Supplementary Information

Oligomers of the ATPase EHD2 confine caveolae to the plasma membrane through association with actin

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Supplementary figure legends

Supplementary Figure S1: EHD2 specifically resides in caveolae, part 1. (A) Confocal image of CV1 cells co-expressing EHD2-EGFP and CAV1-mCherry. Lower row shows enlargement of indicated regions. Arrows highlight co-localizing puncta. (B) TIRF image (110 nm penetration depth) of a CV1 cell co-expressing EHD2-EGFP and CAV1-mCherry. Cells acquired by TIRF microscopy were used to determine the overlap of CAV1 and EHD2 spots at the PM. (C) Confocal images of cells co-expressing EHD2-EGFP and cavin-1-mCherry (together with CAV1-HA, not depicted) or EHD2-mCherry and cavin-2-EGFP show striking co-localization in PM puncta. (D) EHD2-mCherry and Clathrin light-chain (CLC)-GFP or EHD2-mCherry and Flotillin1/2 (Flot1/2)-GFP expressing cells show no over-lap between the proteins. Scale bars 10 μm.

Supplementary Figure S2: EHD2 specifically resides in caveolae, part 2. (A) Confocal images of A549 cells and 3T3-L1 cells stained with anti-EHD2 and anti-CAV1 antibodies. Magnifications highlight co-localization. (B) Confocal images of CV1 cells expressing EHD1-EGFP, EHD3-EGFP, or EHD4-EGFP, immuno-stained for endogenous CAV1. Magnifications show that there is no over-lap of EHD1 and EHD3 with CAV1. 10% of CAV1 spots co-localize with EHD4. Scale bars 10 μm.

Supplementary Figure S3: EHD2 silencing does not affect the integrity of caveolae. (A) HeLa cells were treated with non-targeting siRNA or EHD2 siRNA for 72h. Cells were stained for endogenous CAV1 and analyzed by confocal microscopy. Insets show a magnification of the boxed region. Caveolar spots of the same size and intensity are present in both conditions. (B) HeLa cells, treated with non-targeting siRNA or EHD2 siRNA, were lysed with 0.5% TX100, run through 10-40% sucrose velocity gradients and fractions analyzed by SDS-PAGE/Western blot. CAV1 sediments as 8S and 70S complexes. The 70S complex represents the assembled caveolar domain and is present in both conditions. (C) HeLa cell lysates of control cells or stable cell lines expressing CAV1-EGFP or cavin-1-EGFP were analyzed by SDS-PAGE/ Western-Blot using the indicated antibodies. The expression level of fluorescent fusion proteins does not exceed two-fold in comparison with endogenous protein level. (D) Confocal image of a HeLa cells, expressing CAV1-HA and Rab5-RFP for 16h, immuno-stained for CAV1 and endogenous cavin-1. Highlighted area is enlarged, arrows point to EE. All scale bars 10 μm.

Supplementary Figure S4: Overexpression of wild-type EHD2 and EHD2-I157Q induces clustering of CAV1. (A) CV1 cells expressing wild-type EHD2-EGFP for 16h were immuno-stained with anti-CAV1 antibodies and analyzed by confocal microscopy. Highlighted cell expresses EHD2-EGFP. Side panels of enlarged, boxed regions show increased intensity of CAV1 puncta in EHD2 over-expressing cells in comparison with non-transfected cells. The fluorescence intensity profile of indicated lines are presented in Figure S4C. (B) As in S4A but CV1 cells were transfected with EHD2-I157Q-EGFP. (C) 5 μm CAV1 fluorescence intensity profiles from wild-type EHD2 (left panel) or EHD2-I157Q (right panel) over-expressing cells and neighboring non-transfected cells (highlighted in S4, A and B). High levels of EHD2 significantly increase CAV1 intensity per spot. All scale bars 10 μm.

