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Supplementary Information for

EHD2-mediated restriction of caveolar dynamics regulates cellular fatty acid uptake

Claudia Matthaeus, Ines Lahmann, Séverine Kunz, Wenke Jonas, Arthur Alves Melo, Martin Lehmann, Elin Larsson, Richard Lundmark, Matthias Kern, Matthias Blüher, Hannah Olschowski, Julian Kompa, Britta Brügger, Dominik N. Müller, Volker Haucke, Annette Schürmann, Carmen Birchmeier, Oliver Daumke

Corresponding authors: Claudia Matthaeus, Oliver Daumke

Email: Claudia.matthaeus@nih.gov; oliver.daumke@mdc-berlin.de

This PDF file includes:

Supplementary text: Extended Materials and Methods

Figures S1 to S10

Legends for Movies S1 to S10

SI References

Other supplementary materials for this manuscript include the following:

Movies S1 to S10

Extended Material and Methods

Obesity mouse models. Male NZO/HIBomDife (German Institute of Human Nutrition, Nuthetal, Germany), C57BL/6J (Charles River Laboratories, Sulzfeld, Germany) and B6.V-Lepob/ob/JBomTac (B6-ob/ob) mice (Charles River Laboratories, Calco, Italy) were housed under standard conditions (conventional germ status, 22 °C with 12 hour light /dark cycling). NZO and C57BL/6J mice were fed standard chow diet (Ssniff, Soest). Starting at 5 weeks of age, B6-ob/ob received carbohydrate-free diet. Mice were sacrificed at an age of 20-22 weeks.

Mouse embryonic fibroblast isolation and immortalization. All animals were handled according to governmental animal welfare guidelines. MEFs were obtained from E14.5 EHD2 +/+ or del/del embryos. For this, female, pregnant EHD2 del/+ mice were sacrificed by cervical dislocation, the embryos were dissected and removed from the yolk sac in sterile, cold PBS. For genotyping, a small piece of each mouse embryo tail was harvested followed by complete dissection of the whole embryo. Afterwards, the embryo pieces were treated with 0.25% trypsin/EDTA (Sigma) overnight at 4 °C. After aspiration of the trypsin solution, 10 ml culture medium (DMEM/10% fetal bovine serum (FBS)/5% penicillin/streptomycin (PS)) was added and tissue pieces were broken up by pipetting. The cell suspension was transferred in 75 cm^2 culture flask for cultivation at 37 °C and 5% CO_2. Immortalization of isolated primary MEFs was assured by frequently splitting. From passage 15, an increased growth rate was observed suggesting immortalized MEFs. For all experiments, MEFs between passage 12 and 32 were used. For LD growth, MEFs were either treated with 10 µg/ml insulin, 1 µM dexamethasone, 500 µM IBMX and 10 µM rosiglitazone (all obtained from Sigma) diluted in culture medium (differentiation medium) or 5 µM oleic acid diluted in DMEM.

Primary adipocyte cell culture. Male EHD2 del/+ and EHD2 del/del mice or EHD2 cKO flox/wt or flox/flox were sacrificed by cervical dislocation and gonadal WAT was removed. Adipocytes and stromal vascular fraction (SVF) were isolated after washing the tissue in sterile PBS and digestion by collagenase type II (Sigma C6885). Mature adipocytes floating in the upper phase were transferred into a new flask and diluted with culture medium (DMEM/10% FBS/5% PS), SVF was obtained after 5 min centrifugation at 1,000 rpm. After complete tissue break up, the adipocyte cell suspension was passed through a 270 µm cell strainer and the cells were plated in 75 cm^2 culture flask at 37 °C and 5% CO_2 whereby pre-adipocytes adhere to the flask and mature adipocytes float in the medium. SVF suspension was cleaned by passing through 70 µm cell strainer. The following day, the culture medium was exchanged to remove dead or non-adherent cells. After 5 days, both pre-adipocytes and SVF were split by 0.25% trypsin/EDTA solution and merged for further cultivation. Differentiation to mature adipocytes was induced by 10 µg/ml insulin, 1 µM dexamethasone, 500 µM IBMX and 10 µM rosiglitazone diluted in culture medium. If not otherwise

mentioned, the primary pre-adipocytes were incubated for 5 days with differentiation medium and medium was changed after 2 days. Delipidation of FBS was carried out as described in (1). EHD2 cKO adipocytes were transfected with Cre recombinase-EGFP by using adeno-associated virus particles 8 (AAV8) produced from pAAV.CMV.HI.eGFP-Cre.WPRE.SV40 (Addgene, #105545). The adipocyte cell culture was transfected and differentiated for 5 days.

3T3-L1 differentiation and EHD2 gene silencing. Freshly split 3T3-L1 cells were seeded in DMEM/10% FBS/1% PS supplemented with 10 µg/ml insulin, 0.5 M 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone and 10 µM rosiglitazone (differentiation medium). EHD2 knockdown was performed 6 h after seeding with RNAiMAx (Invitrogen #13778030, 2 different EHD2 stealth siRNAs obtained from Invitrogen), and was repeated every third day. Differentiation medium was removed after 2 days and DMEM/FCS/PS was added. LDs were inspected at day 8.

Histology. EHD2 del/+ and EHD2 del/del mice were anesthetized with 2% ketamine/10% rompun, perfused first by 30 ml PBS and next by 50 ml 4% paraformaldehyde (PFA) and tissues were dissected. After 24 h of fixation in 4% PFA, tissues were dehydrated in 3 steps (each 24h) from 70-100% EtOH and afterwards incubated in xylol (Merck) for 48 h. Next, the tissues were embedding in liquid paraffin at ca. 65 °C and cooled down on ice. 4 µm paraffin sections were obtained, de-paraffinized and hydrated and Masson Trichrome staining (Kit, Sigma) was applied. Briefly, sections were stained with Bouin solution for 15 min at 60 °C, followed by Haematoxylin Gill No. 2 staining for 5 min and incubation in Biebrich-Scarlet-Acid Fuchsin for 5 min. Next, the tissue sections were treated with phosphotungstic/phosphomolybdic acid solution and Aniline Blue solution both for 5 min, and acetic acid treatment (1%) for 2 min. After extensive washing, the sections were dehydrated, incubated in xylol and embedded with Roti Histo Kit (Carl Roth). Images were obtained at Zeiss Axiovert100 microscope.

Immunohistostaining of cryostat sections. Perfused and fixated EHD2 del/+ and EHD2 del/del mice (as described before) were dissected and the investigated tissue pieces were further fixed for 1-4 h in 4% PFA, transferred to 15% sucrose (in PBS, Merck) for 4 h and finally incubated overnight in 30% sucrose. After embedding in TissueTek, the tissue was frozen at -80 °C. 5-15 µm sections were obtained in a cryostat at -20 - -30 °C and stored at -20 °C. For immunostainings, the cryostat sections were incubated with blocking buffer (1% donkey serum/1% TritonX100/PBS) for 1 h at room temperature, and treated overnight at 4 °C with the first antibody diluted in blocking buffer. After washing with PBS/1% Tween, the secondary antibody was applied for 2 h at room temperature. After completion of the staining, the sections were washed and embedded in ImmoMount. The stained sections were analyzed with Zeiss LSM700

microscope provided with Zeiss objectives 5, 10, 20, 40 and 63x. The obtained images were further investigated by ZEN software and ImageJ/Fij.

Transfection and siRNA-mediated knockdown. Cultivated MEFs were transfected with the following plasmids: pEHD2-EGFP, pEHD2-deltaN-EGFP, pEHD2-deltaEH-EGFP, pEHD2-deltaN-EH-EGFP, pEHD2-F322A-EGFP, pEHD2-F122A-EGFP, pCav1-EGFP, pGFP-Dyn2-K44A (Addgene #22301) or pEGFP by lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. Transfected cells were incubated for 48 h and afterwards, treated cells were analyzed by confocal microscopy or TIRF. siRNA knockdown of CD36, Cav1 or Cavin1 was performed in freshly split MEFs by electroporation with the GenePulser XCell (Biorad). Briefly, MEFs were split as described before and the obtained cell pellet was resuspended in OptiMEM (Gibco). After cell counting, the MEF cell suspension was diluted to 1.5×10^6 cells/ml and 300 μl were transferred into electroporation cuvettes (2 mm, Biorad). CD36, Cav1 or Cavin1 stealth siRNA (all obtained from Invitrogen) and siRNA negative control (medium GC content, CD36 siRNA#1 GGAAUUUGUCCUAUUGGCCAAGCUA, CD36 siRNA#2 CCAAGUCUUCUAUGUUCCAAACAAG; CD36 siRNA#3 CCAAUAACUGUACAUCUUAUGGUGC) was added to a final concentration of 200 nM. After careful mixing, the cuvettes were placed into the electroporation device and the pulse (160 μOHM, 500 μF, ∞ resistance) was applied. The electroporated cells were cultivated in DMEM/10% FBS for 48 h before the experiments were started. Successful siRNA knockdown was monitored by CD36 antibody staining, and Cav1 and Cavin1 Western Blots.

Immunocytostaining, LD staining and fatty acid trafficking in fixed cells. Adipocytes or MEFs were seeded on fibronectin (Sigma) coated glass dishes (ThermoFisher). Cells were washed with PBS, treated with 4% PFA for 10 min and blocking buffer (1% donkey serum/1% TritonX100/PBS) for 20 min. The first antibody was incubated for 1 h, followed by secondary antibody and DAPI stain. For LD staining, BODIPY (Molecular Probes #D3922, saturated solution) or Nile Red (Sigma #19123, saturated solution, in EtOH) was diluted to 1:1000 in PBS and applied for 30 min. The stained cells were washed and the glass dishes were placed on conventional microscope slides and embedded in ImmoMount (ThermoScientific #9990402). FA trafficking was investigated by using C12-BODIPY labeled fatty acid (Invitrogen #D3822) or C11-BODIPY594 fatty acid (Invitrogen D3835) in living cells (co-stained with ER tracker Red, Molecular Probes #E34250). Final fatty acid concentration was 1 μM, diluted in HBSS. MEFs were washed with HBSS, FA solution was applied for 1-10 min. To remove excess FAs which interfered with confocal imaging, cells were washed with ice-cold PBS, fixed with 4% PFA and embedded in ImmoMount. Zeiss LSM700 or Zeiss LSM880 confocal microscopes with 40x, 63x and 100x objective (all Oil) were used for fixed samples. ImageJ/Fij was used to analyze images.

