Legends for movies:

Supplementary Movie S1A: Dynamics of Caveolae in Control Cells

HeLa cells stably expressing Cav1-EGFP were imaged at 37°C by TIRF. The osmolarity of the medium was 300 mOsm. Images were captured every 300 ms. Blue arrows show examples of caveolae with slow restricted motion. Red arrows show examples of caveolae with short-range mobility. Yellow lines show partial tracks of caveolae displaying fast long-range mobility.

Supplementary Movie S1B: Dynamics of Caveolae under hypo-osmotic shock

HeLa cells stably expressing Cav1-EGFP were imaged at 37°C by TIRF. The osmolarity of the medium was switched from 300 mOsm to 30m Osm 4 minutes before capturing the movie. Images were captured every 300 ms.

Supplementary Movie S2: Movie of Tether Pulling

Membrane tether formed by adhesive contact between optically trapped bead (3 μm in diameter) and wt MLEC. The tether was elongated by stage displacement. The presence of the sub-micronic membrane tether was checked by observing that the bead snapped back when laser was switched off.

Supplementary Movie S3A: Epifluorescence Movie of Intracellular Pool of Cav1-EGFP

HeLa cells stably expressing Cav1-EGFP were imaged at 37°C with wide-field illumination. Images were captured every 500 ms for 50 sec. Cav1 containing-structures inside the cell appear to be highly mobile.

Supplementary Movie S3B: Effect of ATP Depletion on Caveola Mobility

HeLa cells stably expressing Cav1-EGFP were imaged at 37°C with wide-field illumination after depletion of ATP. Images were captured every 500 ms for 50 sec. Cav1 containing-structures inside the cell show no mobility in contrast with control cells (Supplementary Movie S2A).

SUPPLEMENTARY TEXT

Estimation of the Membrane Area Required to Buffer Membrane Tension

Here, we summarize an estimation of the membrane area that is necessary to avoid an increase in membrane tension upon hypo-osmotic shock as observed in Cav1$^{-/-}$ MLEC and will compare this with the area released by caveola flattening.

Accurate membrane area measurements are generally very difficult to perform from fluorescence confocal micrographs (Boucrot, E., and Kirchhausen,T. (2007) Proc. Natl. Acad. Sci. USA. 104, 7939-7944). As seen from the EM pictures (Figures 2A, 3A, S2A and S6), the membrane profile exhibits numerous undulations of amplitude < ~500 nm. All these undulations are below the spatial resolution of optical microscopy. Determination of the plasma membrane area from the averaged optical profile would thus lead to a strong underestimation. Note that this effect was not as critical for the volume estimation since the volume contained in the undulations is much lower than that of the bulk cytoplasm (Figure 1A and B). Furthermore, it is important to distinguish between large-scale undulations (typically > 100 nm), which are membrane folds, and smaller undulations (of the order of few tens of nm), which are thermal ripples. While the first ones are readily unfolded upon moderate hypo-osmotic shock and do not lead to any increase in membrane tension, the thermal fluctuations of the membrane intrinsically contribute to the membrane tension, as caveolae do. In consequence, starting from a resting situation (where the cell may look like a deflated balloon), large-scale membrane folds would be first smoothened out. This may lead to a significant increase in optically visualized membrane area by more than 10% (as previously reported – see eg. Groulx, N. et al. (2006) J. Membrane Biol. 214, 43–56). However, once the cell membrane is globally unfolded (at a typical scale >100 nm), any additional volume increase will have two concomitant effects if no other membrane reservoir is available: i) a small change in membrane area, and ii) a drastic increase in membrane tension, because further smoothening of the thermal fluctuations and membrane stretching require a significant amount of energy. In the next paragraphs, we will estimate the membrane area change that may effectively contribute to membrane tension and compare it with the added area due to caveolae flattening.

To do it, we exploit the fact that the areal change of a (reservoir-free) lipid bilayer is directly related to its membrane tension through the Helfrich relation (Evans, E, and Rawicz, W. Phys.

