Caveolin-1 deficiency causes cholesterol dependent mitochondrial dysfunction and apoptotic susceptibility

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Running head:
Caveolin regulates mitochondrial function.

Highlights:
1) CAV1 deficiency promotes a mitochondrial and metabolic dysfunction.
2) Without CAV1 free cholesterol accumulates in mitochondrial membranes.
3) Cholesterol increases mitochondrial membrane condensation and ROS accumulation.
4) CAV1⁻/⁻ animals are more vulnerable to mitochondrial toxins and associated diseases.
Abstract

Caveolins (CAV) are essential components of caveolae; plasma membrane invaginations with reduced fluidity, reflecting cholesterol accumulation [1]. CAV proteins bind cholesterol, and CAV’s ability to move between cellular compartments helps control intracellular cholesterol fluxes [1-3]. In humans, CAV1 mutations result in lipodystrophy, cell transformation, and cancer [4-7]. CAV1 gene-disrupted mice exhibit cardiovascular diseases, diabetes, cancer, atherosclerosis, and pulmonary fibrosis [8, 9]. The mechanism(s) underlying these disparate effects are unknown, but our past work suggested CAV1 deficiency might alter metabolism: CAV1+/− mice exhibit impaired liver regeneration unless supplemented with glucose, suggesting systemic inefficiencies requiring additional metabolic intermediates [10]. Establishing a functional link between CAV1 and metabolism would provide a unifying theme to explain these myriad pathologies [11]. Here, we demonstrate that impaired proliferation and low survival with glucose restriction is a shortcoming of CAV1 deficient cells, caused by impaired mitochondrial function. Without CAV1, free cholesterol accumulates in mitochondrial membranes, increasing membrane condensation and reducing efficiency of the respiratory chain and intrinsic anti-oxidant defence. Upon activation of oxidative phosphorylation, this promotes accumulation of reactive oxygen species resulting in cell death. We confirm that this mitochondrial dysfunction predisposes CAV1 deficient animals to mitochondrial related diseases such as steatohepatitis and neurodegeneration.

197 words
Results and Discussion

Establishing a functional link between CAV1 and metabolism would provide a unifying theme to explain the myriad pathologies resulting from CAV deficiency [11]. Thus, mouse embryonic fibroblast cells (MEFs) from wt and CAV1−/− mice [12] were treated with 2-deoxyglucose (2-DG) which inhibits glycolysis. 2-DG reduced proliferation (Figure 1A) and dramatically increased cell death of CAV1−/− but not wt MEFs (Figure 1B). Upon nutrient limitation cells rely primarily on mitochondrial OXPHOS [13]. Thus, we analysed whether the increased apoptosis in CAV1−/− cells upon glycolysis inhibition might be caused by increased demands on mitochondria. We treated cells with DCA to shift glucose metabolism from lactate production to OXPHOS [14] (see also Figure S1). DCA preferentially promoted apoptosis in CAV1−/− MEFs (Figure 1C), supporting the hypothesis that lethality is related to activation of OXPHOS. Since OXPHOS is a major source of ROS, and ROS are apoptogenic triggers, we quantified cellular ROS levels. CAV1−/− MEFs had a significantly higher ROS content (Figure 1D), and DCA treatment enhanced ROS accumulation in CAV1−/− MEFs. The increased ROS was involved in the increased apoptosis, because treatment with the antioxidant BHA reduced the pro-apoptotic effect of DCA (Figure 1C). These results suggest a mitochondrial dysfunction in CAV1−/− cells, which is exacerbated by stimulation of OXPHOS. This sensitivity is not due to unknown additional variations in the genetic background and also occurs in the animal (see also Figures S2 and S3).

