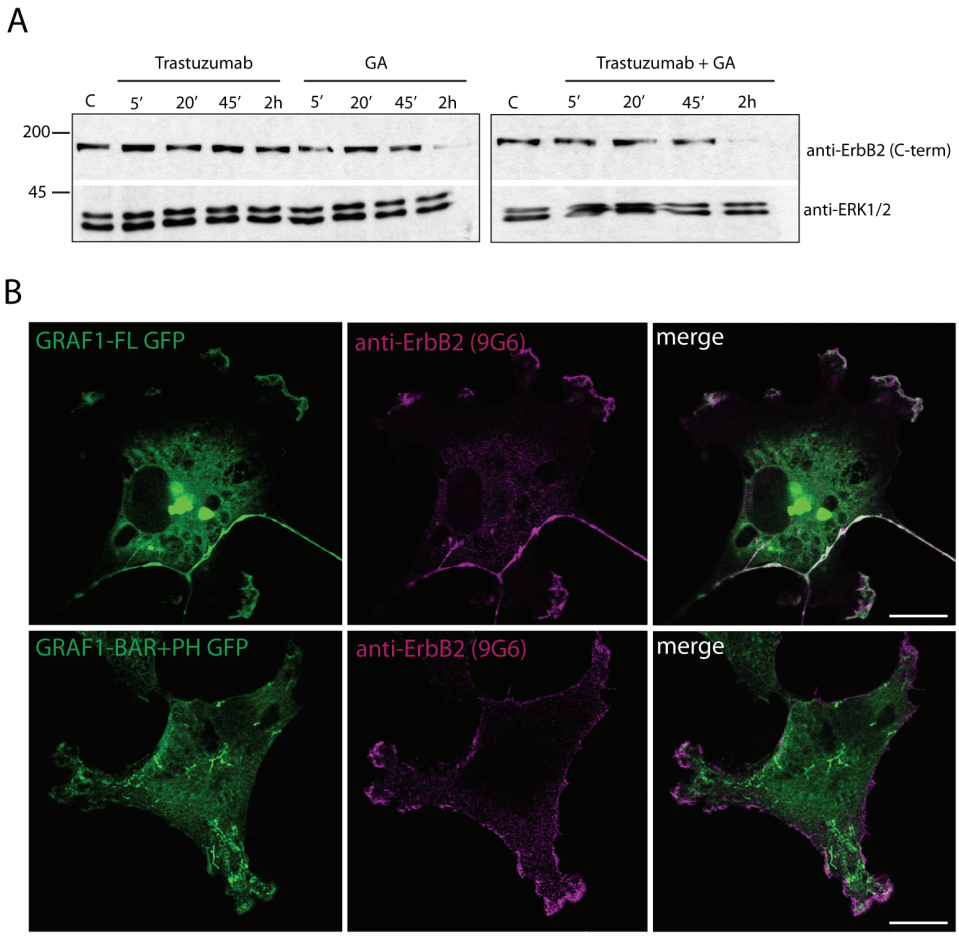

Figure S1 A. SK-BR-3 cells pre-treated with Trastuzumab (10 µg/ml), GA (10 µg/ml), or both for 20 minutes on ice and then warmed for 5, 20, 45, 2 hours before being lysed and analyzed by Western blot with antibodies to ErbB2. ErbB2 is degraded in cells treated with GA or both Trastuzumab and GA, but not in cells treated with Trastuzumab alone. Erk1/2 blot represents the normalization control.B. ErbB2 localization in untreated COS7 transiently transfected with GFP-tagged GRAF1-full length, GFP-tagged GRAF1 BAR+PH and ErbB2. Confocal images of transfected COS7 cells labeled with anti-ErbB2 (N-term, 9G6 Santa Cruz) in magenta. ErbB2 strongly localizes at the cell surface and internalization cannot be detected without GA treatment. Scale bar: 10 µm.