Supplementary Figure S5: EHD2 high MW complexes are independent of CAV1; EHD2 mutants are impaired in oligomerization. (A) HeLa cells expressing EHD2-EGFP were lysed in 60mM octylglucoside and run through 10-40% sucrose velocity gradients. Fractions were analyzed by SDS-PAGE/Western blot. All CAV1 sediments as the low MW species and EHD2 still sediments in oligomers >60S. Fractions were blotted with anti-GFP and anti-CAV1 antibody. (B) CAV1 –/- MEF cells expressing EHD2-EGFP were lysed in 0.5% TX100, run through sucrose velocity gradients and analyzed as in figure S5A with anti-GFP antibodies. EHD2 forms high MW complexes even in the absence of CAV1. (C) Representative sedimentation patterns of EGFP-tagged EHD2 mutants expressed in HeLa cells and run through velocity gradients. Fractions were blotted with anti EHD2 antibody. We noted the occurrence of a double band indicating a cleavage/degradation product of EHD2 seen for all EHD2 constructs but behaving like full-length protein band. (D) Confocal image of CAV1-/- cells transfected with EHD2-I157Q-EGFP and CAV1-mCherry for 6 h. Side panels show enlargement of boxed region. Scale bar 10 μm.

Supplementary Movies

Movie S1: Silencing of EHD2 increases the motility of caveolae. HeLa cells, stably expressing CAV1-EGFP were treated with control siRNA (left panel) or EHD2 siRNA (right panel) and 72h later imaged by TIR-FM. The time-lapse movies were acquired with a penetration depth of 110 nm and a frequency of 0.5 Hz for 100 images. Displayed at 30x real time.

Movie S2: FRAP of caveolae in control cells. HeLa cells, stably expressing CAV1-EGFP were treated with control siRNA. CAV1-EGFP was bleached in a peripheral region of the cell and fluorescence recovery imaged over time by confocal microscopy with an image frequency of 0.125 Hz. 4 min movie displayed at 40x real time.

Movie S3: FRAP of caveolae is elevated in EHD2 depleted cells. HeLa cells, stably expressing CAV1-EGFP were treated with EHD2 siRNA. CAV1-EGFP was bleached in a peripheral region of the cell and fluorescence recovery imaged over time by confocal microscopy with an image frequency of 0.125 Hz. 4 min movie displayed at 40x real time.

Movie S4: EHD2 is absent from moving caveolae. CV1 cells expressing EHD2-EGFP and CAV1-mCherry were imaged by TIR-FM with a penetration depth of 150 nm. Left panel: EHD2-EGFP, middle panel: CAV1-mCherry, right panel: merge of EGD2-EGFP and CAV1-mCherry intensities. The time-lapse movie was acquired with a frequency of 0.5 Hz for 120 images. Displayed at 30x real time.

Movie S5: Lateral movement of EHD2-positive caveolae after CytoD treatment. HeLa cells, stably expressing CAV1-EGFP, transfected with EHD2-mCherry were imaged by TIR-FM with a penetration depth of 110 nm and an image frequency of 0.2 Hz. After 90 sec, medium with CytoD was added to obtain a final concentration of 5 μM CytoD. Displayed at 30x real time.

