were acquired every 3 seconds for 23 minutes with a spinning disk confocal microscope (Zeiss Cell Observer).



Movie 3. VLD endocytosis. Time-lapse of the orthogonal view of the MIP from magnification. Z-stacks of double Flp-In T-REx HeLa cells expressing GFP-GRAF1 (green) and mCherry-Rab8 (red) were acquired every minute using a spinning disk confocal microscope during 10 minutes (Zeiss Cell Observer).