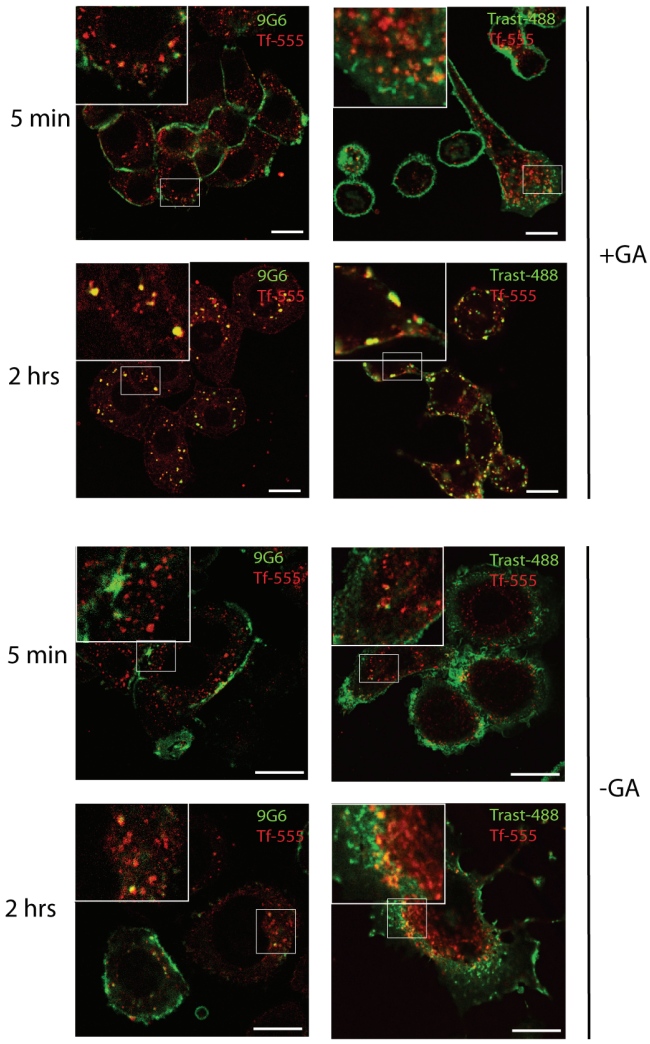

Figure S2. The anti-ErbB2 antibody 9G6 does not influence ErbB2 internalization and trafficking compared to Trastuzumab.
SK-BR-3 cells were pre incubated with/without Geldanamycin (GA, 10 µg/ml) for 20 minutes and next incubated with 9G6 antibody (left column) or Trastuzumab-488 (right column) and with Tf-555 for 20 minutes on ice. Cells were then washed and warmed at 37°C for 5 min and 2 hrs at 37C in the presence/absence of GA. The cells were then acid washed to remove extracellular labeling and fixed. An Alexa 488-conjugated anti-mouse antibody was used to reveal 9G6 anti-ErbB2 staining upon permeabilization (scale bar 10um). Both ErbB2 (9G6) and Trastuzumab-488 are inefficiently internalized and colocalize with Tf-555 after 5 min at 37C. Colocalization beetwen ErbB2 and Tf-555 in more evident after 2 hours in the presence or absence of GA, irrespective of the antibody used. Insets are magnification of boxed areas. Note that even after stringent acid wash (see Materials), some residual anti-ErbB2 9G6 and Trastuzumab-488 labeling is still present on the plasma membrane, making difficult a precise quantification of ErbB2 internalization at early times.

