Hayer et al., http://www.jcb.org/cgi/content/full/jcb.201003086/DC1

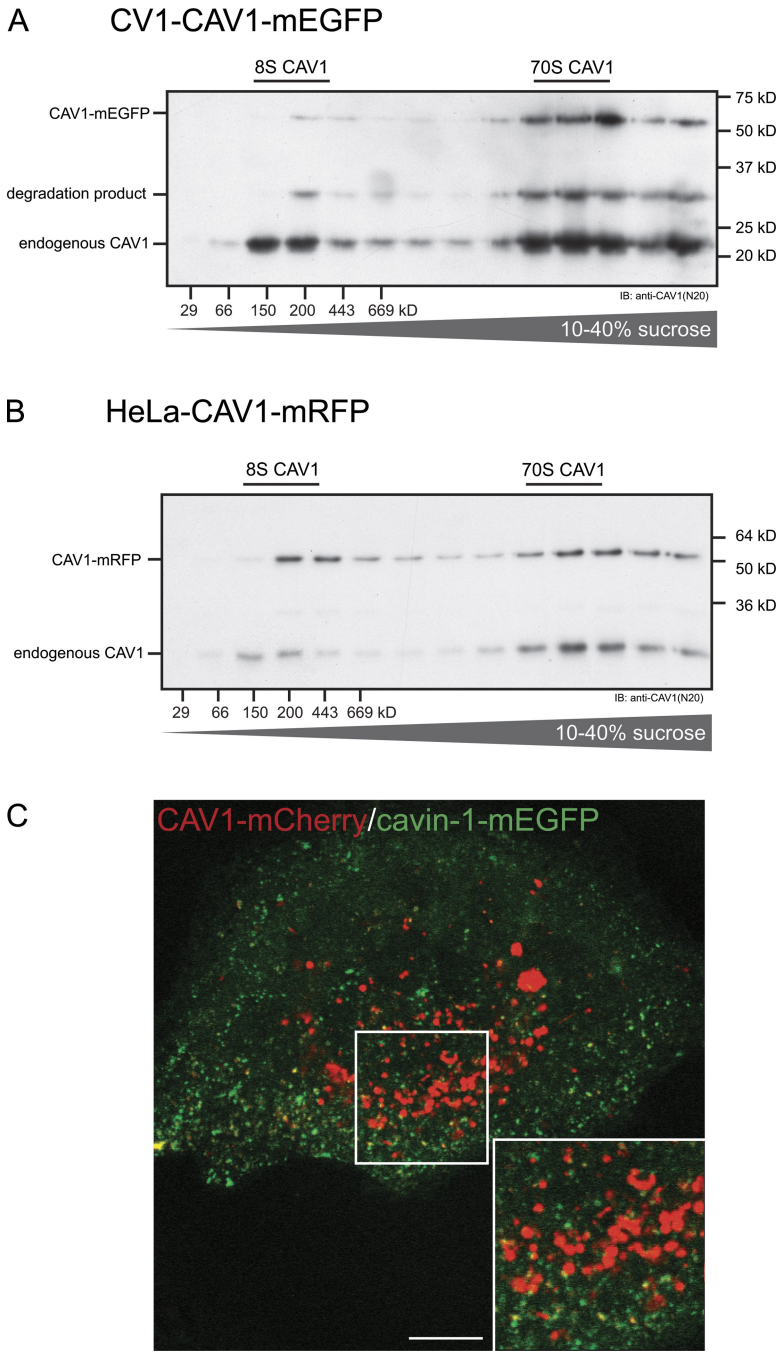


Figure S1. **Expression levels of cell lines stably transfected with fluorescent CAV1 constructs.** (A and B) CV1 cells stably expressing CAV1-mEGFP (A) or HeLa cells stably expressing CAV1-mRFP (B) were lysed, run through sucrose velocity gradients, fractions resolved by SDS-PAGE/Western blotting, and CAV1 detected using anti-CAV1 (N20) antibody. Both CAV1-mEGFP and CAV1-mRFP were efficiently incorporated into 8S/70S-equivalent caveolar complexes and expressed at similar levels as endogenous CAV1, resulting in moderate overexpression. (B) A full scan of the same blot shown in Fig. 2 A (si-control). (C) Co-overexpression of cavin-1 with CAV1 did not reverse targeting of CAV1 to intracellular vacuoles. CAV1-mCherry and cavin-1–mEGFP were cotransfected into CV1 cells and imaged live 12 h after transfection. A single confocal section is shown. Inset shows an enlargement of the boxed area. Bar, 10 µm.