Live imaging of caveolae movement and fatty acid trafficking by TIRF. MEFs transfected with pCav1-EGFP, or 1:1 with pEHD2-EGFP or pGFP-Dyn2-K44A, were incubated for 48 h on fibronectin coated cover slips (25 mm diameter). Samples were mounted in Attofluor Cell Chamber (Thermo) in a physiological buffer (130 mM NaCl, 4 mM KCl, 1.25 mM NaH_2PO_4-H_2O, 25 mM $NaHCO_3$, 10 mM glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.3, 305-315 mOsm/kg). TIRF imaging was performed on an inverted Microscope (Nikon Eclipse Ti) equipped with a 488 laser (Toptica), an dicroic mirror (AHF, zt405/488/561/640 rpc), a 60x TIRF objective (Nikon, Apo TIRF NA 1.49), an appropriate emission filter (AHF, 400-410/488/561/631-640) and a sCMOS camera (mNeo, Andor). All components were operated by open-source ImageJ-based micromanager software. All experiments were performed at 37 °C. To investigate the movement of single caveolae, transfected cells were selected in regions, in which individual Cav1 spots were observed (ROIs illustrated in Fig. 4A, enhanced images). Recordings were obtained with the following imaging settings: image size 1776x1760 pixel, 1x1 binning, 500 frames, 200 ms exposure time/frame. For dual-color imaging (Fig. 6A, B), exposure time for Cav1-EGFP was reduced to 100 ms. Plasma membrane staining by CellBrightA649 was applied 5 min before the recording started to ensure full labelling of the membrane (Fig. 6A). FA tracking was then investigated by applying C12-BODIPY labelled fatty acids (Molecular probes #D3822) or C11-BODIPY591 labelled fatty acids (Molecular probes #D3861) in imaging buffer and TIRF live-imaging was started. Exposure time was 50 ms. Due to the fast FA incorporation into the plasma membrane within seconds and the high background fluorescence from free FAs, it was not possible to precisely quantify the incorporation kinetics. For data analysis, only the first 150 frames were investigated to prevent photobleaching effects. After cropping to the specific ROI, kymograph analysis of several positions within the ROIs were carried out using the Reslice function of ImageJ/Fij to analyse caveolae movement. Investigation of the kymographs revealed a single, straight line for fixed, not moving caveolae and sparks or short lines for fast moving caveolae.

Transmission electron microscopy (TEM). Mice were fixed by perfusion with 4% (w/v) formaldehyde in 0.1 M phosphate buffer and tissues were dissected to 1-2 mm^3 cubes. For morphological analysis, tissue blocs were postfixed in phosphate buffered 2.5% (v/v) glutaraldehyde. Samples were treated with 1% (v/v) osmium tetroxide, dehydrated in a graded series of ethanol and embedded in the PolyBed® 812 resin (Polysciences Europe GmbH). Ultrathin sections (60-80 nm) were cut (Leica microsystems) and stained with uranyl acetate and lead citrate before image acquisition. For immuno-labeling, samples were fixed by perfusion as described above, but postfixed in phosphate buffered 4% (w/v) formaldehyde with 0.5% (v/v) glutaraldehyde for 1 hour. Samples were further processed as described in Slot and Geuze (2). Briefly, samples were infiltrated with 2.3 M sucrose, frozen in liquid nitrogen and sectioned at cryo temperatures.

Sections were blocked and washed in PBS supplemented with 1% BSA and 0.1% glycine. Labeling was performed with an anti-caveolin-1 antibody 1:500 (Abcam #2910) and 12 nm colloidal gold (Jackson Immuno Research). Sections were contrasted with 3% tungstosilicic acid hydrate (w/v) in 2.8% polyvinyl alcohol (w/v) (3). Samples were examined at 80 kV with a Zeiss EM 910 electron microscope (Zeiss). Acquisition was done with a Quemesa CCD camera and the iTEM software (Emsis GmbH).

Electron tomography (ET). For electron tomography, 150 nm sections of EHD2 del/del BAT tissue were prepared. Samples were processed as described for conventional 2D TEM. The samples were tilted from 60 to -60° in 2° steps and examined at 120 kV with an FEI Talos L120C electron microscope. FEI tomography software was used for the acquisition of tomograms, reconstruction and detailed analysis were done with Inspect3D, Amira (both obtained from ThemoFisher) and IMOD (4)).

In situ **hybridization.** Digoxigenin-labeled riboprobes were generated using a DIG-RNA labeling kit (Roche). In situ hybridizations were performed on 14 μm cryosections prepared from E18.5 wt embryos, as previously described (5). To generate an EHD2 specific in situ probe, a 400 bp fragment was amplified from wild type cDNA using PCR and the primers listed below. The PCR product was cloned into pGEM-Teasy (Promega). T7 and sp6 polymerases were used to generate Ehd2-sense and antisense probes, respectively. EDH2_ISH_FWD: 5'-CAGGTCCTGGAGAGCATCAGC-3'; EDH2_ISH_REV: 5'-GAGGTCCTGTTCCTCCAGCTCG-3'

Western Blot. EHD2 protein levels in different tissues were examined by Western Blot. For this, EHD2 +/+, EHD2 del/+ and EHD2 del/del mice were sacrificed by cervical dislocation and organs were dissected and snap-frozen in liquid nitrogen. After homogenization of the tissue in 1x RIPA buffer (Abcam) with a glass homogenizer, the tissue lysate was incubated for 1 h on ice followed by 15 min centrifugation at 15,000 rpm. Supernatant was transferred into a fresh tube and protein concentration was measured by NanoDrop. At least 10 μg protein/lane was applied to 4-12% SDS-PAGE NuPage (Invitrogen) and SDS-PAGE was performed according to the manufacture's protocol. Afterwards, proteins were blotted on nitrocellulose membrane (Amersham) at 80 V for 1 h, followed by blocking of the membranes with 5% milk powder (in TBST, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.1% Tween20) for 2 h at room temperature. To detect EHD2 protein levels, rabbit-anti-EHD2 (1:2,000) was applied over night at 4 °C. After washing with TBST, the secondary antibody goat-anti-rabbit-HRP was added to the membrane for 2 h at room temperature. Detection of EHD2 bands results from ECL detection solution and intensities were obtained by ChemiDoc XRS (Biorad).

Antibodies and dyes. Anti-EHD2-Rb (self-made), anti-Cav1-Mouse (BD Biosciences #610407), anti-Rabbit IgG HRP (Dianova), anti-Mouse IgG HRP (Dianova), anti-Perilipin1-Rb (Cell signaling #9349), anti-CD36-Rb

(Novus Bio #NB400-144 and Abcam #ab133625), anti-Cavin1-Rb (Abcam, #ab48824), anti-Dyn2-mouse (Sigma), anti-Rb-Cy3 (Dianova), anti-mouse IgG-Alexa488 (ThermoScientific #R37114), DAPI (Sigma D9542), CellBrightA649, C12-BODIPY labelled fatty acid (Molecular probes #D3822), C11-BODIPY591 labelled fatty acid (Molecular probes #D3861), ER–TrackerRED (Molecular Probes #E34250), Nile Red (Sigma #19123), BODIPY (Molecular Probes #D3922).

Blood plasma analysis. To measure distinct blood plasma parameter related to metabolic changes like adiponectin, insulin or free fatty acids, blood was taken from EHD2 del/+ and EHD2 del/del mice immediately after cervical dislocation. All blood samples were taken at 10.00 am. Briefly, mice were opened and the thorax was partly removed to get access to the left heart ventricle, a cannula was inserted and blood samples were taken. After short centrifugation at high speed, the plasma fraction was transferred to a fresh tube and snap frozen in liquid nitrogen. The following assays were used to measure the described blood plasma markers: Plasma insulin levels were measured by Mouse Ultrasensitive Insulin ELISA (80-INSMSU-E10, Alpco). Plasma adiponectin and leptin levels were measured by Mouse Adiponectin/Acrp30 (DY1119) and Mouse/Rat Leptin (MOB00) ELISA kits (R&D Systems). Plasma lipids were quantified with commercially available kits: cholesterol (cholesterol liquicolour colorimetric assay, Human, Wiesbaden, Germany), triglycerides/glycerol (triglyceride/glycerol colorimetric assay, Sigma) and non-esterified fatty acids (Wako Chemicals). All measurements were done according to manufacturers' recommendations.

Fatty acid uptake assay by fluorescence-activated cell sorting (FACS) analysis. EHD2 del/+ and EHD2 del/del pre-adipocytes were seeded in 6-well plates (100.000 cells/well) and differentiated into mature adipocytes as described above. The fatty acid uptake assay was performed as described elsewhere (6). Briefly, after 5 days of differentiation, adipocytes were starved for 1 h with serum-free DMEM. Next, 2 µM dodecanoic acid (FA12) labelled with BODIPY (Molecular probes #D3822) diluted in serum-free DMEM + 10 µg/ml insulin was added to the adipocytes and incubated for 5, 10, 20, 30 and 60 min at 37 °C. After washing twice with ice-cold PBS, 150 µl 0.25% trypsin/EDTA/PBS was applied to detach the cells. The adipocytes were treated with 500 µl ice-cold FACS buffer (HBSS/10% FBS/10 mM EDTA) and the cell solution was transferred to FACS tubes. Shortly before measurement, 1 µl/ml propidium iodide was added. FACS experiments were performed at LSR Fortessa 5 Laser with the following parameters: FSH: A, H, W, Voltage 255; SSC: A, H, W, Voltage 203; A488: A, Voltage 198; PE: A, Voltage 341. For each FACS sample, 30.000 cells were investigated. As negative control, unstained EHD2 del/+ and EHD2 del/del adipocytes were examined at first and the obtained BODIPY intensity values were used as a reference for unstained cells. To exclude adipocytes which did not show any positive fatty acid uptake, all unstained

cells were removed resulting in only positively stained population (R1, illustrated in red in Fig. 2E). Within this R1 population, adipocytes with strongly increased BODIPY intensity values were gated to population R2 (blue, Fig. 2E). Detailed analysis/gating and statistics was done by using FlyingSoftware2.5.1 (Perttu Terho, Cell Imaging Core, Turku Center for Biotechnology). For each experiment, 15.000 cells were analyzed and gated to the unstained, R1 or R2 population. Next, the percentage of cells gated to the populations were calculated for every time point and illustrated in the bar graph (Fig. 2F). The R2 population was investigated in more detail by normalization to the R2 cell number of EHD2 del/+ adipocytes (FI Appendix, Fig. S6L).