Rev. Lett. 64, 2094-2097 (1990) and references therein): $\frac{\sigma}{\sigma_0} = \exp\left(\frac{8\pi\kappa}{k_B T}\frac{\Delta A}{A_0}\right)$ where σ_0 is the initial (resting) membrane tension, σ is the membrane tension of the stressed membrane, $k_B T$ is the thermal energy, κ is the bending rigidity (~40 $k_B T$ as measured on plasma membrane vesicles) and A_0 and ΔA are the initial membrane area and the change in area respectively. This relation is an approximation for small area strains and describes the entropic regime, in which bending ripples are progressively smoothened out. We neglect here the elastic regime, in which the membrane exhibits a limited microscopic extensibility, which then leads to cell lysis (Note that the outcome remains unchanged within errors if this effect is considered). In order to find experimental conditions in which this relation can be used, we selected ATP-depleted or CD-treated Cav1$^{-/-}$ MLECs, for which the membrane-cytoskeleton adhesion energy is minimal (or negligible). Under these conditions, we thus assume that the tether force directly yields the bilayer tension . Cav1$^{-/-}$ MLEC exhibit a significant tether force increase during shock. By measuring f_0 and f, the tether forces in iso-osmotic conditions and after shock, we derive σ_0 and σ (since $\sigma = \frac{f^2}{8\pi^2\kappa}$), and thus $\Delta A/A_0$,which corresponds to the fractional change in area that Cav1$^{-/-}$ MLEC experience during hypo-osmotic shock. Using the numbers obtained for the tube force before and after the shock (Figure S7), we calculate $\Delta A/A_0$ =**0.28±0.08 %** for ATP-depleted cells (CD treated cells reveal 0.18±0.06 % change). Since wt MLEC do not exhibit any tether force increase upon hypo-osmotic shock, our assumption at this stage is that the calculated area change observed with Cav1$^{-/-}$ MLEC is assigned to caveolae flattening.

In order to check whether this value is close to the effective area released by all the lost caveolae, we used both data from EM experiments and TIRF imaing although by TIRF we expect a underestimation of the cell membrane area and thus a overestimation of the fraction (or percentage) of membrane released. Analysis of EM pictures on wt MLEC gave us the number of caveolae per µm of membrane section (see Figure 2B). Calculating the expected number of caveolae (for example) per 100µm^2 both in iso-osmotic and hypo-osmotic conditions, and then assuming the area per caveolae to be 0.008 µm^2 ((Richter et al., 2008), we find that caveolae flattening leads to 0.15±0.1 % change in area. Here, we still need to correct for differences of osmolarity between tether force and EM experiments; tether forces have been measured at 150 mOsm while EM was done on samples at 30mOsM. Using the measured relation between caveolae loss and osmolarity (plot from Figure 1E), we expect that

caveolae will release a membrane area that corresponds to **0.12±0.1 %** of the total membrane area at 150m Osm. The same analysis could be done from TIRF imaging: we have observed a loss of 400 caveolae for a region of 1400 μm^2 in MLECs under 30m Osm shock. This leads to a membrane release of 0.23 ± 0.05 % in 30 mOsm hypo-osmotic shock or a release of **0.18 % ± 0.05** for 150 mOsm hypo-osmotic shock, which is consistent with the EM estimate. Therefore, the estimated area change in Cav1$^{-/-}$ MLEC and the fraction of area added by caveolae in wt MLEC are similar taking into accounts the errors. Although the derivation of these numbers is not direct, we think that the agreement is not coincidental, which supports our proposed mechanism.

EXTENDED EXPERIMENTAL PROCEDURES

Materials

Lipofectamine 2000, HGF, FBS and DMEM were purchased from Invitrogen (Carlsbad, CA). Dexamethasone, collagen type I, gentamycin, bovine Insulin, human Apotransferrin, dynasore, Cytochalasin D, Latrunculin A, Jasplalinolide, BFA, m-β-cyclodextrin were purchased from (Sigma-Aldrich, St. Louis, MO). The following products were purchased from the indicated commercial sources: EGM-2 and AMAXA HUVEC nucleofector kit (Lonza, Basel, Switzerland), FuGENE (Roche Diagnostics, Meylan, France), MG132 (Calbiochem, Darmstadt, Germany).