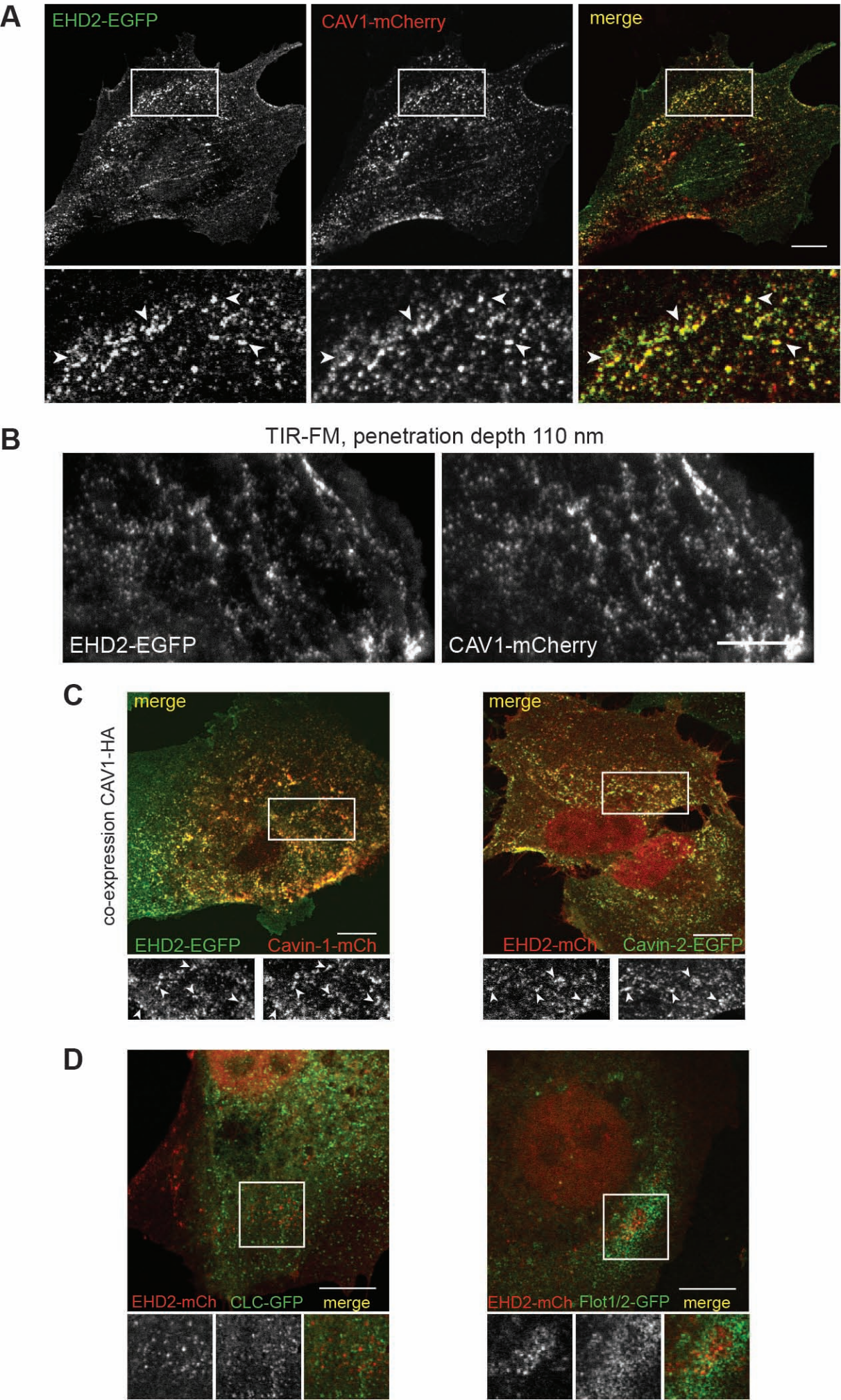
Movie S6: Caveolae retract with actin after CytoD treatment. HeLa cells, stably expressing CAV1-EGFP were treated with control siRNA and transfected with mRFP-β-actin. Cells were imaged by TIR-FM with a penetration depth of 110 nm and an image frequency of 0.2 Hz. After 90 sec, medium with CytoD was added to obtain a final concentration of 5 μM CytoD. Displayed at 30x real time.

Movie S7: Caveolae retraction with actin after CytoD treatment is decreased after EHD2 depletion. HeLa cells, stably expressing CAV1-EGFP were treated with EHD2