Glucose uptake assay. 2-NBDG ((2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) uptake of EHD2 del/+ and EHD2 del/del adipocytes was measured as recommended (Glucose uptake assay from BioVision). Briefly, adipocytes were treated as described for the fatty acid uptake assay. However, after starvation, 200 µM 2-NBDG (molecular probes #N13195) diluted in serum-free DMEM + 10 µg/ml insulin was applied to the cells followed by incubation times from 5-60 min. Staining analysis was done as mentioned for fatty acid uptake with the same FACS parameters and gating procedure whereby only one positive stained cell population was examined (R1, illustrated in SI Appendix, Fig. S6O).

Triglyceride measurement. Triglyceride levels were measured in 100 mg WAT or BAT tissue pieces from EHD2 del/+ and del/del mice or in 100.000 MEFs as previously described (7) by using the commercial kit RandoxTR-210 (Crumlin).

Cholesterol measurement. Total cholesterol and cholesterol ester levels were determined of 100 mg WAT or BAT pieces freshly obtained from EHD2 del/+ and del/del mice, or from 100.000 EHD2 +/+ and del/del MEFs by using Total Cholesterol Assay Kit (Fluorometric, Cell Biolabs #STA-390). The protocol was followed as described by the manufacturer, detection was done in an Infinite 200 TECAN plate reader with excitation wavelength 550 nm, emission wavelength 595 nm.

Free fatty acids quantification. Free fatty acid levels in EHD2 +/+ and del/del MEFs were determined by using the Free Fatty Acid Quantification Kit (Abcam, #ab65341). 500.000 cells were seeded in DMEM/10% FBS/ 1% PS and 5 µM oleic acid was applied for 24 h. MEFs lysates were prepared as described by the manufacturer, followed by the colorimetric assay and detection in Infinite 200 TECAN plate reader at a wavelength of 570 nm.

TLC and MS lipid analysis. 100.000 EHD2 +/+ and del/del MEF cells were cultured for 2 days in DMEM/10%FBS/1%P/S, followed by overnight treatment of oleic acid. After washing with DMEM, 2 µM dodecanoic acid (FA12) labelled with BODIPY (Molecular probes #D3822) diluted in HBSS was applied for 10 or 20 min. After washing twice with ice-cold PBS, cell pellets were resuspended in methanol and

subjected to Bligh and Dyer lipid extraction as described (8). Organic solvent was evaporated by a gentle stream of nitrogen. Dried lipids were resuspended in 100 µl methanol. A 5 ul-aliquot of each sample was spiked with 25 pmol PC standard (PC 13:0/13:0, 14:0/14:0, 20:0/20:0; 21:0/21:0, Avanti Polar Lipids) and analyzed on a QTRAP6500+ (Sciex) in positive ion mode, applying precursor ion scanning to select for choline phosphate head group fragment ions at m/z 184 (7). Data evaluation was performed with LipidView (Sciex). Based on PC as bulk membrane lipid, equal amounts of extracted lipids (280 pmol) were subjected to TLC analysis on Silica G60 plates with concentration zone. C12:0 BODIPY-FA, TopFluor-DAG (18:1/C11 TP) and TAG (18:1/18:1/C11 TP) were used as TLC standard. TLC separation was performed in an automatic developing chamber (ADC2, Camag) using hexane:ethyl acetate (1:1, vol:vol). Fluorescence detection on dried TLC plates was performed with an Amersham Image 600 unit (GE Health Care). To test for BODIPY-C12 incorporation into cellular lipids, a 10 µl aliquot of each fraction was diluted 1:10 in 10 mM ammonium acetate in methanol and subjected to MS analysis on a QTRAP6500+, scanning for the neutral loss of fatty acids, including [C12 BODIPY-FA]$^-$, as NH_4^+ adducts.

Gene expression analysis. EHD2 del/+ and EHD2 del/del adipocytes were differentiated for 5 days, washed twice with ice-cold PBS, and RNA was isolated according to the Qiagen protocol (RNeasy Mini Kit, Qiagen). SuperscriptIII First Strand Synthesis Kit (Invitrogen #18080051) was used to obtain corresponding cDNA, which then was used for real-time PCR. Gene expression levels were analyzed by GoTaq q-PCR (Promega, #A6001) Master Mix in Fast real time PCR cycler (Applied Biosystems) was used according to the manufacturer's protocol. To measure the relative fold change of genes in EHD2 del/del adipocytes compared to EHD2 del/+, the comparative real-time PCR method was applied whereby actin was used as reference gene.

Total mRNA from murine gonadal adipose tissue (gWAT) was extracted with RNeasy Mini Kit (QIAGEN GmbH, Hilden) according to manufacturer's instructions. RNA was transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega) according to manufacturer's recommendations. Expression of mRNA was determined by quantitative real-time PCR on LightCycler 480 II/384 (Roche, Rotkreuz, Switzerland) using GoTaq Probe qPCR Master Mix (Promega, Madison, USA) applying TaqMan Gene Expression Assays. Target gene expression of was normalized to the mean expression of *Eef2*, *Ppia* and *Actb* in murine samples.

Gene	Description	TaqMan Assay
EHD2	EH-domain containing 2	Hs.PT.58.4969281
Ehd2	EH-domain containing 2	Self-designed

Actb	Actin, beta	Self-designed
Eef2	Eukaryotic translation elongation factor 2	Self-designed
Ppia	Peptidylprolyl isomerase A	Mm.PT.39a.2.gs

Actb Left primer: TACGACCAGAGGCATACAG, Right primer: GCCAACCGTGAAAAGATGAC, Probe: TTGAGACCTTCAACACCCCAGCCA, **Eef2 (Integrated DNA Technologies)** Left primer: CACAATCAAATCCACCGCCA**,** Right primer: TGAGGTTGATGAGGAAGCCC**,** Probe: TAAGCAGAGCAAGGATGGCT**, Ehd2 (UPL, Roche)** Left primer: CAGCTGGAGCACCACATCT**,** Right primer: TCATGTGCCATCAACAGCTC**,** UPL probe: #80

Statistical analysis. At first, a normality distribution test (Kolmogorov-Smirnov test) was carried out for all experimental values. If the data was normally distributed, Student t-Test (two-tailed P-value) was applied, otherwise Mann-Whitney-Rank-Sum (two-tailed P-value) test was used to calculate the significant difference between two groups. Two-way-Anova tests were used to investigate LD size after CD36 siRNA knockdown, whereby for EHD2 wt and KO MEFs each CD36 siRNA#1-3 treated cells were compared to nonsense siRNA (negative control, Fig. 5G). Box plots, if not otherwise indicated in the figure legends, always represents median with whiskers from minimum to maximum, column bar graphs and line graphs represent mean with mean standard error of the mean (SE). Statistical calculations were carried out by using Prism (GraphPad software). Distribution of LD sizes represented in histograms as well as Pearson correlation (SI Appendix, Fig. S9) were also obtained by using Prism. For all experiments including the examination of mice or mouse tissue or human samples, n represents the number of mice/patients which were used (Fig. 1, 3, 7, SI Appendix, Fig. S1-6) and all analyzed cryo/paraffin sections or caveolae are also indicated (e.g.: n = 80 caveolae/3 mice). In cell culture experiments (Fig. 2, 4-6, SI Appendix, S5, S7-10), n represents the number of investigated events (e.g.: lipid droplet area, fatty acid accumulation/cell) and the number of independently performed experiments (e.g.: n = 80 lipid droplets/3 independent experiments). The following P-values were used to indicate significant difference between two groups: * $P<0.05$; ** $P<0.001$; *** $P<0.0001$.
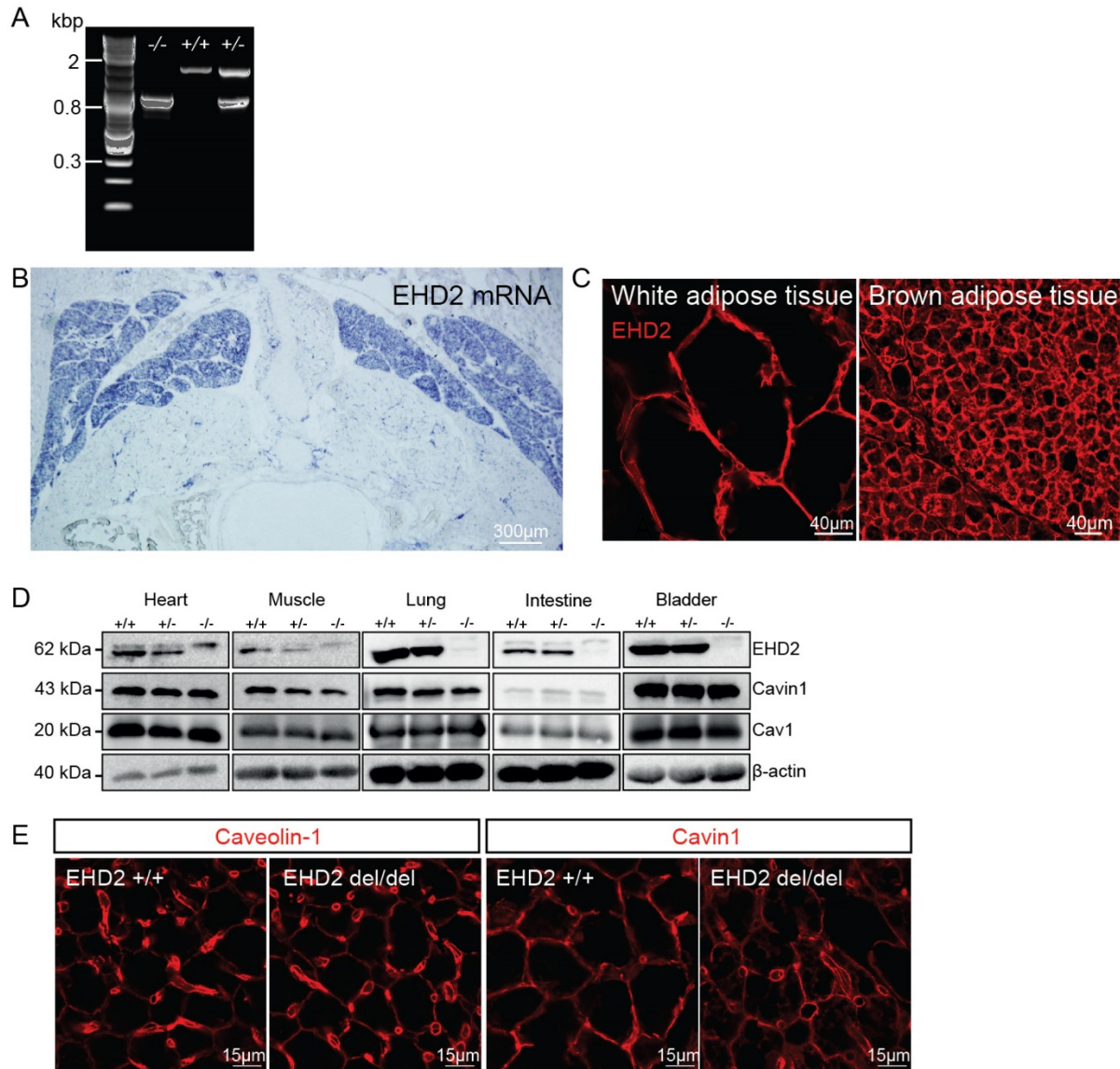
**Fig. S1: EHD2, Cav1 and Cavin1 expression in BAT (related to Fig. 1)**