Cell culture and Treatments

Wild type (wt) and caveolin negative ($Cav1^{-/-}$) mouse lung endothelial cells (MLEC) (Murata et al., 2007) were transiently transfected with plasmid encoding Cav1-EGFP (Pelkmans et al., 2001) or Cavin1-mCherry (Hill et al., 2008) or clathrin heavy chain siRNA (Fisher Scientific Dharmacon) with AMAXA HUVEC nucleofector kit following the manufacturer's instructions, and used for experiments 24-72 hrs post transfection. The Cav1-Dendra2 plasmid was prepared by replacing EGFP in the Cav1-EGFP plasmid by Dendra2 from pDendra2-C plasmid (Evrogen, Moscow, Russia) using Mfe1 and Age1 restriction sites. Human muscle cells were immortalized; one control cell line expressing wt Cav3, and one obtained from a biopsy of a patient with hyperCKaemia expressing the mutation Cav3-P28L (Muscle Tissue Culture Collection, Munich, Germany; biopsy reference: Bonn 18/03, female *1969, heterozygote mutation 27C>A (27 D>E).

Membrane Tether Extraction and Force Measurements

Plasma membrane tethers were extracted from cells by using optically trapped beads. The optical tweezers were created by steering a 1064 nm laser beam (Coherent, Santa Clara, CA) into a high numerical aperture objective (100X, 1.3 NA) on an inverted microscope (Zeiss Axiovert 200, Carl Zeiss, Jena, Germany). Polystyrene beads (3 μm in diameter) coated with concanavalin A (Sigma-Aldrich) were used as a handle to extract membrane tethers from the cell surface. Images were acquired with a CCD camera (XC-ST70CE, Sony, Tokyo, Japan) at 25 Hz, recorded on a computer with a video capture card (Piccolo Pro 2, Euresys, Angleur, Belgium) after contrast enhancement (Argus image processor, Hamamatsu Photonics, Hamamatsu, Japan) and analyzed offline using MatLab (Mathworks, Natick, MA). Trapping

stiffness was calibrated by measuring the fluctuations of a captured bead at different laser powers (Cuvelier et al., 2005). Since the position of beads was detected with a resolution of 10 nm, the force resolution was of the order of 1 pN for a trap stiffness of ~100 pN/µm. In a typical experiment, an optically trapped bead was brought into contact with the plasma membrane for 2-20 sec to ensure an adhesive contact. The cell was then displaced linearly with a piezo-stage (PI, Karlsruhe, Germany) at 0.1 µm/s to extract a membrane tether of length between 5 and 150 µm. The membrane tether was then held at constant length to measure the static force, independently of dynamical effects related to the speed of extraction. After extraction of a tether in iso-osmotic conditions ($0 \text{ min} < t < \sim 1 \text{ min}$), for assaying membrane tension changes due to hypo-osmotic shock, the medium is diluted with water until the osmolarity reaches 150 mOsm The observation chamber containing cells was mounted on a temperature controlled stage of the microscope (Tempcontrol 37-2 digital, Carl Zeiss), and temperature was set to 37ºC through the whole course of experiments.

PMS Formation and Micropipette Aspiration

For plasma membrane spheres (PMS) generation, cells were grown on glass cover slides (25 x 40 mm^2) to 60-80 % confluence, and then incubated for 6-8 hrs in PBS (pH 7.4) supplemented with 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 10 µM MG132. Tether force measurements and PMS aspiration were performed by placing the sample on a modified confocal microscope equipped with optical tweezers and a pipette micromanipulator (see Sorre et al., 2009 for technical details).