How are the CAV1−/− mitochondria altered? Measured by flow cytometry using Mitotracker FM (data not shown) and cellular cytochrome C content (Figures 2G and 4E), mitochondrial content is similar in both cell types. In contrast, the mitochondrial membrane potential (ΔΨ) was markedly higher in CAV1−/− cells (Figure 1E). The routine flux control ratio reflects how close
the routine respiration operates to the respiratory capacity of the electron transport system, and was markedly higher in CAV1<sup>−/−</sup> cells (Figure 1F). We then purified mitochondria [15] from CAV1<sup>−/−</sup> and wt murine liver [16] and quantified function in identical environments. The fraction was enriched in cytochrome C and free of extramitochondrial contamination (Figure 1G). CAV1 was absent in wt mitochondria, though present in a crude fraction containing mitochondria and associated endoplasmic reticulum (ER). We determined the respiratory capacity of the purified mitochondria by examining substrate-driven oxygen consumption. The acceptor control ratio (ACR) was calculated to determine the tightness of the coupling between respiration and ATP production, and the uncoupling control ratio (UCR) calculated as the index of oligomycin-inhibited respiration and FCCP stimulated respiration. ACR was markedly lower in CAV1<sup>−/−</sup> mitochondria, while the UCR was unaffected (Figure 1H). Thus, CAV1<sup>−/−</sup> mitochondria show reduced flux between the respiratory chain and the production of energy. The apparent discrepancy of higher mitochondrial potential and higher oxygen consumption observed in CAV1<sup>−/−</sup> cells deserves further analysis but since the UCR is unaffected it is not caused by changes in membrane permeability.

How might CAV1 loss result in mitochondrial impairment? CAV1 contributes to intracellular cholesterol homeostasis [1-3]. CAV1 deficiency might alter mitochondrial cholesterol levels, which regulate the organelle’s function and apoptotic susceptibility [15]. CAV1<sup>−/−</sup> mitochondria had a significant increase (39%) in free cholesterol (Figure 2A), that could not be accounted for the presence of other cholesterol-enriched organelles (Figure 1G). This deficiency is generic: a mitochondrial fraction isolated from CAV1<sup>−/−</sup> MEFs had a similar increase of 33% (Figure 3A). Mass spectrometry analysis of major lipids revealed no other significant changes in the total amount of phospholipids or in the relative enrichment of each phospholipid (Table S1). Thus,
only the cholesterol/phospholipid ratio was altered from 0.79 in wt to 1.00 in CAV1−/− mitochondria.

Mitochondria are cholesterol-poor organelles and little is known about regulation of their cholesterol influx/efflux [17]. Cholesterol likely reaches mitochondria through specialised ER domains called mitochondrial associated membranes (MAM) [18]. Since it is a MAM resident protein [19] and transports cholesterol from the ER to the plasma membrane [20], CAV1 could control MAM cholesterol levels. If so, CAV1 loss would influence steroid synthesis. In steroidogenic cells, after synthesis in the ER, cholesterol is transported into mitochondria and the P450 side chain cleavage enzyme (CYP11A1) converts it to pregnenolone, the steroid precursor. Mitochondrial cholesterol availability is the rate-determining step in steroid biosynthesis [21], so pregnenolone levels indicate the rate of mitochondrial cholesterol influx. Reduction of CAV1 levels in steroidogenic F2-CHO cells stably transfected with CYP11A1 caused a significant increase in pregnenolone biosynthesis (Figure 2B). Similarly, serum steroid concentrations were significantly higher in CAV1−/− mice (Figure 2C), confirming at the systemic level that CAV1 deficiency promotes higher mitochondrial cholesterol influx and thus increases steroid biosynthesis.

In general, cholesterol decreases membrane fluidity, so the mitochondrial cholesterol increase could alter mitochondrial membrane properties. We developed a new technique to measure mitochondrial membrane fluidity and found by di-4-ANEPPDHQ that purified CAV1−/− mitochondria had increased membrane condensation (Figure 2D and see supplement). Reduced membrane fluidity impairs import of glutathione into the mitochondria (mGSH) [15]. GSH is a key antioxidant that modulates the oxidative state of the cell and ultimately apoptosis [22].
Indeed, purified CAV1<sup>−/−</sup> hepatic mitochondria had a 28% reduction in mGSH content (Figure 2E). A mitochondrial fraction isolated from CAV1<sup>−/−</sup> MEFs also showed a reduction of 59% (Figure 3B). Decreased mGSH partially explains the ROS accumulation in CAV1<sup>−/−</sup> cells. Mitochondrial GSH reduction predisposes cells to apoptosis [15, 22] and indeed CAV1<sup>−/−</sup> MEFs displayed significantly higher apoptosis when challenged with TNFα (Figure 2F). The increased apoptosis was confirmed by measuring cytochrome C release into the cytosol (Figure 2G). Using cell permeable GSH ethyl ester (GSH-EE) to increase mGSH levels eliminated the difference in apoptotic sensitivity between wt and CAV1<sup>−/−</sup> fibroblasts (Figure 2F).