A Genotyping of EHD2 delta E3 offspring (wild type – band size 1,700 bp; EHD2 KO – band size 830 bp).

B *In situ* hybridization against EHD2 mRNA of BAT in an E18 C57BL6/N embryo.

C Cryostat section of adult C57BL6/N white adipose or brown adipose tissue stained against EHD2.

D Western Blot analysis of different tissues from EHD2 +/+, +/- and -/- mice showing EHD2, Cav1 and Cavin1 protein level.

E Cavin1 and Cav1 protein level in BAT cryostat sections from EHD2 +/+ and del/del mice.
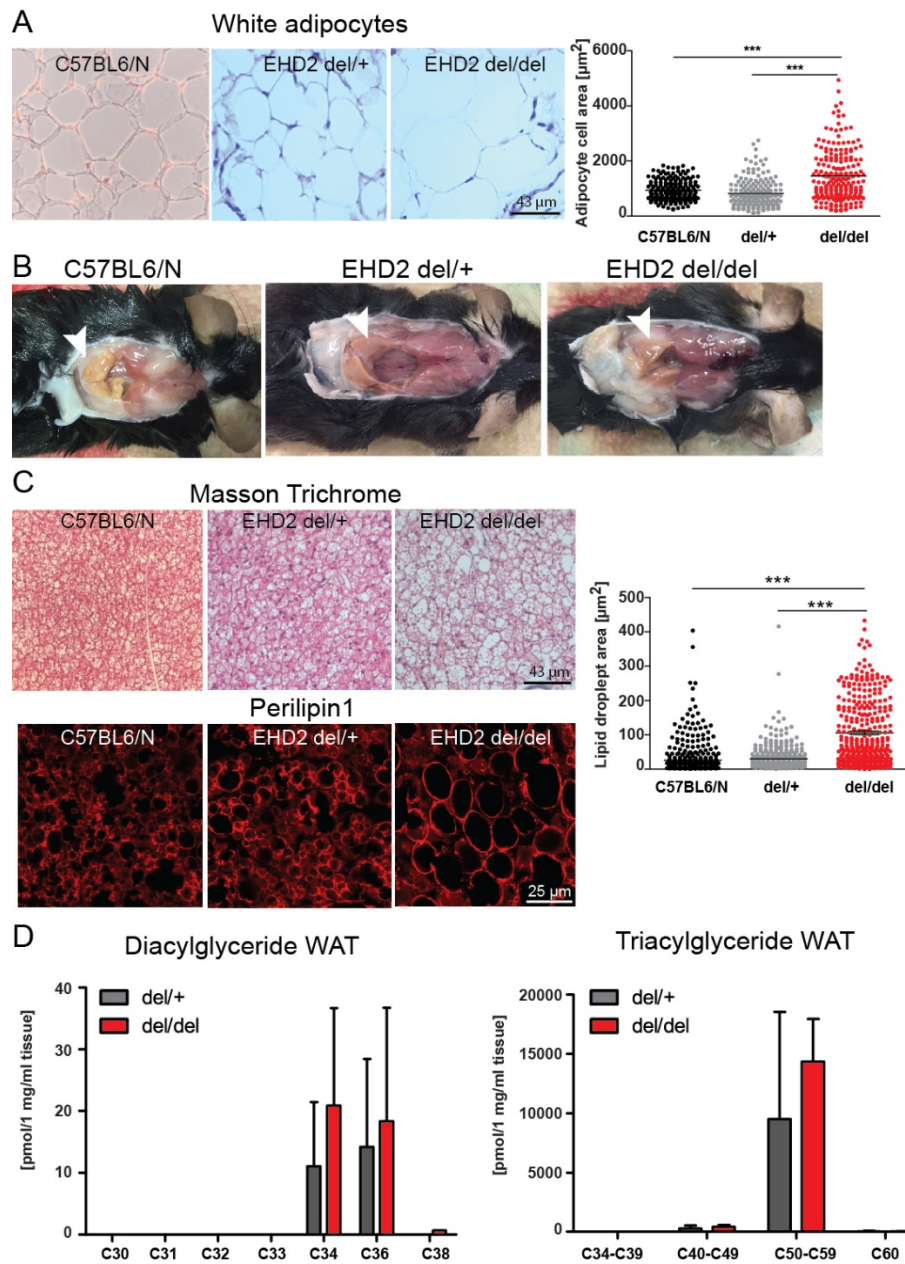
Fig. S2: C57BL6/N and EHD2 del/+ mice did not reveal differences in lipid accumulation (related to Fig. 1)

A WAT paraffin sections from C57BL6/N mice stained with Masson Trichrome and analyzed by adipocyte cell size (n(C57BL6/N) = 186/3; n(EHD2 del/+ = 172/3; n(EHD2 del/del) = 199/3).
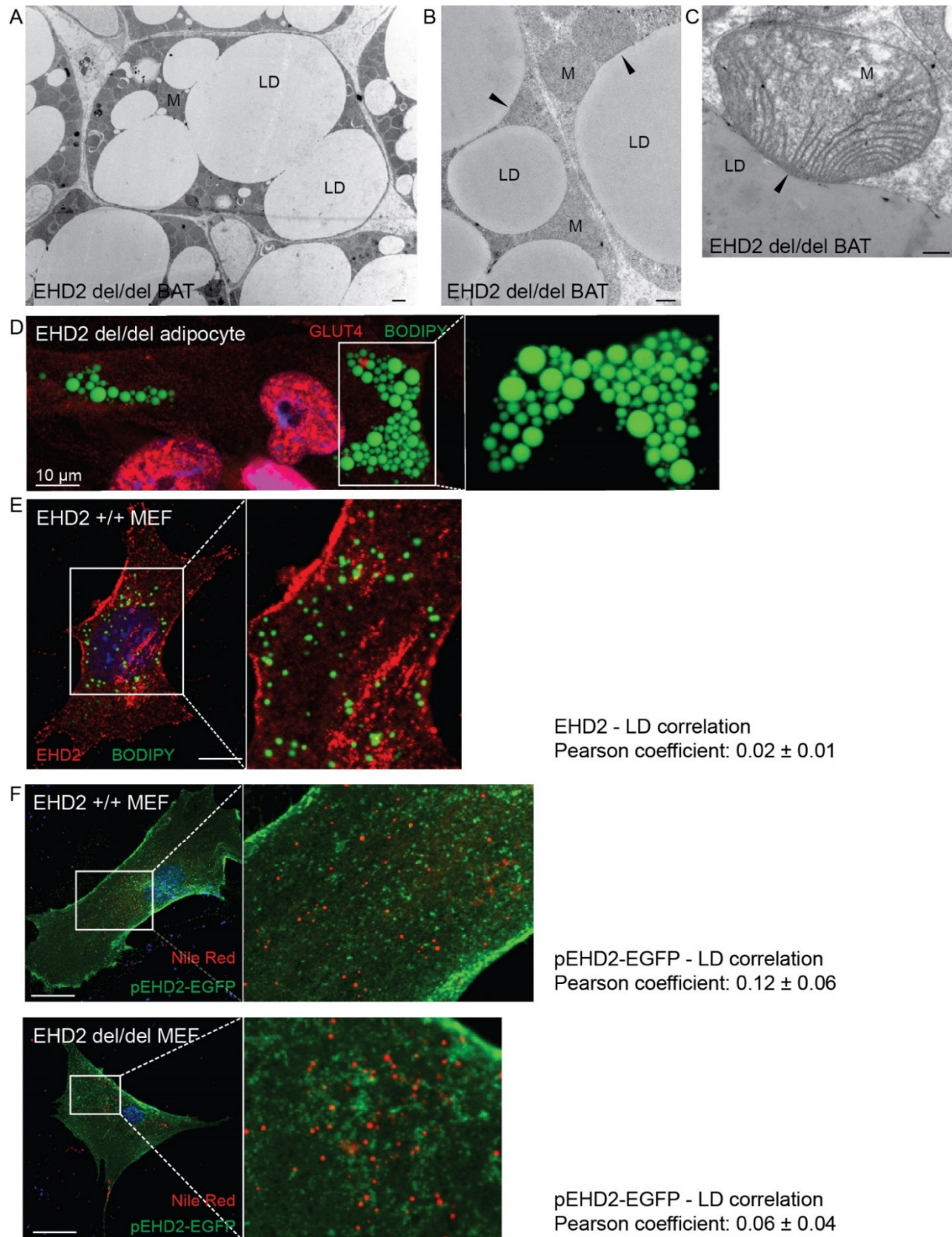
B BAT examples of C57BL6/N, EHD2 del/+ and del/del mice.