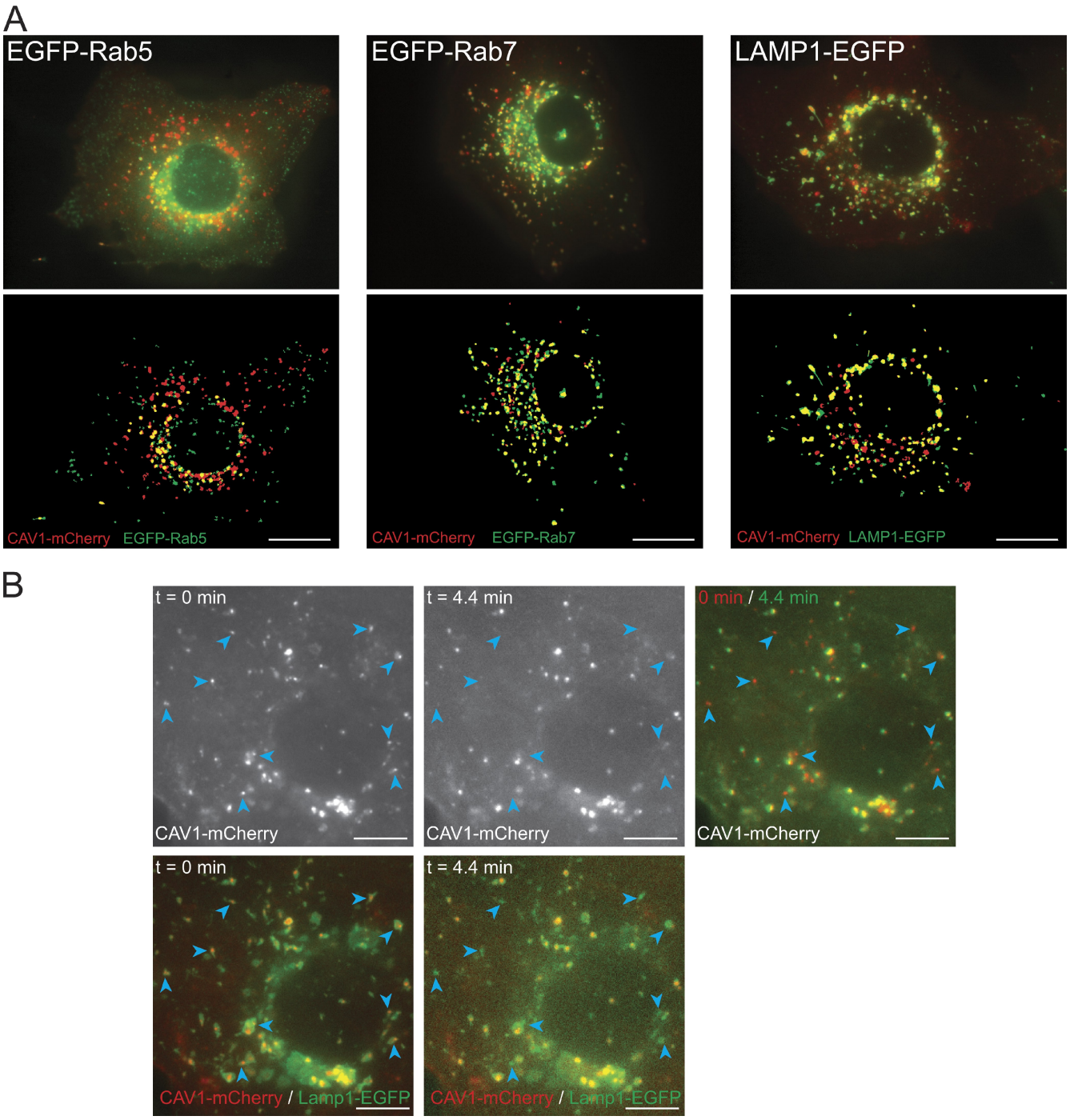


Figure S2. **Colocalization analysis and loss of late endosomal CAV1 during fixation.** (A) For quantitative colocalization analysis, CV1 cells cotransfected with CAV1-mCherry and either EGFP-Rab5, EGFP-Rab7, or Lamp1-EGFP were imaged live using an epifluorescence microscope. Examples of original images (contrast enhanced) and corresponding automatically detected endosomes are shown. (B) A time-lapse series of a CV1 