This data thus supports the hypothesis that CAV1 deficiency promotes cholesterol accumulation in mitochondria, reducing membrane fluidity and causing organelle dysfunction by i) reducing respiratory chain efficiency and increasing ROS levels, and ii) reducing uptake of mGSH and thus mitochondrial anti-oxidant defence. To directly test cholesterol’s role, we treated purified CAV1<sup>−/−</sup> mitochondria with beta-cyclodextrin to extract cholesterol. This restored the cholesterol/phospholipid ratio of CAV1<sup>−/−</sup> mitochondria to the wt levels without affecting the amount of phospholipids (4.30 ± 1.15 ng cholesterol/mg protein and 6.06 ± 0.76 nmol Pi/µg protein) (Figure 2A). Critically, these CAV1<sup>−/−</sup> mitochondria treated with cyclodextrin had reduced membrane order as showed by di-4-ANEPPDHQ (Figure 2D) and their ACR index recovered to wt mitochondria levels (Figure 2H). Further, their susceptibility to mitochondrial toxins was reversed (Figure 4I). Conversely, when purified wt mitochondria were loaded with an additional 25% of cholesterol, they demonstrated increased membrane order (Figure 2I), reduced ACR index (Figure 2J) and reduced entry of mGSH (Figure 2K). Re-expression by retroviral infection of CAV1 in CAV1<sup>−/−</sup> MEF [23], recovered mitochondrial cholesterol and mGSH levels
(Figures 3A and 3B), reduced the routine flux control ratio especially after OXPHOS activation by DCA (Figure 3C), and decreased the oxidative stress caused by DCA as measured by oxidation of DHE (Figure 3D). In summary, dysfunction in the CAV1−/− mitochondria largely results from increased mitochondrial cholesterol.

Mitochondrial impairment should make CAV1-altered animals sensitive to diseases involving mitochondrial malfunction. Because cholesterol loading of mitochondria is known to sensitize the liver to steatohepatitis [15], CAV1−/− mice should be particularly sensitive to this disease. We treated mice with the agonistic anti-Fas antibody Jo2. Injury was minimal in wt liver but in CAV1−/− mice Jo2 caused appearance of serum transaminases reflecting hepatic damage (Figure 4A). Steatohepatitis progression was shown by hematoxiline/eosin and inflammatory cell infiltration of liver sections (Figures 4B and 4C). The increased susceptibility of CAV1−/− hepatocytes to Jo2 was reproduced in isolated primary hepatocytes (Figures 4D, 4E and 4F). Importantly, increasing cellular GSH levels by the cell permeable GSH ethyl ester rescued CAV1−/− hepatocytes from Jo2-induced cell death (Figure 4F).

Mitochondrial impairment and oxidative stress contribute to neuronal death in multiple forms of neurodegeneration [24], and CAV1−/− brain mitochondria also have increased cholesterol levels and reduced mGSH (Figures 4G and 4H). To test whether CAV1 loss also sensitized these mitochondria to typical neurodegenerative insults, we incubated mitochondria with oligomeric human recombinant Aβ1-42 (the amyloid beta peptide (Aβ) characteristic of Alzheimer’s disease and a potent mitochondrial toxin [25]). CAV1−/− brain mitochondria had higher ROS generation
(Figure 4I) and enhanced cytochrome C release (not shown). This effect was reversed by extracting mitochondrial cholesterol with cyclodextrin (Figure 4I).

Finally, we tested for mitochondrial dysfunction/sensitivity in the intact brain, by injecting 3-Nitropropionic acid (3-NP). This is a mitochondrial toxin, used extensively as a model of Huntington’s disease; its toxicity is associated with oxidative stress [26]. 3-NP was injected in the striatum of wt and CAV1−/− mice and degenerating cells were visualized 24 hours later. In the CAV1−/− striatum we found a much larger lesion (Figure 4J) (volume quantified in serial sections) and by staining with TUNEL (Figure 4K), we calculated twice the apoptotic neurons per lesion (62.3×10^3 ±7.6×10^3 in wt and 133×10^3 ±13.5×10^3 in CAV1−/−, **P<0.01).