C BAT paraffin, and cryostat sections stained against Perilipin1. Lipid droplet size was measured by Perilipin1 staining. n(C57BL6/N) = 461/3; n(EHD2 del/+) = 398/3; n(EHD2 del/del) = 352/3.

D Diacylglyceride and triglyceride levels in EHD2 del/+ and del/del WAT (n = 2). Graphs illustrate each replicate with mean +/- SE, column bar graphs show mean + SE, normally distributed groups were analyzed by t-test, not normally distributed values with Mann Whitney U test, *** P<0.0001.

Fig. S3: Lipid droplets of EHD2 del/del cells exhibit normal shape and organelle contact sites

EHD2 - LD correlation
Pearson coefficient: 0.02 ± 0.01

pEHD2-EGFP - LD correlation
Pearson coefficient: 0.12 ± 0.06

pEHD2-EGFP - LD correlation
Pearson coefficient: 0.06 ± 0.04

Fig. S3: Lipid droplets of EHD2 del/del cells exhibit normal shape and organelle contact sites (related to Fig. 1 and 2)

A-C Overview of EHD2 del/del brown adipocyte (**A**) obtained by EM. EHD2 del/del LDs showed contact sites with several mitochondria (**B**, **C**, arrowheads: M – mitochondria; LD – lipid droplet, scale bar 200 nm).

D EHD2 del/del differentiated adipocytes revealed large LDs that showed the expected shape and form.

E EHD2 staining in wild type MEFs illustrate the localization of EHD2 mainly at the plasma membrane. Rarely, single EHD2 spots were found at LDs (BODIPY staining, scale bar 20 µm, Pearson coefficient n = 13).

F EHD2 +/+ and del/del MEFs were transfected with EGFP-tagged EHD2 and LDs were stained with Nile Red (scale bar 20 µm, Pearson coefficient n = 13).
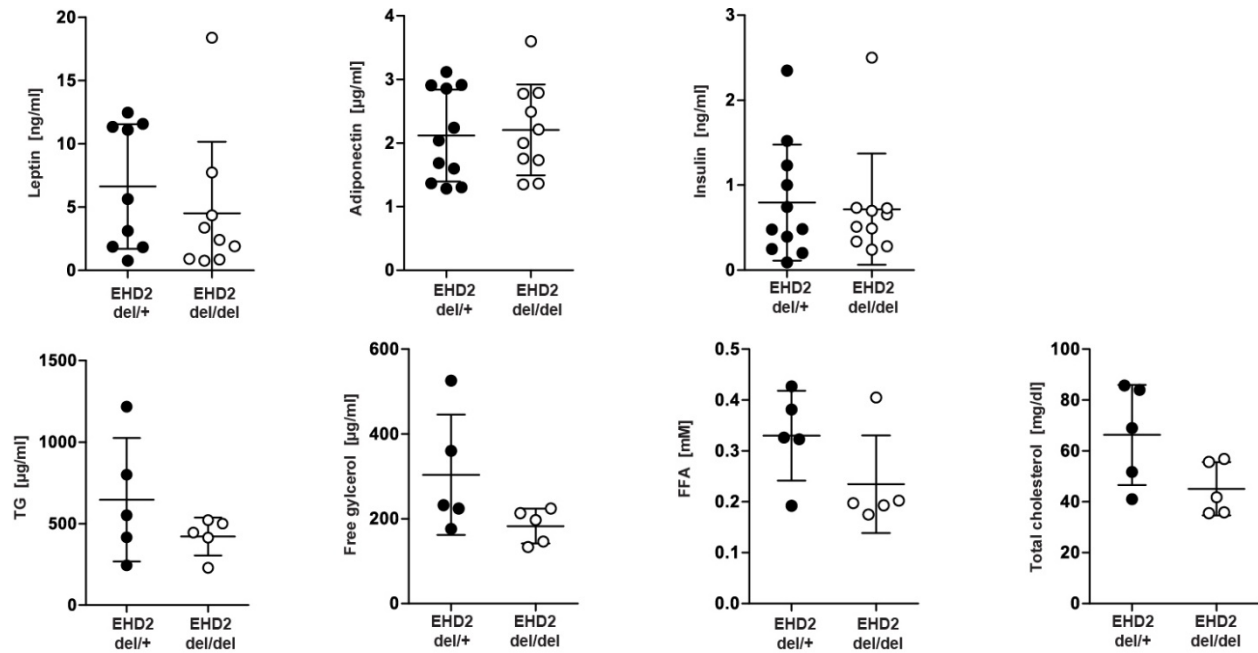
Fig. S4: Blood plasma analysis of EHD2 del/+ and EHD2 del/del mice did not reveal any significant differences (related to Fig. 1)

Blood samples obtained from EHD2 del/+ and del/del mice (n(EHD2 del/+) = 10 or 5; n(EHD2 del/del) = 10 or 5; graph illustrates each replicate with mean +/- SE). FFA – free fatty acid, TG – triglycerol.
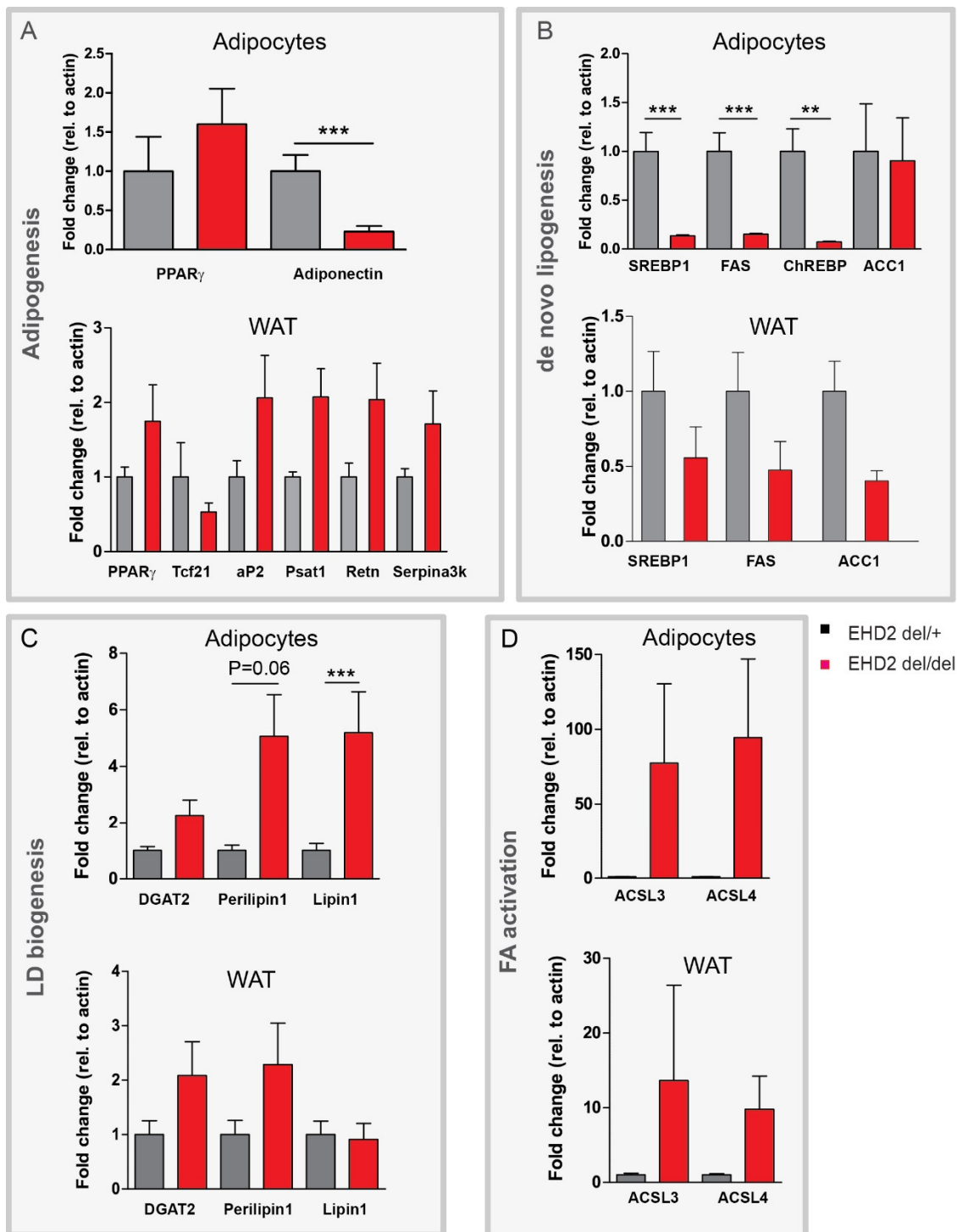
Fig. S5: Gene expression analysis in EHD2 del/+ and del/del white adipocytes (related to Fig. 1 and 2)
A-B Gene expression analysis of genes involved in adipogenic genes (**A**) or *de novo* lipogenesis (**B**) in EHD2 del/+ and del/del differentiated adipocytes or WAT (n = 8).
C-D Gene expression analysis of LD biogenesis genes (**C**) or FA activation genes (**D**, n = 4).
Column bars show mean + SE, t-test or Mann Whitney U test was used to calculate significance, ** $P<0.001$, *** $P<0.0001$.