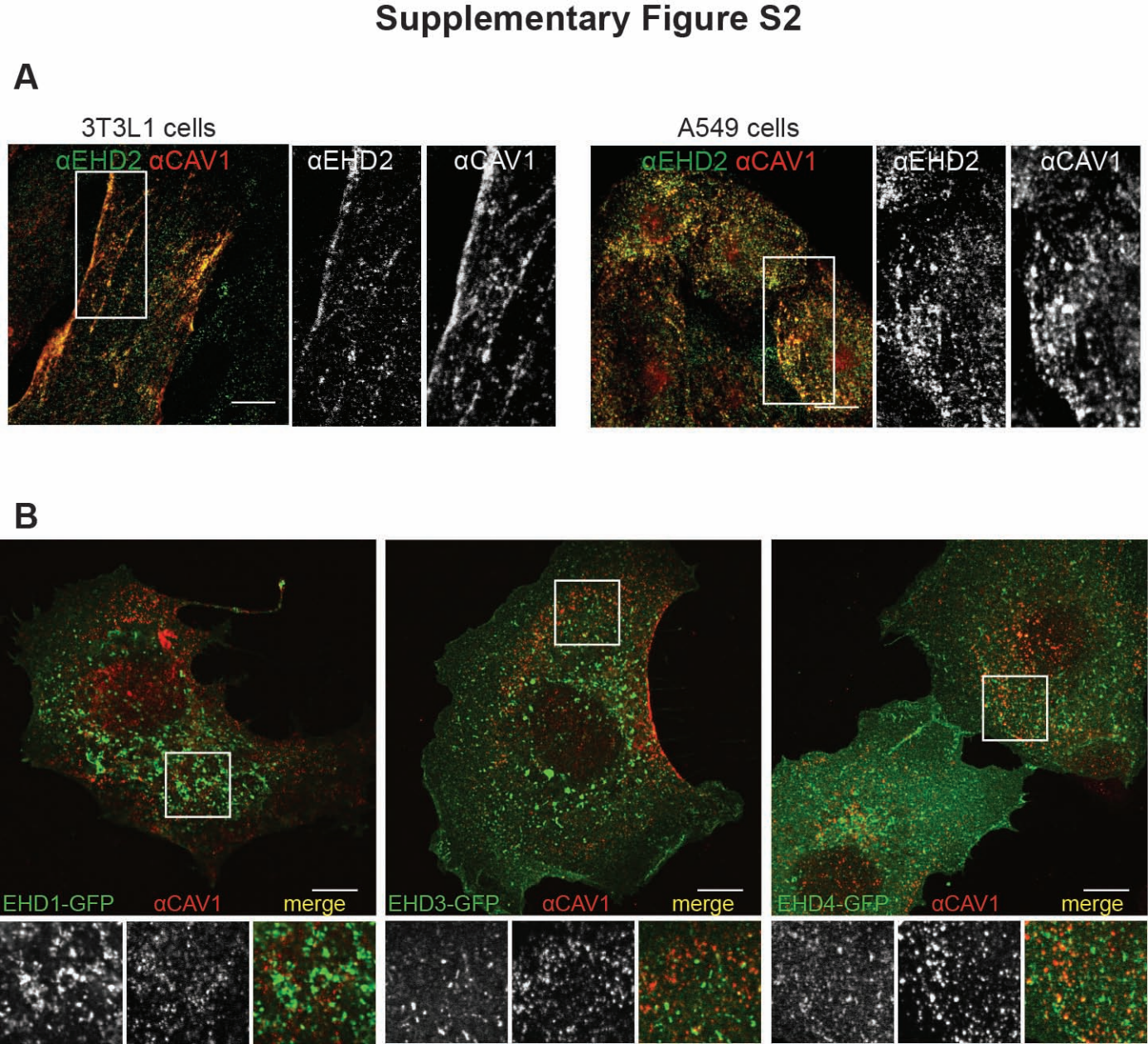
siRNA and transfected with mRFP-β-actin. Cells were imaged by TIR-FM with a penetration depth of 110 nm and an image frequency of 0.2 Hz. After 90 sec, medium with CytoD was added to obtain a final concentration of 5 μM CytoD. Displayed at 30x real time.

Movie S8: EHD2-I157Q is linked to actin in the absence of caveolae. CAV1-/- MEF cells, were transfected with EHD2-I157Q-EGFP and mRFP-β-actin. Cells were imaged by TIR-FM with a penetration depth of 110 nm and an image frequency of 0.2 Hz. After 90 sec, medium with CytoD was added to obtain a final concentration of 5 μM CytoD. Displayed at 30x real time.