In summary, CAV1 deficiency impairs mitochondria by promoting an increased influx and accumulation of free cholesterol in mitochondrial membranes. This increases membrane condensation, decreasing efficiency of the respiratory chain and the intrinsic anti-oxidant defence. Upon activation of OXPHOS, the combination of these factors promotes accumulation of ROS, resulting in cell death. While we only investigated the effect of the mitochondrial failure caused by CAV1 deficiency in liver, brain and fibroblasts, naturally occurring CAV1 deficiencies in humans cause disease in other tissues as well. The precise contribution of the mitochondrial dysfunction in the appearance and/or progression of the pathologies attributed to the loss of CAV should now be addressed in each specific case. In this respect, we have confirmed organismal vulnerability to mitochondrial perturbations occurring during progression of steatohepatitis and neurodegeneration. In a physiological context, cells are continuously exposed to changes in the balance between aerobic glycolysis and mitochondrial oxidative
metabolism, so our findings more generally suggest that CAV deficiency will progressively result in mitochondrial failure, sustained oxidative stress, and apoptosis, casually contributing to disease pathogenesis.
Experimental Procedures

Reagents and antibodies. BHA (B1253), GSH-EE (G1404), DCA (347795), 2-DG (31060, Fluka), insulin (I9278), EGF (E1557), PDGF (P4056), EGF (E1557), collagenase type IV (C5138), glucose (8270), fatty acids (L9655) and 3-NP (N5636) were from Sigma (St. Louis, MO, USA). Jo2 (554254) was from Pharmingen (San Diego, CA, USA), Hoechst-33258, Deep Red Mitotraker (M22426), Mitotraker green FM (M-7514) and DHE (D11347) were from Molecular Probes (Eugene, OR, USA), Trypsin/EDTA from Life Technologies, TNFα (300-01A) from PeproTech (Bionova, Madrid, Spain). Monoclonal anti-cytochrome C (6H2B4) was from BD Pharmingen (San Diego, CA, USA), anti-smac/DIABLO from Calbiochem (La Jolla, CA, USA), anti-GFP (ab290) from Abcam (Cambridge, UK) and anti-CAV1 (C13630) and anti-actin from Transduction Labs (Lexington, KY, USA).

Cells and animals. MEFs [12] were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% foetal bovine serum, L-glutamine (2mM), penicillin (50U/ml) and streptomycin sulfate (50μg/ml) (Biological Industries, Ltd. Israel). F2-CHO, 3T3L1 cells, CAV1⁻/⁻-reconstituted MEFs and CAV1⁻/⁻ MEF stably transfected with the empty vector were obtained and cultured as described [23, 27, 28]. CAV1⁻/⁻ and wt mice [16] were kept under a controlled humidity and lighting schedule with a 12h dark period. All animals received human care in compliance with institutional guidelines regulated by the European Community. A complete description of the experimental procedures can be found in supplemental.

Statistical analysis. The statistical significance of differences were determined using the Student’s t test, *P<0.05, **P<0.01.
Acknowledgments.

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References.


Legends.

**Figure 1. Mitochondrial dysfunction in CAV1<sup>−/−</sup> cells.** (A) Wt (white bars) and CAV1<sup>−/−</sup> MEF (black bars) were cultured with 2-DG. After 48 hours cell number was determined and expressed with respect to the initial number of cells (t<sub>0</sub>). (B) Apoptosis, analysed by flow cytometry via binding of annexin V and staining with propidium iodide, promoted by 5mM 2-DG. (C) Apoptosis promoted by DCA, some cells were pre-treated with the antioxidant BHA. (D) Levels of ROS in cells incubated during 24 hours with DCA. The results are expressed as the relative H2DCFDA fluorescence with respect to untreated wt cells. (E) ΔΨm of CAV1<sup>−/−</sup> with respect to wt MEF. (F) Oxygen consumption by wt and CAV1<sup>−/−</sup> MEF expressed as the routine flux control ratio. (G) Western blotting analysis of CAV1 (plasma membrane), Rab11 (recycling endosomes), GM130 (Golgi complex), Sec61 (ER) and cytochrome C (Cyt C, mitochondria) in purified wt and CAV1<sup>−/−</sup> mitochondria (M), homogenates (H) and in a crude fraction that contains mitochondria and associated ER (cM). (H) Ratios of oxygen consumption in wt (white bars) and CAV1<sup>−/−</sup> mitochondria (black bars) purified from mice liver. Statistical significances were determined in at least 5 independent experiments or 10 mice using the Student’s t test, *P<0.05, **P<0.01.