Fig. S6: Cellular investigation of EHD2 del/del adipocytes and 3T3-L1 cells
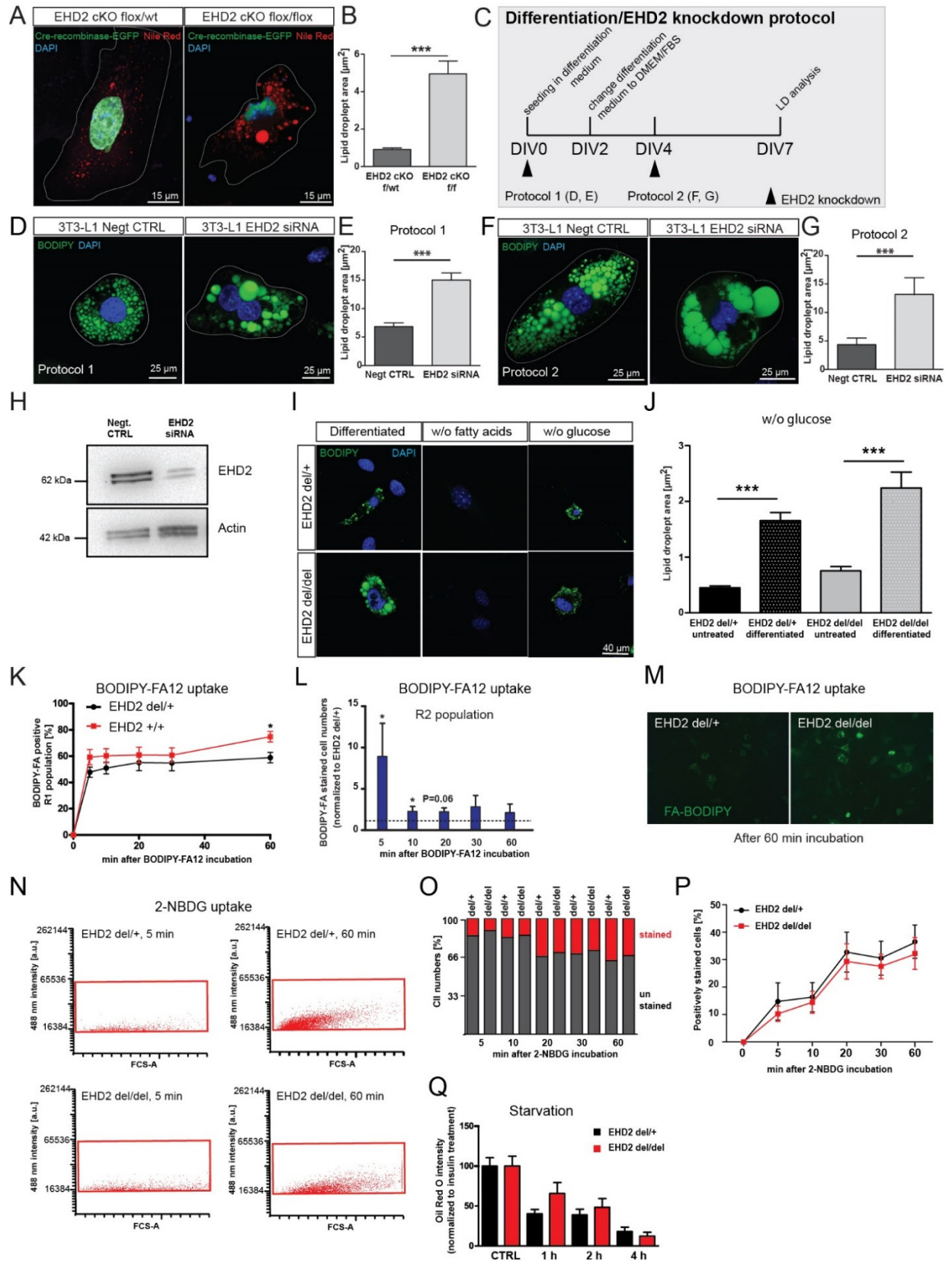
Fig. S6: Cellular investigation of EHD2 del/del adipocytes and 3T3-L1 cells (related to Fig. 2)

A-B Cultivated EHD2 cKO flox/wt or flox/flox adipocytes were infected with a Cre recombinase-EGFP virus to induce EHD2 deletion and differentiated for 5 days. LDs were stained with Nile Red (n(flox/wt) = 74/2, n(flox/flox) = 82/2).

C-H LD analysis in differentiated 3T3-L1 after EHD2 siRNA knockdown. The applied differentiation and EHD2-knockdown strategies are shown in **C**. In protocol 1 (**D, E**), EHD2 was treated with EHD2 siRNA or non-sense siRNA concomitantly with differentiation. In Protocol 2 (**F, G**, Morén et al. ref (9)), 3T3-L1 cells were treated with EHD2 siRNA or non-sense siRNA at day 4 following differentiation. LDs were stained with BODIPY. **D**: n(Negt CTRL) = 208/2; n(EHD2 siRNA) = 187/2). **E**: n(Negt CTRL) = 340/3; n(EHD2siRNA) = 348/3. Successful EHD2 knockdown was confirmed by Western Blot (**H**).

I-J Pre-adipocytes were treated for 5 days with either differentiation medium containing delipidated FBS or without glucose followed by BODIPY staining illustrating LDs. Summary of LD size in EHD2 del/+ and del/del differentiated adipocytes without glucose (untreated: n(del/+) = 49/2 ; n(del/del) = 46/2; differentiated: n(del/+) = 75/2 ; n(del/del) = 62/2).

K-L Analysis of FA uptake in differentiated EHD2 del/+ and EHD2 del/del adipocytes (see also Fig. 2). Dodecanoic acid-BODIPY uptake was measured after 5, 10, 20, 30 or 60 min. R1 population indicates positively stained cells (illustrated in red in Fig. 2E). R2 populations (blue) correspond to higher BODIPY staining intensity in cells and represent adipocytes with an increased uptake of dodecanoic acid (shown in blue in Fig. 2E). **L** shows the normalization of EHD2 del/del R2 population relative to EHD2 del/+ R2. n(del/+) = 6/3 experiments, n(del/del) = 8/3 experiments).

M Example images of differentiated adipocytes treated with BODIPY-FA12 for 60 min.

N-P Following 5 days of differentiation, glucose uptake (2-NBDG glucose) in cultured adipocytes was measured after 5-60 min. Example graphs for EHD2 del/+ and del/del after 5 min or 60 min illustrating positively stained cell population (**N**). Overview of glucose uptake in percent cell number (**O**) and time dependence (**P**, n = 6/3 experiments).

Q Differentiated EHD2 del/+ and EHD2 del/del adipocytes were starved for 1-4 h and Oil Red O staining was applied to illustrate lipid accumulation (compared to Oil Red O staining intensity after 5 days of differentiation, before starvation, n(EHD2 del/+) = 53/3; n(EHD2 del/del) = 50/3).

Line graphs show mean +/- SE, column bar graphs show mean + SE, t-test or Mann Whitney U test was used to calculate significance, * P<0.05, *** P<0.0001.

Fig. S7: Caveolar mobility in MEFs
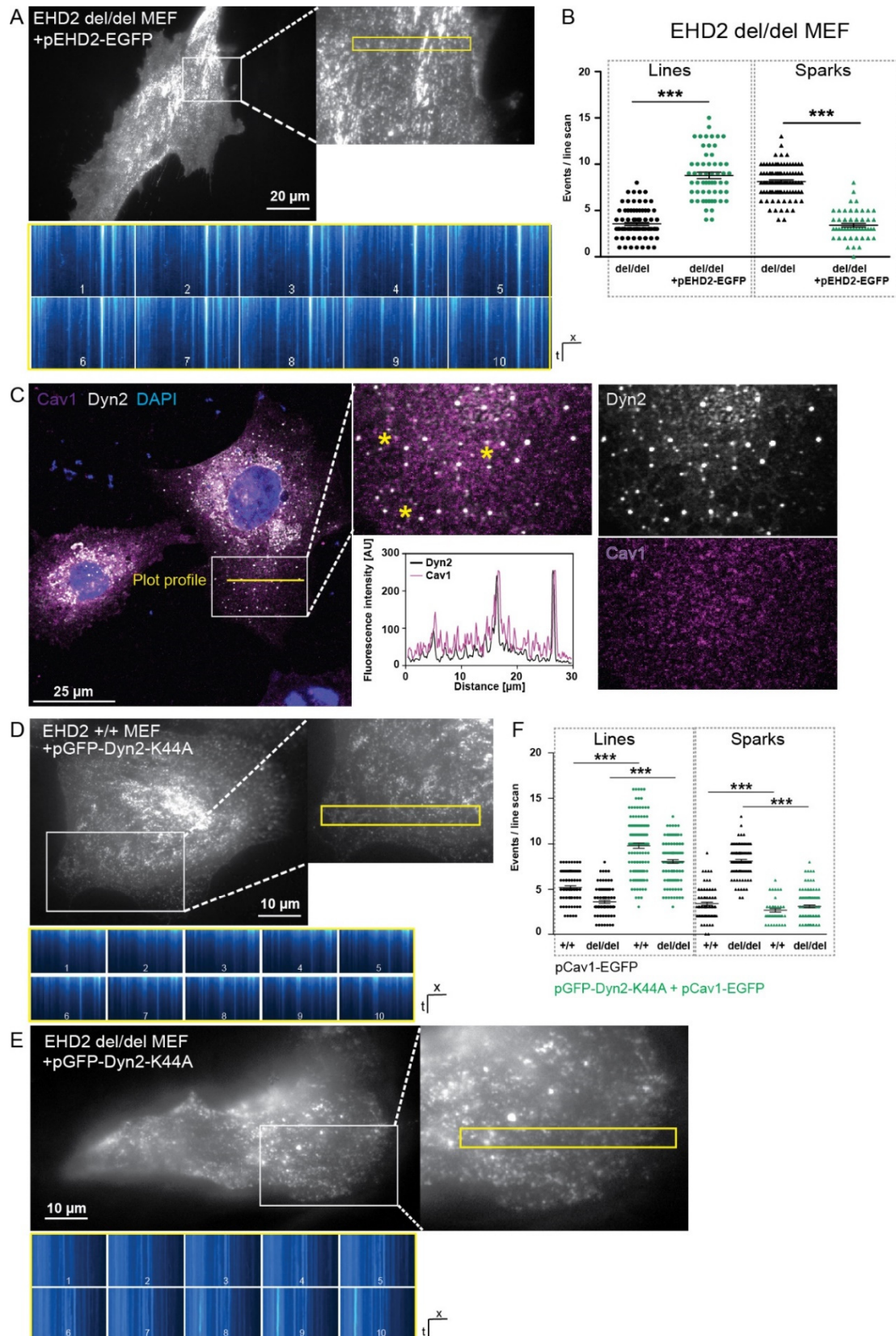
Fig. S7: Caveolar mobility in MEFs (related to Fig. 4 and 5)

A-B TIRF live-imaging in EHD2 del/del MEFs transfected with pEHD2-EGFP and pCav1-EGFP (1:1) to investigate single caveolae movement. Non-moving caveolae correspond to vertical lines within the line scan, moving caveolae can be related to single sparks (**B**, n = 30). See also SI Appendix, Movie S4.