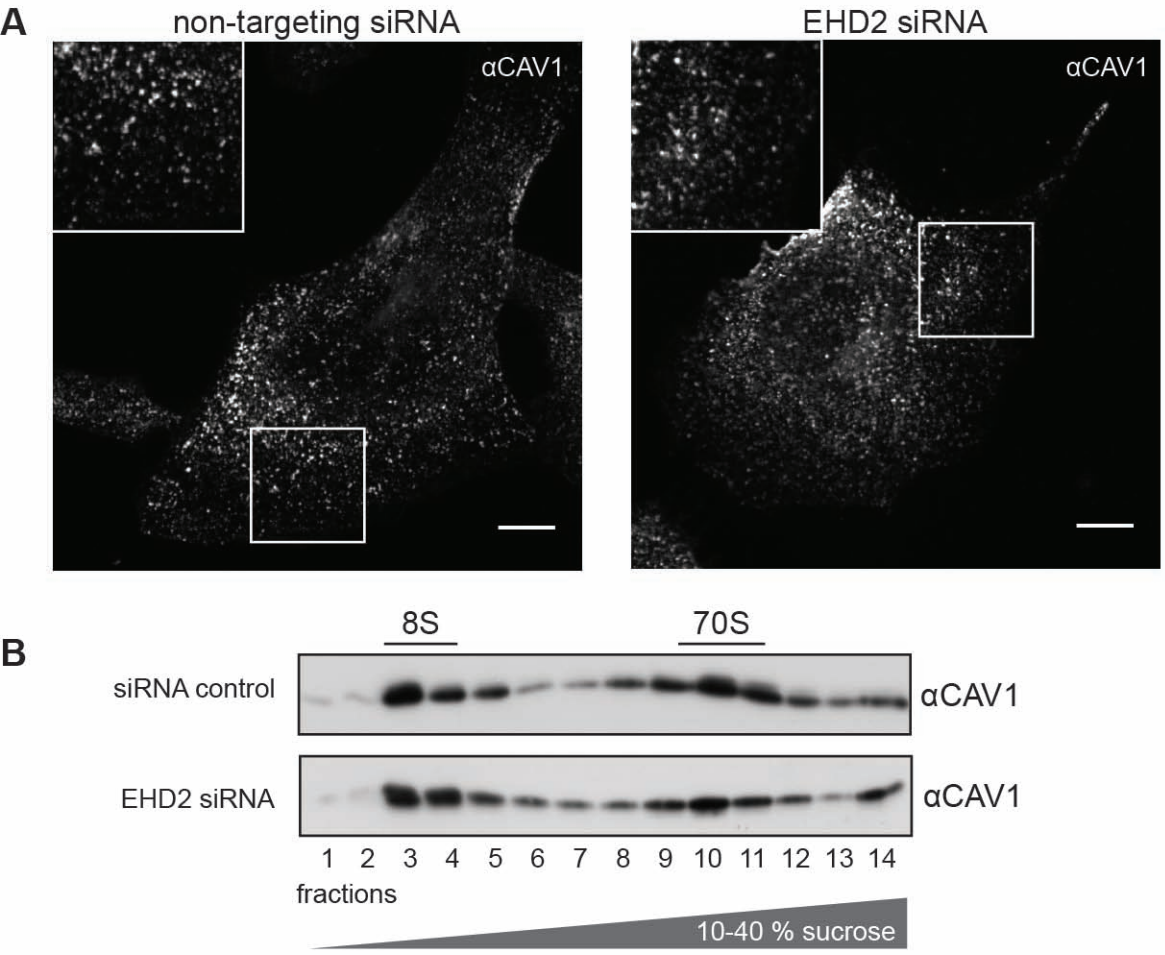
Supplementary Figure S1

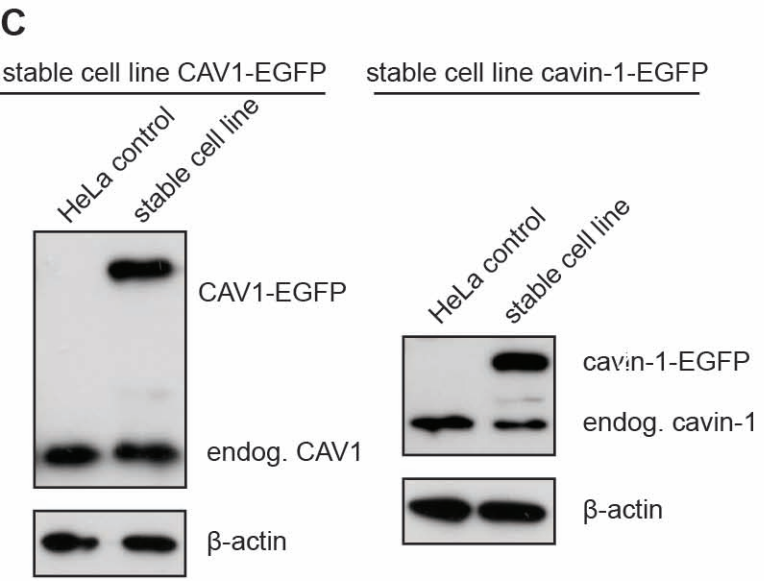


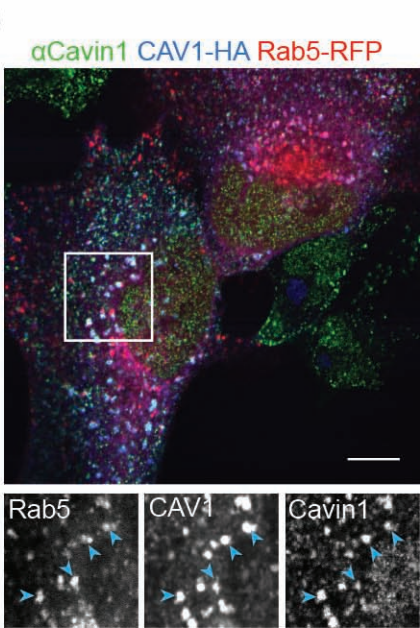