**Figure 2. Cholesterol accumulation promotes dysfunction of CAV1<sup>−/−</sup> mitochondria.** (A) Free cholesterol in wt (white bars) and CAV1<sup>−/−</sup> (black bars) mitochondria purified from mice liver. In some experiments mitochondria were pre-treated with cyclodextrin to extract cholesterol (slashed bars). (B) Expression of CAV1 in F2-CHO cells was reduced by RNA interference during 48 hours (Western blotting of CAV1 is shown in the bottom) and production of pregnenolone was measured during the next 24 hours. (C) Pregnenolone, corticosterone and testosterone levels in the serum of CAV1<sup>−/−</sup> (black bars) and wt mice (white bars). (D) Membrane order analysed with ANEPPDHQ of wt (white bars), CAV1<sup>−/−</sup> (black bars) and cyclodextrin-treated wt white bars) and CAV1<sup>−/−</sup> purified mitochondria (slashed bars). (E) Mitochondrial GSH in wt (white bars) and CAV1<sup>−/−</sup> (black bars) mitochondria purified from mice liver. (F) Apoptosis promoted by 24 hours of TNFα in untreated wt (white bars) and CAV1<sup>−/−</sup> MEF (black bars) or in cells treated with GSH-EE. (G) Cytochrome C (Cyt C) in cytosolic supernatants and homogenates (homog) corresponding to TNFα treated MEFs. (H) Purified mitochondria from wt (with bars) and CAV1<sup>−/−</sup> (slashed bars) were treated with cyclodextrin and the rates of oxygen consumption measured. (I-J) Purified wt mitochondria (white bars) were enriched with 25% of
cholesterol (slashed bars) and membrane condensation (I) and rates of oxygen consumption (J) were measured. (K) Influx of a radio-labelled GSH into wt mitochondria untreated (white bars) or enriched with 25% of cholesterol (slashed bars).

**Figure 3. Re-expression of CAV1 recovers mitochondrial function.** (A and B) Free cholesterol and GSH in mitochondria purified from wt (white bars), CAV1−/− (black bars), CAV1−/−-reconstituted MEFs (slashed bars) and CAV1−/− MEF infected with an empty vector (black bars). CAV1 levels are shown by Western blotting. (C) Routine flux control ratio in untreated MEFs and in cells incubated with DCA for 5 hours. (D) Oxidative stress caused by mitochondrial function in MEFs. Results are expressed as the ratio between the fluorescence intensity of DHE after treating the cells with DCA for 5 hours with respect to the initial intensity.

**Figure 4. Dysfunction of CAV1−/− mitochondria enhances pathogenesis.** (A-C) To model steatohepatitis wt (white bars) and CAV1−/− mice (black bars) were treated with Jo2. Liver damage was evaluated 24 hours later by appearance of transaminases in serum (AST and ALT). Inflammation was visualised in liver sections of wt (left) and CAV1−/− mice (right) with hematoxilin/eosin and myeloperoxidase staining. (D-E) Wt and CAV1−/− primary hepatocytes were treated with Jo2 for 24 hours. Apoptosis in wt (left panel) and CAV1−/− hepatocytes (right) was visualized with a Hoechst staining (D) and released cytochrome C (CytC) into the cytosol quantified by Western blot (E). (F) MTT cell viability assay of wt and CAV1−/− hepatocytes treated with Jo2 or with Jo2/GSH-EE. (G-H) Free cholesterol and mGSH in wt (white bars) and CAV1−/− (black bars) purified brain mitochondria. (I) ROS generation in wt (white bars) and CAV1−/− (black bars) purified brain mitochondria (some treated with cyclodextrin, slashed bars) incubated with Aβ1-42. (J-K) 3-NP was injected in the striatum of wt and CAV1−/− mice and the volume of the lesion measured 24 hours later in serial Fluoro-Jade-stained sections and apoptotic nucleus were visualised in TUNEL-stained sections (K) of wt (left) and CAV1−/− striatum (right).
Figure 1 Bosch et al.