C Dynamin2 (Dyn2) and Cav1 immunostaining in EHD2 +/+ MEFs. Plot profile represents fluorescence intensity of Cav1 and Dyn2 in respect to cellular location, yellow stars illustrate colocalization sites.

D-F TIRF live-imaging in EHD2 +/+ and del/del MEFs transfected with pCav1-EGFP and pGFP-Dyn2-K44A (1:1) to investigate single caveolae movement. Non-moving caveolae correspond to vertical lines within the line scan, moving caveolae can be related to single sparks (F, n(+/+) = 9 cells/3; n(del/del) = 12 cells/3). See also SI Appendix, Movies S5-6.

Graphs indicate each replicate from maximal to minimum value with mean, t-test or Mann Whitney U test were used to calculate significance, *** P<0.0001.

Fig. S8: Determinants of fatty acid uptake in MEFs
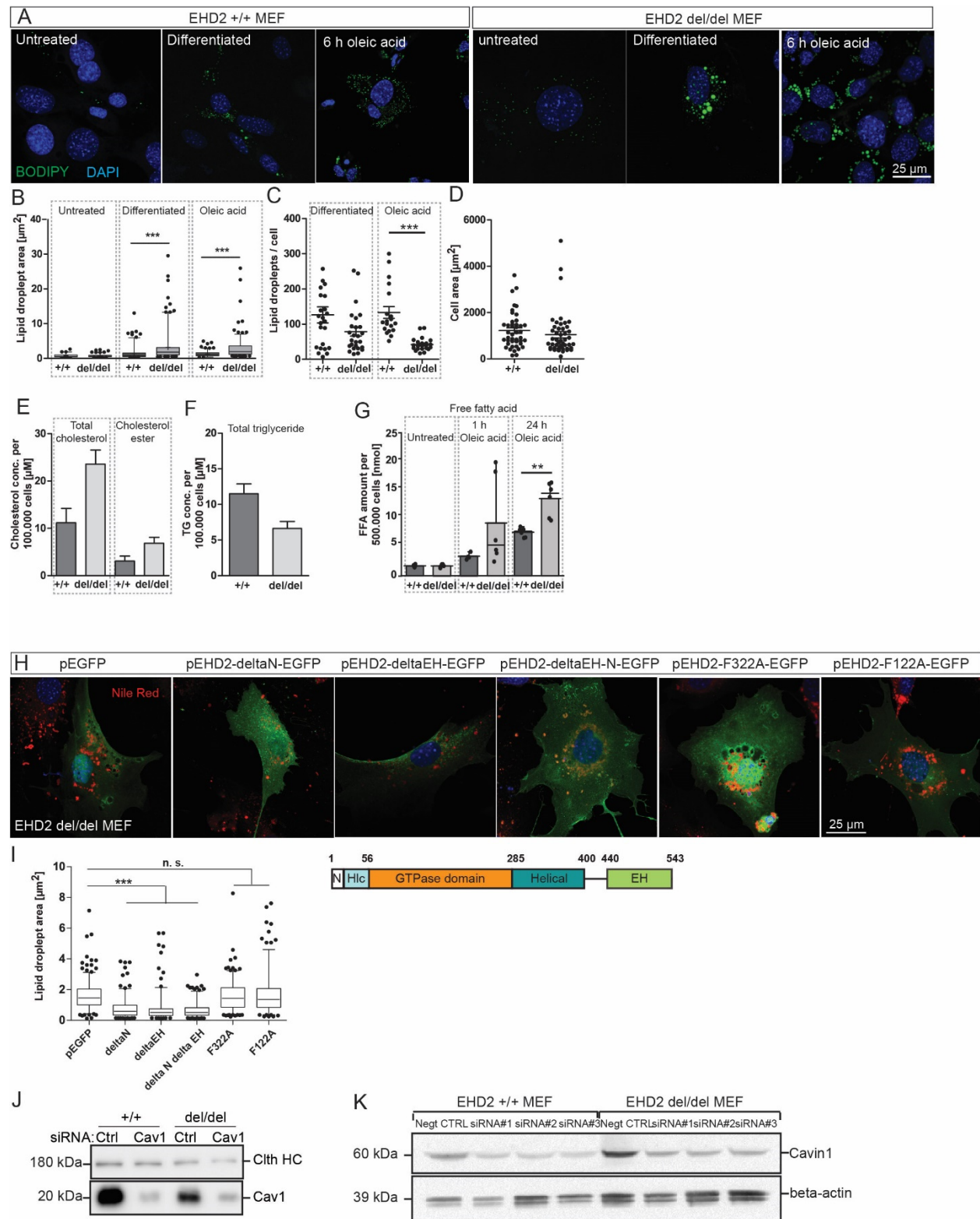
Fig. S8: Determinants of fatty acid uptake in MEFs (related to Fig. 5)

A-B LD analysis after 5 days of differentiation or 6 h of oleic acid in EHD2 +/+ or del/del MEFs by BODIPY staining (**B**, untreated: n(+/+) = 61/2, n(del/del) = 139/4); differentiated: n(+/+) = 148/3, n(del/del) = 200/4; oleic acid: n(+/+) = 146/3, n(del/del) = 217/3).

C Lipid droplet number per cell (differentiated: n(+/+) = 24/3; n(del/del) = 26/3; oleic acid: n(+/+) = 19/3; n(del/del) = 23/3).

D Cell area of EHD2 +/+ and del/del MEFs (n(+/+) = 43/5; n(del/del) = 47/5).

E-F Total cholesterol, cholesterol ester (**E**) and triglyceride content (**F**) was determined in differentiated EHD2 +/+ and del/del MEFs (n = 4).

G Free fatty acid levels were measured in 500.000 EHD2 +/+ and del/del MEFs untreated, or treated for 1 h and 24 h with oleic acid (n = 6/3).

H-I EHD2 constructs were transfected in EHD2 del/del MEFs. After 48 h, cells were treated with oleic acid followed by Nile Red staining to determine LD size (n(pEGFP) = 275/3; n(pEHD2-deltaN-EGFP) = 193/3; n(pEHD2-deltaEH-EGFP) = 197/3; n(pEHD2-deltaN-EH-EGFP) = 196/3; n(pEHD2-F322A-EGFP) = 204/3; n(pEHD2-F122A-EGFP) = 212/3).

J-K Western Blot of MEFs treated with Cav1 siRNA (**I**) or Cavin siRNA (**J**), Clth HC - clathrin heavy chain.

Box plots indicate mean +/- SE and single replicates of 5% of maximal and minimum values are illustrated, or graphs illustrate each replicate from maximal to minimum value with mean, column bar graphs show mean + SE, t-test or Mann Whitney U test were used to calculate significance, *** P<0.0001.
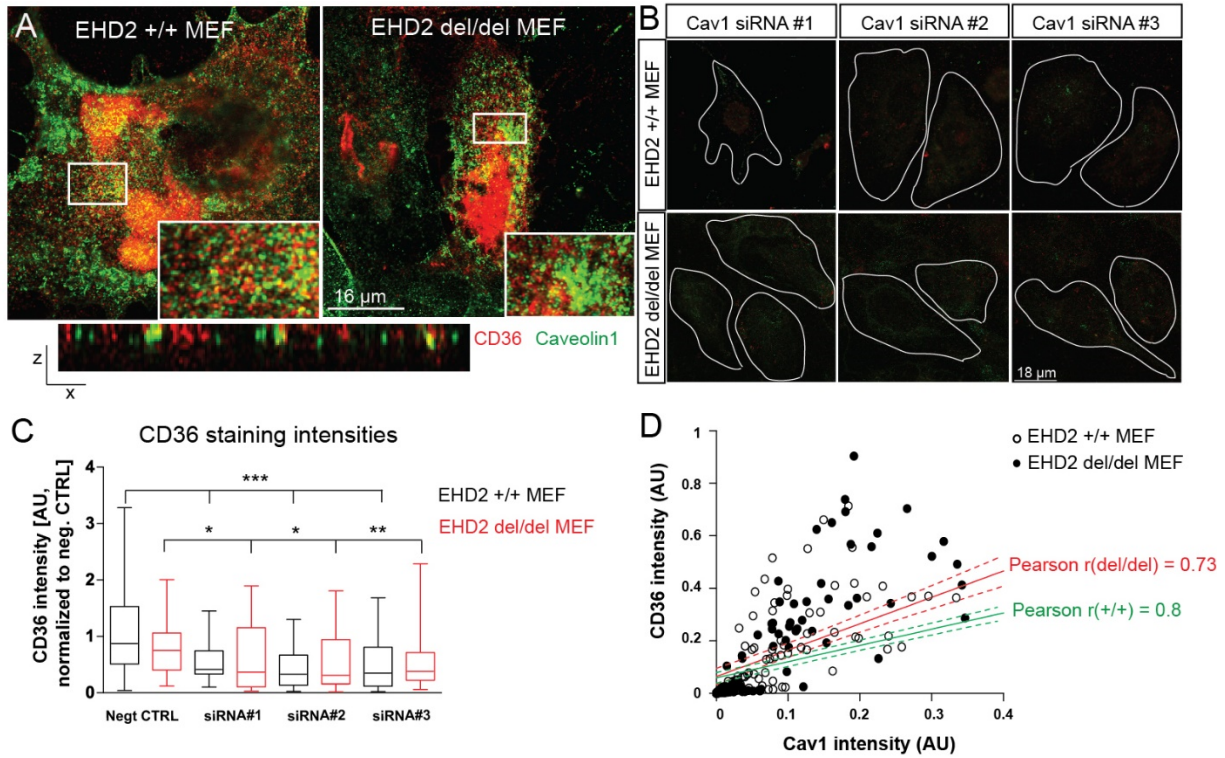
Fig. S9: CD36 plasma membrane localization requires Cav1 (related to Fig. 5)

A CD36 and Cav1 immunostaining in EHD2 +/+ and del/del MEFs.