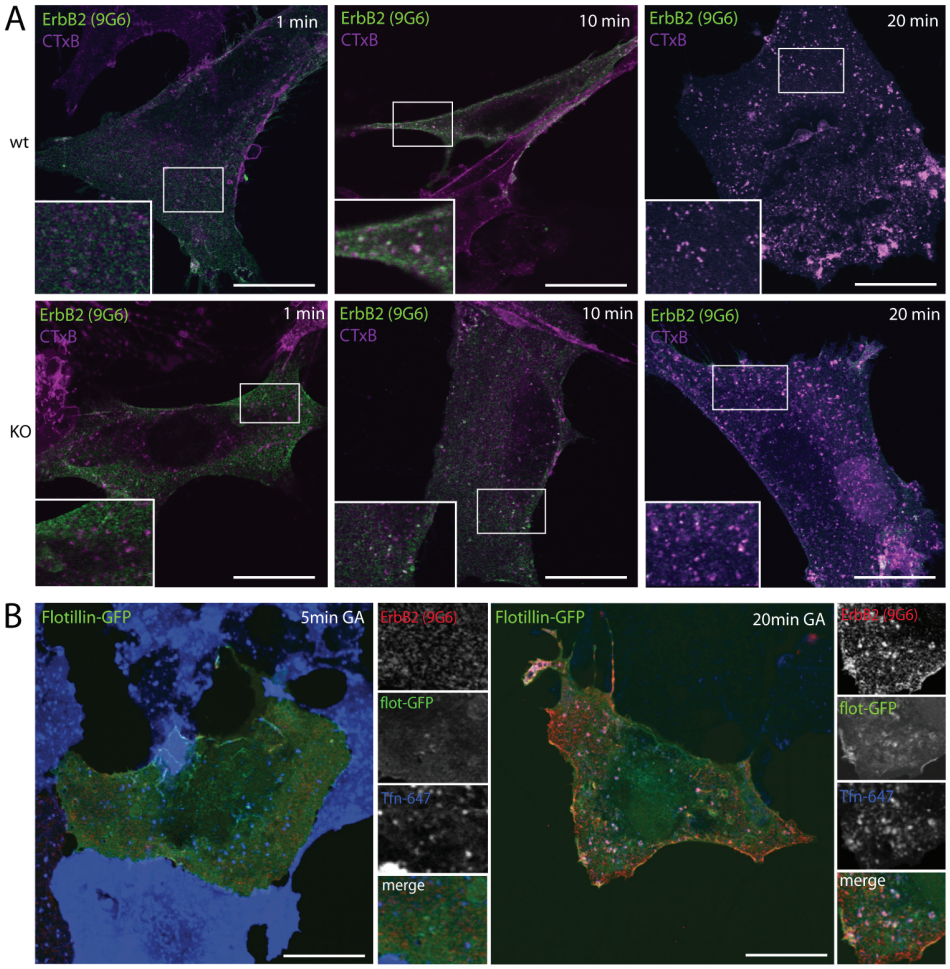

Figure S3 A. Internalization of ErbB2 (9G6) in Mefs wild-type and caveolin-1 KO cells. Mefs cells were transfected with untagged ErbB2 wild type for 24 hours. Upon GA pre-treatment (10 µg/ml) for 20 min 37°C, cells were incubated with 9G6 anti-ErbB2 Ab (extracellular domain, green) and CTx-B-Alexa555 (magenta) on ice for 15 minutes and then warmed at 37°C to allow for internalization for 1, 10 and 20 minutes. Anti-ErbB2 9G6 was visualized in green with an anti-alexa488 antibody. In contrast to CTx-B, ErbB2 is found within internal vesicles only after 10- 20 min at 37°C, whereas after 1 minute is mainly found at the plasma membrane. B. COS7 cells were co-transfected with untagged ErbB2 wild type and GFP-tagged Flotillin-1 for 24 hours. Cells on coverslips were incubated with GA (10 µg/ml) for 20 minutes before being incubated with anti-ErbB2 antibody 9G6 and Tf-alexa 647 for 5 and 20 minutes at 37°C. Anti-ErbB2 antibody 9G6 was visulized with anti-alexa555. After cold acid wash and fixation, cells were processed for immunofluorescence and analyzed by confocal microscopy.
Scale Bars, 10 µm.