Single, free, and fluctuating PMS were selected for micropipette aspiration experiments as described previously for artificial lipid vesicles (Sorre et al., 2009). In brief, PMS were held with a micropipette (of diameter ~3µm) under slight aspiration. Aspiration was tuned by moving vertically a water reservoir connected to the pipette. By partially entering the pipette, the membrane is strained. As a consequence, the portion of the vesicle outside the pipette is pressurized. To some extent, this assay is similar to a gentle and controlled hypo-osmotic shock. Membrane tethers were formed using an optically trapped bead, as explained above for cells. The tether force at constant length was measured while the aspiration of the PMS was increased in a 3 Pa stepwise manner.

Fluorescence Imaging and Analysis

For Total Internal Reflection Fluorescence (TIRF) microscopy, the different wavelengths (488 nm, 568 nm, 614 nm) of an argon-krypton mixed gas laser (Melles Griot, Carlsbad, CA)

were selected by an acoustic-optical tunable filter unit, routed to the microscope by a single-mode polarization-maintaining fiber optic and the TIR mode generated by illumination through the periphery of a high NA objective (100X, 1.45 NA) in a Zeiss Axiovert 200 inverted microscope. Images were acquired by an EM CCD camera (8 μm pixel dimension, 1000×1000 pixels, C 9100-02, Hamamatsu Photonics, Japan). For dual wavelength imaging, the emission light was passed through a dual-view optical unit (Photometrics, Tucson, AZ) that split the separate wavelengths to different parts of the CCD chip.

Caveolae were detected from TIRF images by first applying on the raw image a local intensity threshold of window size varying from 8×8 to 64×64 pixels depending on the quality of the image. Pixels clustering together were detected as particles depending on their connectivity. Holes within particles, if any, were filled, connected particles were disconnected by eroding boundaries and finally particles were selected by size. In order to track caveolae mobility, the centers of mass was first calculated for each caveolae and subsequently at any given time, starting from the first frame, the closest caveola (distance < 5 pixels) was detected in the subsequent frame and the trajectory of the center of mass was thus obtained. Confocal imaging of cells was performed on a Nikon A1R confocal microscope (Nikon Imaging Centre, Institut Curie) with a high NA objective (100X, 1.4 NA) and at 1 airy unit pinhole aperture. For determining colocalization of Cav1-EGFP with Cavin1-mCherry imaged by dual-TIRF in HeLa cells, images were analyzed as described before to obtain the center of mass (CM) of Cav1 or Cavin1 punctuated structures. Next using Labview, the number of Cav1 structures having proximal Cavin1 structures was calculated (distance between CM_{Cav1} and CM_{Cavin1} < than 3 pixels). The percentage of Cav1 structures having very proximal Cavin1 structure was treated as the percentage of colocalization). For volume measurement, 3D confocal imaging was performed on a Nikon A1R confocal microscope with 1μm z-step. The volume for each time-point was calculated by programmed edge detection of the cell at each z-plane and integrating the cell-area for all planes. TIRF-FRAP experiments were performed on a Nikon Eclipse 2000 (Nikon Imaging Centre, Institut Curie) microscope equipped with an EM CCD (16 μm pixel dimension, 512×512 pixels, Roper Scientific, Tucson, AZ). A region (5×5 μm) was chosen for bleaching. Pre-bleaching and post-bleaching acquisitions were performed with a time interval of 1 or 0.5 sec. During analysis, for any time point, first the background fluorescence was subtracted, next a normalization was done to compensate for photo-bleaching (with intensity of a region-of-interest (ROI) covering a large part of the cell), and then a normalization with the pre-bleach intensity was done. Mobile fractions (A) were estimated by fitting the averaged FRAP curves to a standard recovery

equation ($I(t)= A(1-exp(-t/\tau))$ where $I(t)$ is the recovered intensity at time t and τ is the half-life for recovery. Lines show fit for the curves for iso- and hypo-osmotic conditions. For checking the statistical significance, A for each cell was derived from individual fits and the mobile fractions (A) for iso-osmotic conditions compared with that of hypo-osmotic conditions. Cells were always maintained at 37ºC during imaging. Image analysis for all experiments was done using Labview 8.5 and Vision 8.5 (National Instruments, Austin, TX). FLIM microscopy experiments were performed as described previously (Hill et al., 2008). RICM was performed with an inverted microscope (Axiovert 200, Carl Zeiss, Germany) equipped with an EMCCD camera (CoolSnap, Roper Scientific, NJ) using a 100x objective. A HBO mercury lamp and an interference filter (λ=546 nm, $\Delta\lambda$=5 nm) was used for creating the interference image.