cell coexpressing Lamp1-EGFP and CAV1-mCherry was acquired during and after FA fixative addition. t = 0 min marks the time when FA was added. Note the disappearance of the lumenal content (CAV1-mCherry) of LE/LYS short after addition of FA (arrowheads), whereas the limiting membrane (Lamp1-EGFP) remained intact (Video 2). Bars, 10 µm.

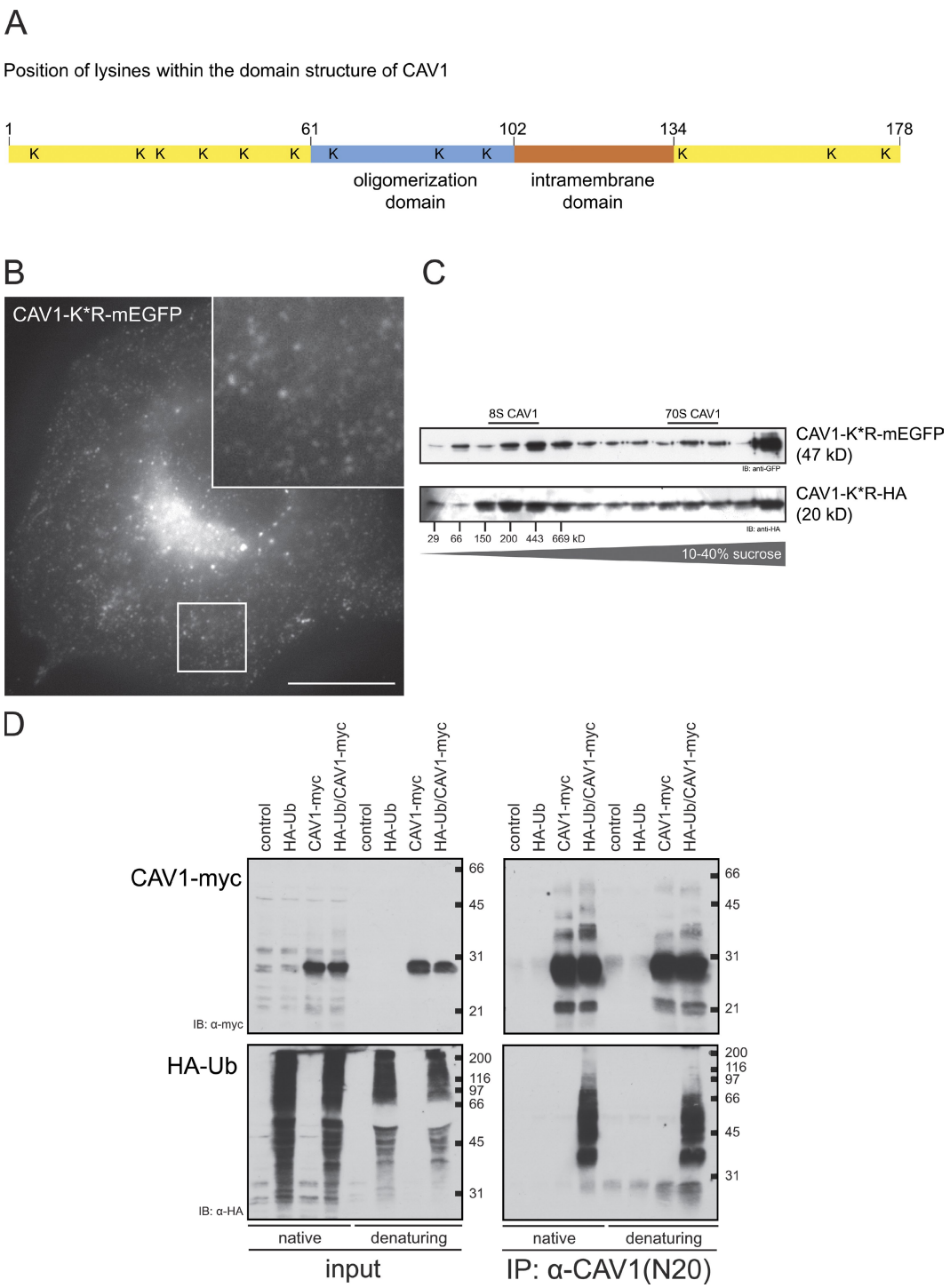
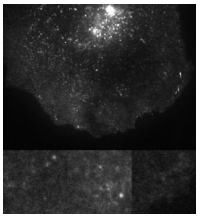
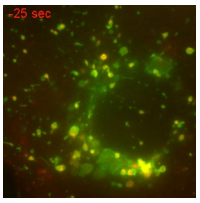