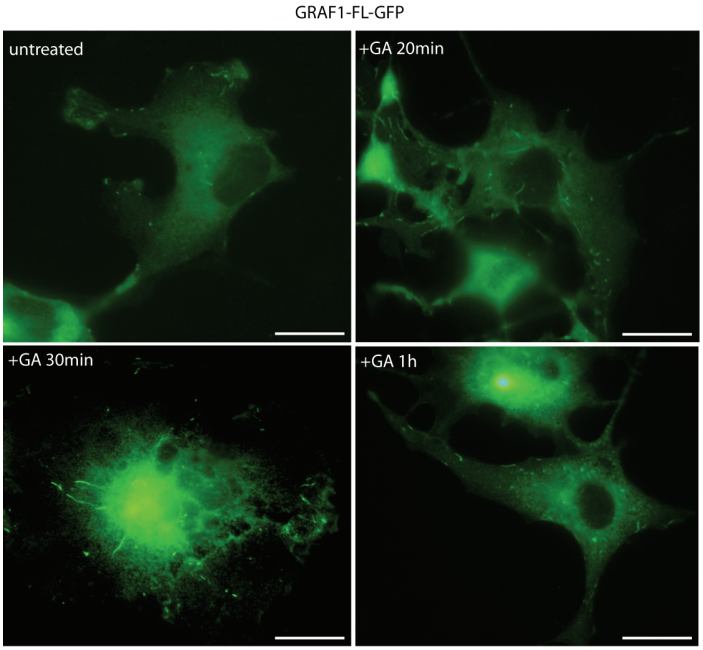

Figure S4. GA affects GRAF1 tubules morphology. COS-7 transiently transfected with GRAF1-FL GFP were treated with GA (10ug/ml) for the indicated times.
Note that GRAF1 tubules assume an elongated configuration under GA treatment.
Scale Bars, 10 µm.

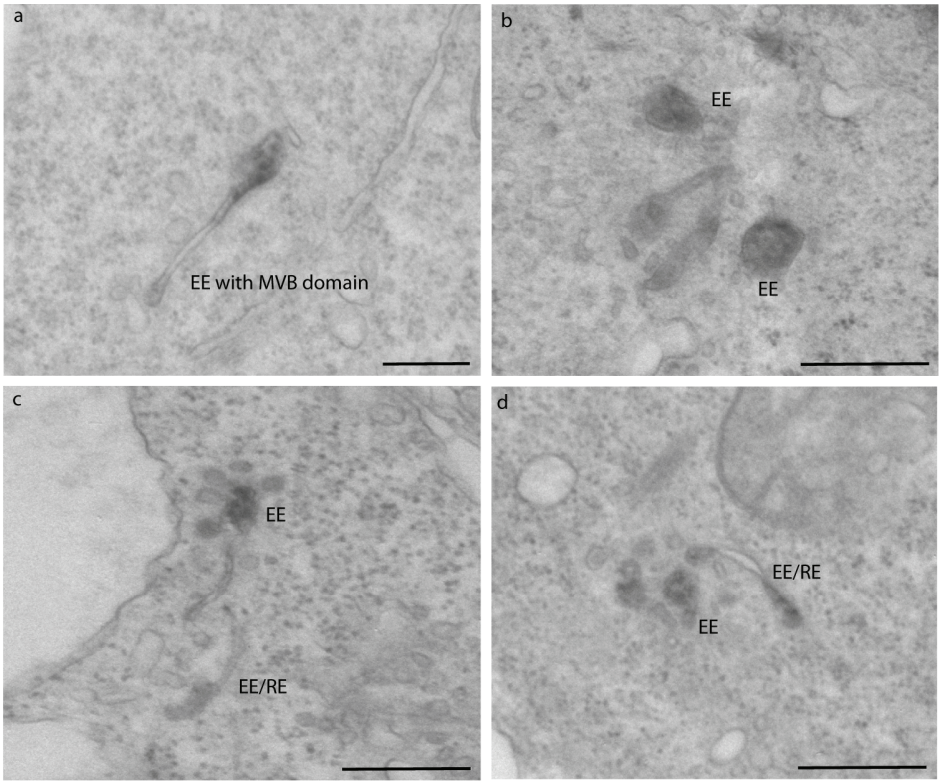

Figure S5. Examples of GA-modified endosomal structures upon 20 minutes of internalization of Trastuzumab-HRP. In a, is shown an elongated EE with MVB domain (enlargement of Fig.4, e). In b are shown vacuolar early endosomes, in c-d are reported EE/RE tubules along with early endosomes. Scale Bars: 500 µm.