A) Number of cells after 2-DG treatment

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C) Apoptosis after DCA

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D) ROS levels

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E) Relative TMRM fluorescence

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F) O₂ consumption

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G) Homogenate/mito ratio

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H) O₂ consumption

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Figure 2 Bosch et al.
Figure 3 Bosch et al.
Figure 4 Bosch et al.

A) AST and ALT levels in primary hepatocytes treated with Jo2.

B) Jo2 promoted inflammation in liver (hematoxylin/eosin).

C) Jo2 promoted inflammation in primary hepatocytes (myeloperoxidase).

D) Jo2 promoted apoptosis in primary hepatocytes (Hoechst).

E) Cytochrome C release from mitochondria.

F) Hepatocyte viability assay.

G) Cholesterol content in mitochondria.

H) Mitochondrial GSH content.

I) Mitochondrial ROS promoted by Aβ1-42.

J) Lesion volume in striatum.

K) 3-NP promoted apoptosis in brain (Tunel).
Inventory to supplemental data.

A single Word document contains the next items:

1 - Figure S1, text and legend. It contains additional data to Figure 1.

2 - Figure S2 text and legend. It contains additional data to Figure 1.

3 - Figure S3 text and legend. It contains additional data to Figure 1.

4 - Table S1 and legend. It contains additional data to Figure 2.

5- Supplementary text for Figure 2. It is a detailed explanation of the experiment showed in Fig2D.

6 - Additional Methods.

7 - Supplemental References.
Supplementary Data.

Figure S1 (related to Figure 1): Supplementary Figure 1A shows an example of the massive apoptosis promoted by 2-DG in CAV1⁻/⁻ MEF when compared with wt MEF. In Supplementary Figure 1B cells were treated with DCA (a pyruvate dehydrogenase kinase inhibitor [1]), to shift glucose metabolism from lactate production to OXPHOS, and the oxidation of ¹⁴[C]-glucose into CO₂ was measured. Supplementary Figure 1C shows an example of the increased membrane potential displayed by CAV1⁻/⁻ MEF. Figures in the main text show the average of different experiments.

Legend S1. (A) Representative example of apoptosis, analysed by flow cytometry via binding of annexin V and staining with propidium iodide, promoted by 5mM 2-DG. (B) Stimulatory effect of DCA on ¹⁴[C]-glucose oxidation into CO₂. (C) Representative analysis of the ΔΨm measured with TMRM and flow cytometry of CAV1⁻/⁻ with respect to wt MEF.
**Figure S2** (related to Figure 1). In principle, some of the effects demonstrated here might be due to a particular genetic background of the CAV1<sup>−/−</sup> mice, rather than due to the loss of CAV1 function. To evaluate this, we generated an entirely independent polyclonal 3T3 L1 cell line stably expressing a lentivirus-mediated siRNA targeting of CAV1 (CAV1<sup>−</sup>) [2] that showed a reduction of more than 90% of RNA and protein corresponding to CAV1 (Supplementary Figure 2). Similar to CAV1<sup>−/−</sup> MEFs, CAV1<sup>−</sup> 3T3 cells showed higher ΔΨ, increased apoptosis and ROS accumulation when grown in the presence of 2-DG or DCA, and higher apoptosis sensitivity when exposed to the TNFα challenge (Supplementary Figure 2). We conclude that these changes in mitochondrial function result from loss of CAV1 activity, rather than the result of a hidden background mutation, or from a particular genetic adaptation to the loss of CAV.

**Legend S2.** (A) Total levels of CAV1 were analyzed by Western blotting in 3T3L1 wt, CAV1<sup>+</sup> and CAV1<sup>−</sup> cell homogenates. GFP expression correlates with the presence of the lentivirus expressing a scramble siRNA (CAV1<sup>+</sup>) or siRNA of CAV1 (CAV1<sup>−</sup>). Actin levels were measured as a loading control. (B) Proliferation of 3T3L1 CAV1<sup>+</sup> (white bars) and CAV1<sup>−</sup> (black bars) cells
after 48 hr treatment with 2-DG (5mM). Number of cells was counted by trypan blue exclusion. (C and D) Apoptotic cell death promoted by 5mM 2-DG treatment during 48 hours. (E) Cellular ROS levels after 24 hours treatment with increasing concentrations of DCA. (F and G) Mitochondrial membrane potential of CAV1+ and CAV1− 3T3L1 cells was quantified by flow cytometry. F shows representative fluorescence distribution of the probe TMRM and G, the average value of independent experiments. (H) Effect of the antioxidant GSH-EE pre-treatment (5mM, 30 min) on the apoptosis promoted by TNFα. The statistical significance were determined using the Student’s t test, *P<0.05, **P<0.01.