Supplementary Figure S2



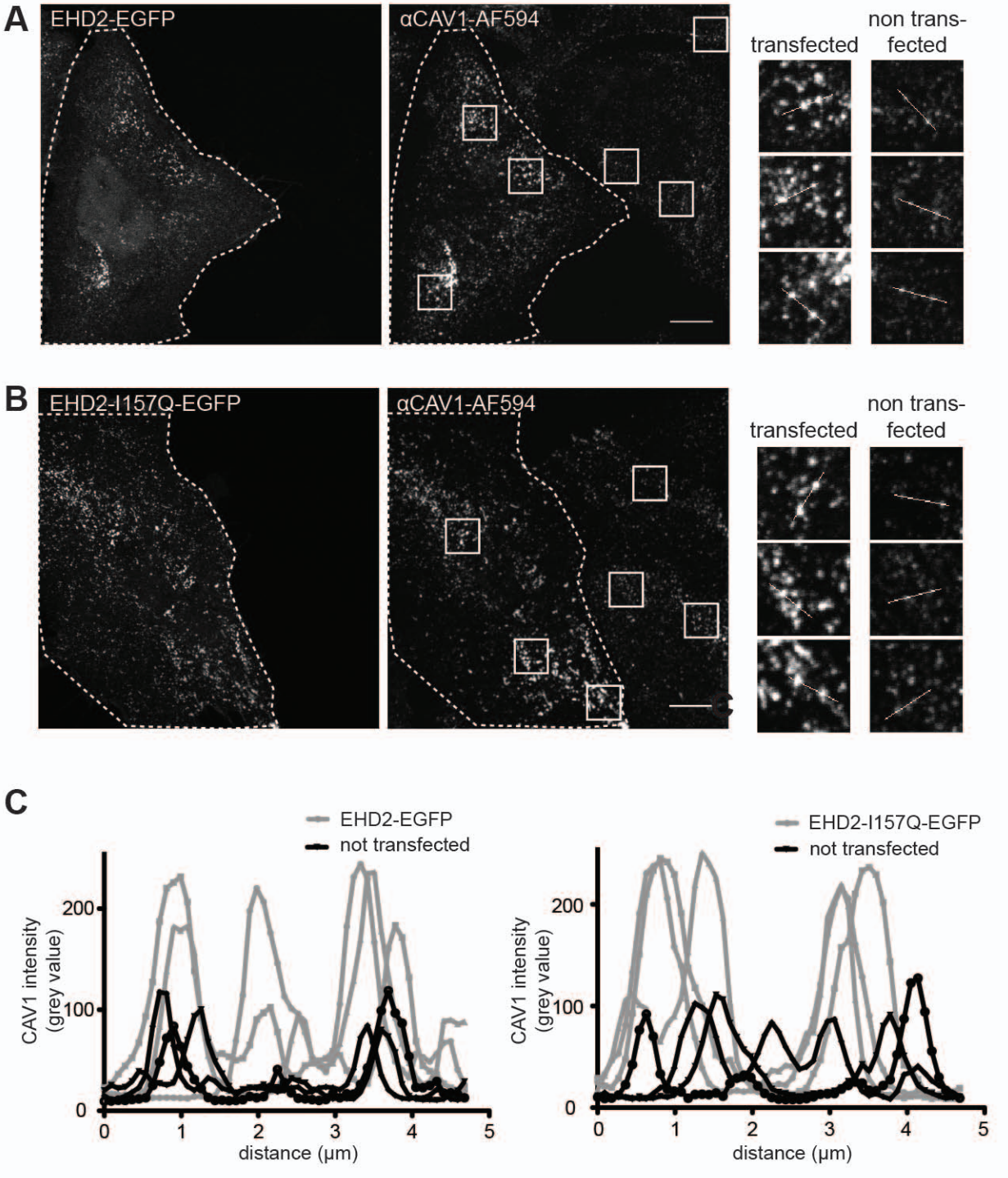
Supplementary Figure S3







Supplementary Figure S4



Supplementary Figure S5