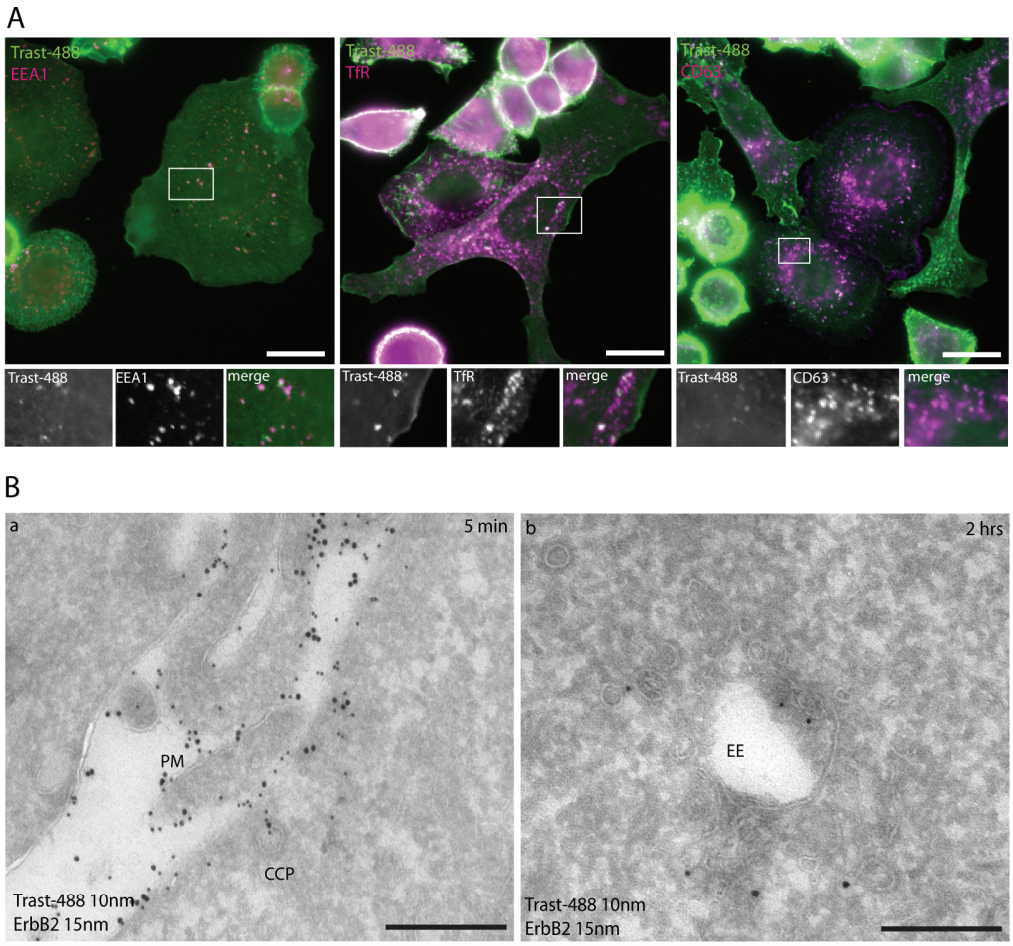

Figure S6. Trastuzumab does not modify the ErbB2 internalization. SK-BR-3 cells treated with Trastuzumab-488 for 30 minutes on ice and warmed for 2 hour at 37°C. Subsequently, cells were processed for immunofluorescence and imaged by confocal microscopy. Anti-EEA1 and anti-TfR (magenta) were used to detect early and recycling compartments, respectively. Anti-CD63 (magenta) was used as marker for late/lysosomal compartments. Note the colocalization of Trastuzumab-488 with early and recycling markers and the partial overlap with CD63. SK-BR-3 treated with Trastuzumab-488 were in parallel processed for Tokuyasu cryosectioning (a, b). 65 nm cryosections were double labeled with rabbit anti-Alexa antibodies (10nm Protein A gold) and 9G6 anti-ErbB2 antibodies (15nm Protein A gold). Note the labeling for ErbB2 and Trastuzumab in highly ruffled regions of the plasma membrane and within clathrin-coated pits. After 2 hours of Trastuzumab treatment, we observed ErbB2/Trastuzumab localization in early/recycling compartments. We did not observe elongated endosomes in the absence of GA. Scale bars: (a) 200nm, (b) 500nm.