Figure S3 (related to Figure 1). Mitochondrial malfunction also occurs in the animal. Primary hepatocytes isolated from liver of wt and CAV1−/− mice [3] also displayed higher ΔΨm (Supplementary Figure 3A and 3B), increased cellular ROS (Supplementary Figure 3C) and amplified susceptibility to Jo2, a Fas agonistic antibody that induces apoptosis (Figure 3). To determine whether mitochondrial malfunction causes decreased proliferation, wt and CAV1−/− hepatocytes were isolated from untreated livers and cultured with glucose. Then, levels of ROS were determined after 24 hours and cell number after 48 hours. CAV1−/− hepatocytes proliferated like wt cells in the presence of glucose and ROS levels were comparable (Supplementary Figure 3C). To confirm that CAV1−/− hepatocytes do not proliferate when mitochondrial function is required, hepatocytes were cultured as above with glucose but were now constrained to use OXPHOS by means of DCA. CAV1−/− hepatocytes had highly reduced proliferation and the expected accumulation of ROS (Supplementary Figure 3D). Thus, CAV1−/− hepatocytes do not proliferate when mitochondrial function is required. Since MEF and hepatocytes came from mice with different genetic backgrounds [3, 4], these results provide strong evidence of a general mechanism by which loss of CAV1 contributes to mitochondrial impairment.
Legend S3. (A) Wt (left panel) and CAV1−/− primary hepatocytes (right panel) loaded with TMRM. (B) Mean ΔΨm of CAV1−/− (black bars) with respect to wt hepatocytes (white bars). (C-D) Hepatocytes isolated from untreated livers and cultured for 48 hours in the presence of glucose or glucose and DCA. Levels of ROS were determined 24 hours after isolation and number of hepatocytes after 48 hours. Statistical significances were determined in at least 5 independent experiments using the Student’s t test, *P<0.05, **P<0.01.

Table S1 (related to Figure 2).

<table>
<thead>
<tr>
<th>Major lipids in purified liver mitochondria</th>
<th>WT</th>
<th>CAV1−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>cholesterol</td>
<td>4.71 ± 0.71</td>
<td>6.35** ± 0.94</td>
</tr>
<tr>
<td>inorganic phosphate (Pi)</td>
<td>5.98 ± 0.83</td>
<td>6.34 ± 0.89</td>
</tr>
<tr>
<td>phosphatidylcholine (PC)</td>
<td>477.48 ± 45.35</td>
<td>441.81 ± 12.30</td>
</tr>
<tr>
<td>phosphatidylethanolamine (PE)</td>
<td>332.86 ± 22.68</td>
<td>358.28 ± 15.88</td>
</tr>
<tr>
<td>cardiolipin (CL)</td>
<td>109.56 ± 7.33</td>
<td>99.81 ± 5.64</td>
</tr>
<tr>
<td>phosphatidylinositol (PI)</td>
<td>62.18 ± 5.59</td>
<td>52.40 ± 5.30</td>
</tr>
<tr>
<td>phosphatidylglycerol (PG)</td>
<td>6.64 ± 0.94</td>
<td>7.73 ± 0.28</td>
</tr>
</tbody>
</table>

Legend Table S1. A mitochondrial fraction was isolated from wt and CAV1−/− mice liver and major lipids were analysed by mass spectrometry (see methods). Cholesterol is expressed in ng of cholesterol/mg de protein; total phospholipids (Pi, inorganic phosphate) in nmol Pi/µg de protein; PI, PE and PC pmol/µg protein; CL and PG in arbitrary units. The statistical significances in cholesterol levels was determined in 3 independent experiments using the Student’s t test, **P<0.01. No other significant differences were observed.

Supplementary text for Figure 2D.