Colocalization Analysis in PMS

Confocal images of Cav1-EGFP and Cavin1-mCherry in a z-section of width 0.4 µm around the equatorial plane of PMS were analyzed for colocalization by first fitting an ellipse (close to a circle) to the membrane contour and finding the fluorescence profile of both Cav1 and Cavin1 along the membrane. From these fluorescence profiles, peaks consisting of at least 5 data points and having an intensity of at least 1 standard deviation above the mean fluorescence were detected as peaks. Next, colocalization of Cav1-EGFP and Cavin1-mCherry was quantified by counting the peaks that overlap within 2 pixels (1 pixel = 0.055 µm). Several confocal z-planes at the equatorial region of the PMS were separately analyzed to find the average number of colocalized structures. To find the probability of random overlap of peaks, the fluorescence profile of a curved line out of the membrane but similar in contour length to that of the PMS was used. On average, the noise level was found to be on the order of 5 ± 1 coincidental colocalizations per confocal plane. Image analysis was performed using Labview 8.5 and Vision 8.5.

Cell Stretching

The PDMS sheets were prepared by mixing at 10:1 ratios, the PDMS and the cross-linker (Sylgard 184, Dow Corning, Midland, MI) and baking at 60 ºC (12 hrs) in a 90 mm diameter Petri-dish placed at a vertical geometry allowing flow-through of excess PDMS and formation of thin sheets. A chamber for cells was created by coupling the rectangular section (~12×7 mm) of the thin sheet to another PDMS sheet with a rectangular hole (inner dimensions: ~6×4 mm) using silicon grease. The surface modification consisted of first, attachment of PLL-g-

PEG, followed by selectively destruction, using photo-masks, of the PLL-g-PEG layer by UV irradiation and finally incubation with fibronectin, leading to the creation of adhesive patches (Aziounne et al., 2009, Lab On Chip *9*, 1640-1642). Specifically, rectangular patches of size 13×60 μm were used. The strain was calculated by the change in linear dimensions of the rectangular cell footprint visible by the Cav1-EGFP fluorescence. The choice of rectangular (elongated) patterns ensured that the transverse contraction was minimized (and negligible in practice) with respect to longitudinal extension.

Immunofluorescence Studies

For immunofluorescence experiments, cells were washed in PBS and fixed in 4% paraformaldehyde for 15 min at room temperature, then washed once in PBS, and quenched for 10 min in 50 mM NH_4Cl in PBS at room temperature. For permeabilization of the plasma membrane, cells were first incubated in permeabilizing buffer (PB, PBS with 1 mg/ml BSA and 0.05% saponin) for 45 min at room temperature, then for 60 min with supplemented primary antibody (rabbit polyclonal anti-caveolin) (BD, Franklin Lakes, NJ). After two washes in fresh PB, the cells were incubated in PB supplemented with secondary antibody (anti rabbit Cy-3) for 45 min at room temperature. After successive washes in PB, PBS, and H_2O, the cells were mounted on microscope slides in 25 mg/ml Dabco (Sigma-Aldrich), 100 mg/ml Mowiol (Calbiochem-Novabiochem, La Jolla, CA), 25% glycerol (vol/vol), 100 mM Tris-HCl, pH 8.5. Cells were then studied on an inverted microscope (Leica, Wetzlar, Germany).