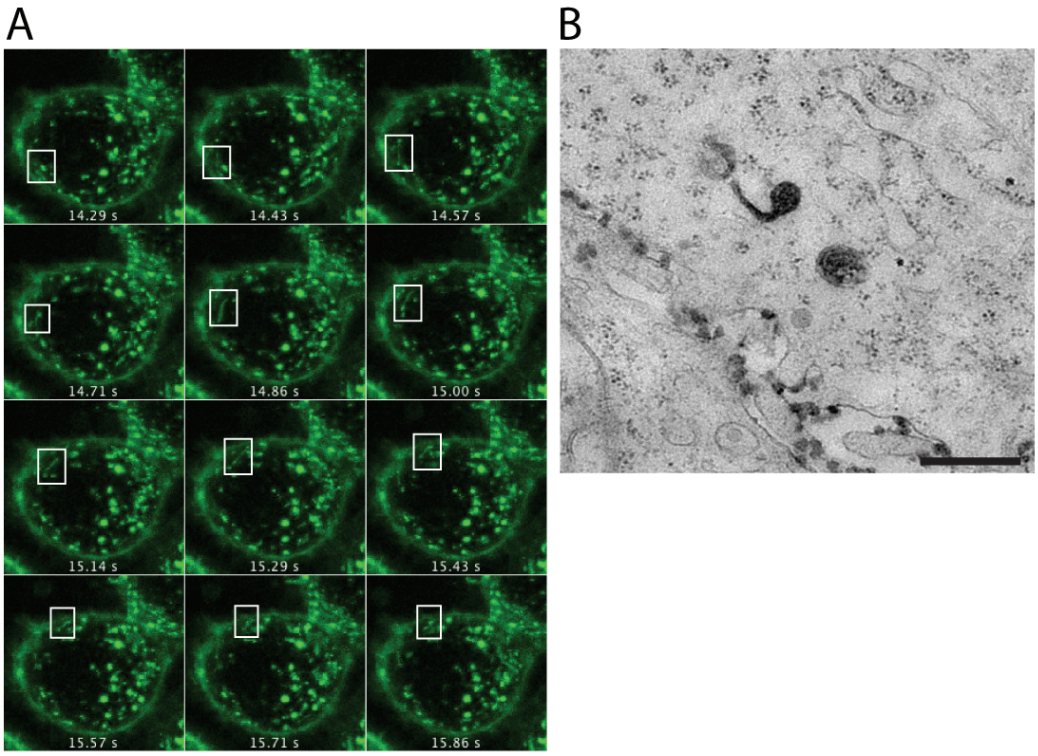

Figure S7. Long term (24 hrs) internalization assay of Trastuzumab-488 in SK-BR-3 cells. Trastuzumab-488 localizes within vesicular and dynamic tubular compartments. In the boxed area is shown a dynamic tubule travelling from a region close to the plasma membraneinto the cytoplasm, and then moving again toward the cell surface. B. Trastuzumab-HRP internalization after 24 hrs revealing HRP reaction product within endosomal structures. Scale bar: 500 nm.

Movie S1. Dual-axis tomogram of internalized Trastuzumab-HRP in GA-modified endosomes in SKBR3 cells, processed by chemical fixation and embedded in Epon.

Movie S2. 3D model of GA-modified endosome in SKBR3 cells.

Movie S3. Tomogram of internalized CLIC (labeled with CTx-HRP) in 300nm thick section of Mefs cav1-KO cells.

Movie S4. 3D model of CLIC carrier visualized in Movie S3.

Movie S5. Long-term (24 hrs) internalization assay of Trastuzumab-488 in SK-BR-3 cells. Note that Trastuzumab-488 localizes within vesicular and dynamic tubular compartments. In the boxed area is shown a dynamic tubule travelling from a region close to the cell surface into the cytoplasm, and then moving again toward the cell surface.