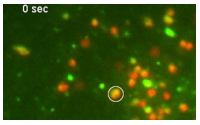
Figure S3. **The lysine-null mutant CAV1-K*R and denaturing immunoprecipitations to detect ubiquitinated CAV1.** (A) To generate CAV1-K*R constructs, all 12 lysines in CAV1 were mutated to arginines. (B) CV1 cells transfected with CAV1-K*R-mEGFP displayed normal cellular distribution of expressed mutant protein in the Golgi, in caveolar spots at the cell surface, and associated with endosomal structures. Inset shows an enlargement of boxed area. (C) When expressed in CV1 cells, both CAV1-K*R-mEGFP and CAV1-K*R-HA assembled into 8S- and 70S-equivalent caveolar complexes, indicating successful caveolae assembly. (D) HEK293 cells were transfected with HA-ubiquitin (HA-Ub), CAV1-myc, or cotransfected with both constructs (left). CAV1 was immunoprecipitated using anti-CAV1 (N20) antibody either under native or denaturing conditions (see Materials and methods). Probing blots with anti-HA antibody revealed ubiquitinated CAV1 species in immunoprecipitates prepared from cells overexpressing CAV1-myc. Migration of molecular mass standards is indicated in kilodaltons. Bar, 10 μm.



Video 1. **Surface delivery of CAV1-mEGFP in U18666A-treated cells.** CV1 cells treated with 5 µM U18666A (16 h) were transfected with CAV1-mEGFP using electroporation (see Materials and methods) and incubated in the presence of 1 mM cycloheximide during cell attachment (2 h). Expression of CAV1-mEGFP was induced by washout of cycloheximide, and cells were imaged by TIR-FM 60 min after washout. The evanescent field was adjusted such that both cell surface and part of the Golgi were visible. CAV1-mEGFP can be observed in tubular post-Golgi carriers. Upon arrival at the cell surface, they release their cargo, visible as burst of fluorescence diffusing laterally into the plasma membrane. Boxed areas showing examples are enlarged below. Time-lapse sequences were acquired at 1 Hz with an integration time of 500 ms/frame. Video is displayed at 15× real time.



Video 2. **The limiting membranes but not the lumenal contents of LE/LYS can be fixed by FA.** CV1 cells coexpressing CAV1-mCherry (red channel) and Lamp1-EGFP (green channel) were mounted in a live cell chamber. FA was added to final 4% at t = 0 s, and time-lapse imaging was performed on an epifluorescence microscope at 0.2 Hz. Note the disappearance of CAV1-mCherry from numerous endosomal structures within the initial minutes of fixation (red circles), leaving empty Lamp1-EGFP organelles behind. Video is displayed at 10× real time.



Video 3. **Targeting of CAV1 to the acidic lumen of endosomes.** CV1 cells coexpressing CAV1-mEGFP and CAV1-mCherry (12 h) were imaged on an epifluorescence setup equipped with a dual-view beam splitter at 0.2 Hz. The endosome marked by a circle is both red and green initially but loses green fluorescence as the endosome progresses toward the perinuclear area. Because mEGFP fluorescence is sensitive toward acidic pH, the loss of mEGFP fluorescence is consistent with targeting of CAV1 to the acidic lumen of LE/LYS (Fig. 5). Video is displayed at 50× real time.