Deep-Etched EM of the Cytoplasmic Surface of MLEC

MLEC were grown on glass coverslips and used for the experiment 2 days after plating the cells. The cells were treated with three different osmotic conditions – iso-osmotic (300 mOsm), hypo-osmotic (30 mOsm, 5 min), and recovered iso-osmotic (30 mOsm, 5 min, followed by 300 mOsm, 5min). Immediately after being unroofed of the apical cell membrane by a small powered ultrasonic probe, the basal cell membrane was fixed for 15 min in 2.5% glutaraldehyde in the inside buffer (70 mM KCl, 5 mM MgCl2, 3 mM EGTA, 30 mM HEPES buffer adjusted at pH 7.4 with KOH), and washed with the inside buffer for 10 min three times. The specimen was washed in distilled water for 1 min before rapid freezing. The cover slip attached to the basal side of the cells was set on the plunger tip of the rapid freezing device (Polaron Instruments. Inc., Hatfield, PA) with the cytoplasmic surface of the plasma membrane down. The cover slip was fallen onto a polished pure copper block, which was pre-

cooled by liquid helium. The frozen cover slip was immersed in liquid nitrogen and was transferred into the freeze etching shadowing chamber (BAF060, Bal-TEC AG, Balzers, Liechtenstein). The cytoplasmic surface was deeply etched and rotary shadowed with platinum/carbon at an angle of 22ºC from the surface and with carbon from the top. The replica was removed from the cover slip in aqueous solution of 10% hydrofluoric acid. After the replica was washed with distilled water, the replica was mounted on mesh copper grid coated with polyvinyl formvar (Nisshin EM Co., Tokyo, Japan). Finally, the sample grid was observed by transmission electron microscopy (TEM) (Tecnai Spirit 120KV, FEI, Hillsboro, OR) equipped with CCD camera (4K×4K pixels, Gatan Ultrascan, Pleasanton, CA).

Electron Microscopy

For conventional EM, cells were fixed with a mixture of 2% PFA and 1.5% glutaraldehyde in phosphate buffer, post fixed with Osmium tetroxide, dehydrated in ethanol and embedded in epon. For ultrathin cryosectioning and immunogold labeling we fixed cells with 2% PFA or with a mixture of 2% PFA and 0.2% glutaraldehyde in phosphate buffer, pH 7.4. Cells were processed for ultracryomicrotomy and immunogold labeled using an anti-Cav1 polyclonal antibody (BD) and Protein A conjugated to 10 nm gold (PAG10) (Cell Microscopy Center, Utrecht University, The Netherlands) as reported previously (Slot, J.W., and Geuze, H.J. (2007) Nat. Protoc. *2*, 2480-2491)(Slot and Geuze, 2007). Sections were observed under an electron microscope (Philips CM120; FEI Company, Eindhoven, The Netherlands), and digital acquisitions were made with a numeric camera (Keen View; Soft Imaging System, Munster, Germany).

Cholesterol Measurement

For cholesterol measurement in PMS, two confluent 150 mm Petri dishes (4-5 x 10^6 cells) were used. PMS were detached through gentle shake, transferred into a 15 ml tube and concentrated through centrifugation for 5 min at 1000 rpm in a table top centrifuge (5810, Eppendorf, Germany). The concentrated PMS (100-150 µg of proteins) were dissolved in 0.2M NaOH (1 ml). Total cholesterol was extracted with methanol (2.5 ml) followed by hexane (5 ml). About 4.5 ml of the hexane phase was evaporated under vacuum and dissolved in mobile phase. Separation of free cholesterol (FC) and cholesterol esters (CE) was done by reverse phase HPLC on a C-18 column (25 x 0.46 cm length, 5-µm pore size, Sigma, St Louis, MO, USA) by detecting 205 nm absorbance after elution with acetonitrile/isopropanol

(30/70, v/v). The amount of proteins present in PMS preparations was measured using the bicinchoninic acid (BCA) method.

Statistical Tests

Significant differences were assessed for each data set by first testing them for normality and by using a two sample t-test to calculate the P value using Origin (OriginLab Corporation, Northampton, MA).