B-C CD36 plasma membrane staining intensity was determined after Cav1 siRNA treatment in EHD2 +/+ and del/del MEFs (negative CTRL: n(+/+) = 64/3; n(del/del) = 61/3; siRNA#1: n(+/+) = 30/3; n(del/del) = 25/3; siRNA#2: n(+/+) = 44/3; n(del/del) = 24/3; siRNA#1: n(+/+) = 44/3; n(del/del) = 39/4).

D Pearson correlation of Cav1 and CD36 staining intensities obtained in Cav1 siRNA treated MEFs (n(+/+) = 116/3; n(del/del) = 95/3).

Box plot illustrates mean with whiskers from minimal to maximal value, Mann Whitney U test were used to calculate significance, *** P<0.0001.

Fig. S10: Fatty acid processing in MEFs
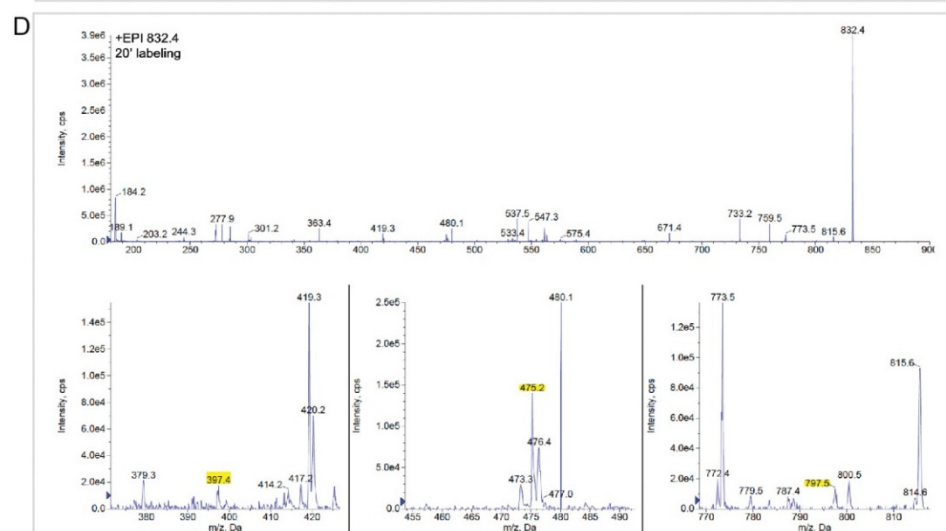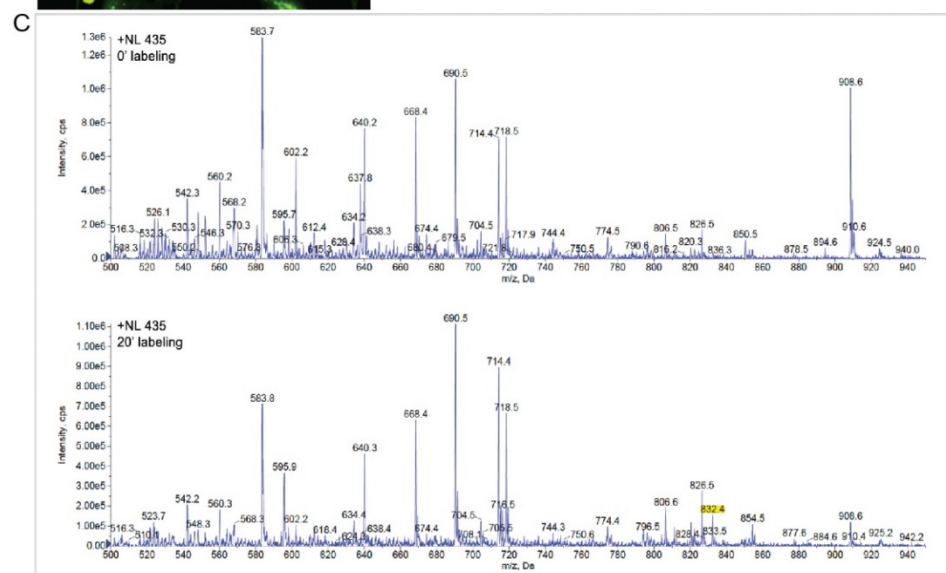
Fig. S10: Fatty acid processing in MEFs (related to Fig. 6)

A LDs of EHD2 +/+ MEFs were pre-stained with Nile Red (red) and BODIPY-labelled C12 fatty acid (FA – green) was applied for 3 min.

B Incorporation of BODIPY-FA into diacylglycerol. EHD2 +/+ and del/del MEFs were treated with oleic acid overnight, followed by BODIPY-labelled C12 fatty acid treatment for 10 or 20 min. Cellular lipids were extracted, analyzed by TLC using the solvent system hexane/ethyl acetate (1:1) and detected by fluorescence imaging. For cellular lipids, loading volumes were adjusted to apply same amounts of total PC (as bulk membrane lipid). Fluorescent fatty acid (FA), triacylglycerol (TAG) and diacylglycerol (DAG) were loaded as TLC standards.

C Positive ion mode neutral loss (+NL) scan for m/z 435 to monitor ammonium adducts of BODIPY-C12 FA in the absence (upper panel) or presence (lower panel) of BODIPY-C12 FA. A molecular peak at m/z 832.4 (yellow) corresponding to DAG BODIPY-C12/22:0 was subjected to MS enhanced product ion experiment.

D Positive ion mode enhanced product ion (+EPI) scan of m/z 832.4 (upper panel) from m/z 150 – 900. Zoom ins show fragment ions resulting from neutral loss of ammonium adducts of BODIPY-C12 FA (lower panel, left, m/z 397.4), FA 22:0 (lower panel, middle, m/z 475.2) and neutral loss of water (lower panel, right, m/z 797.5).

Movie Legends

Movie S1: Electron tomography of EHD2 del/del BAT (related to Fig. 3). Caveolae in a 150 nm EHD2 del/del BAT section were investigated by electron tomography. The movie includes all images obtained during the tilting from -60° to 60° (image acquisition every 2°). Both, detached and plasma membrane bound caveolae can be observed. For better handling during the segmentation and reconstruction in IMOD, the tomogram was reflected horizontal.

Movie S2: TIRF live imaging of caveolae in EHD2 +/+ MEFs (related to Fig. 4). EHD2 +/+ MEFs were transfected with pCav1-EGFP to detect single caveolae and afterwards TIRF live imaging was performed.

Movie S3: TIRF live imaging of caveolae in EHD2 del/del MEFs (related to Fig. 4). EHD2 del/del MEFs were transfected with pCav1-EGFP to detect single caveolae and afterwards TIRF live imaging was performed. Notably, Cav1 spots in MEFs lacking EHD2 showed increased caveolar dynamics.

Movie S4: TIRF live imaging of caveolae in EHD2 del/del MEFs transfected with pEHD2-EGFP (related to Fig. 4). EHD2 del/del MEFs were co-transfected with pCav1-EGFP and pEHD2-EGFP and caveolae movement was observed by TIRF live imaging. Re-expression of EHD2 in EHD2 del/del MEFs strongly reduced the dynamics of caveolae.

Movie S5: TIRF live imaging of caveolae in EHD2 +/+ MEFs expressing pGFP-Dyn2-K44A (related to Fig. 4 and 5)

EHD2 +/+ MEFs were co-transfected with pCav1-EGFP and pGFP-Dyn2-K44A and caveolae movement was observed by TIRF live imaging. Overexpression of dominant negative Dyn2 mutant in EHD2 +/+ MEFs strongly reduced the dynamics of caveolae.

Movie S6: TIRF live imaging of caveolae in EHD2 del/del MEFs expressing pGFP-Dyn2-K44A (related to Fig. 4 and 5). EHD2 del/del MEFs were co-transfected with pCav1-EGFP and pGFP-Dyn2-K44A and caveolae movement was observed by TIRF live imaging. Overexpression of dominant negative Dyn2 mutant in EHD2 del/del MEFs strongly reduced the dynamics of caveolae.

Movie S7: TIRF live imaging fatty acid incorporation in plasma membrane in EHD2 +/+ MEFs (related to Fig. 6). TIRF imaging of EHD2 +/+ MEFs that were pre-stained with plasma membrane dye (CellbrightA647) and BODIPY-labelled C12-fatty acids were applied.

Movie S8: TIRF live imaging fatty acid incorporation in plasma membrane in EHD2 del/del MEFs (related to Fig. 6). TIRF imaging of EHD2 del/del MEFs that were pre-stained with plasma membrane dye (CellbrightA647) and BODIPY-labelled C12-fatty acids were applied.

Movie S9: TIRF live imaging of fatty acid accumulation in caveolae in EHD2 +/+ MEFs (related to Fig. 6B, C). TIRF imaging of EHD2 +/+ MEFs to which BODIPY-labelled C12-fatty acids were applied. FA intensity histogram was adapted to better illustrate caveolae and FA.

Movie S10: TIRF live imaging of fatty acid accumulation in caveolae in EHD2 del/del MEFs (related to Fig. 6B, C). TIRF imaging of EHD2 del/del MEFs to which BODIPY-labelled C12-fatty acids were applied. FA intensity histogram was adapted to better illustrate caveolae and FA.

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