To test for structural consequences of the mitochondrial cholesterol increase, we analyzed purified mitochondria in a flow cytometer by imaging di-4-ANEPPDHQ, whose fluorescence emission undergoes a spectral blue-shift between the liquid-ordered (cholesterol enriched) and the liquid-disordered (cholesterol poor) phases [5]. Mitochondria were identified by co-labelling with Deep-Red Mitotracker, ruling out contamination by other organelles. Emission intensities at 530 and 670nm corresponding to at least 30,000 mitochondria per condition were converted into a general
polarization index (GP) [6], with a GP of -1 being very fluid and +1 being very condensed. Consistent with cholesterol enrichment, CAV1<sup>-/-</sup> mitochondria had a larger GP (Figure 2D), reflecting increased membrane condensation. A direct consequence of reduced mitochondrial membrane fluidity is impaired transport of glutathione into the mitochondria (mGSH) [7]; mGSH is a key antioxidant imported from the cytosol to modulate the oxidative state of the cell and ultimately apoptosis [8].

**Supplementary methods:**

**Animals and primary hepatocytes isolation.** CAV1<sup>-/-</sup> and wt mice [3] were kept under a controlled humidity and lighting schedule with a 12h dark period. All animals received human care in compliance with institutional guidelines regulated by the European Community. Food and water were available *ad libitum*. Primary hepatocytes from wt and CAV1<sup>-/-</sup> mice (8–12 weeks old) were isolated by collagenase perfusion with a flow rate of 7-9ml/min, and cultured in DMEM+10% FBS at a density of 0.5×10<sup>6</sup> cells/well on 6-well plate coated with rat tail collagen.

**Cell proliferation.** Proliferation of MEF cells was determined by trypan blue exclusion of cells plated (5×10<sup>4</sup>/well) during 24 hours in 6-well plates in a haemocytometer. For hepatocyte proliferation the media was supplemented with 10nM insulin, 20ng/ml EGF, and 10ng/ml PDGF and additionally, 6h after isolation, hepatocytes were incubated in a nutrient-free media or in a media supplemented with glucose (4.5mg/l) or glucose and DCA (25mM) and number of cells determined by crystal violet. The percentage of cells in each well was calculated with respect to the initial number of cells (6h after isolation). The effect of Jo2 (0.05µg/ml) was assessed by MTT (Promega, Madison, WI). When indicated cells were pre-treated for 30min with 5mM GSH-EE.

**Assessment of cell death.** MEF cells were plated at 10<sup>5</sup>/well in 6-well culture plates. Following overnight growth, experimental media containing 5mM 2-DG or control media was added and incubation continued for 24 hours or 48 hours. For the DCA and TNFα treatments, cells where incubated either with DCA (25mM) or TNFα (6nM) during 24 or 48 hours. In some experiments cells were pre-treated with antioxidants for 30min (100µM BHA or 5mM GSH-EE). Adhered and detached cells were then pooled, washed and labelled with annexin-V-FITC and propidium iodide using the annexin-V-FLUOS Staining Kit (Roche) according to manufacturer’s
instructions. To determine the percentage of cells displaying annexin V plus PI staining, samples were subsequently analyzed by flow cytometry with a FACSCalibur equipped with a 488nm Argon laser and a 635nm red diode laser (Becton & Dickinson, San Jose, CA, USA). Data from the experiments were analysed using CellQuest software (Becton & Dickinson). Apoptosis is defined as the percentage of cells contained in the lower and upper right quadrants (Supplementary Figure 1).

**Assessment of hepatocyte death.** Hepatocellular apoptosis promoted by Jo2 treatment (0.05μg/ml) was determined by nuclear DNA staining with Hoechst-33258. Cells were fixed with 3.7% paraformaldehyde in PBS, stained with Hoechst-33258 (1μg/ml) for 15min and apoptotic nuclei were determined by visualizing chromatin condensation under a fluorescent microscope. The images were acquired with the appropriate filters for Hoechst. At least 250 cells in six different high-power fields were counted and expressed as a percentage of total cells. Liver samples following Jo2 administration were processed for H&E and myeloperoxidase staining using standard procedures (Servicio Anatomía Patológica, Hospital Clinic). The cells were observed using an oil-immersion Plan-Apo63x/1.4 objective in an Axio-plan or Axiovert 200M Zeiss microscope (Zeiss, Göttingen, Germany). Images were captured with an AxioCam HRc camera and then digitally treated with AxioVision 3.1 software. Image analysis was performed with Adobe-Photoshop 5.5 software (Adobe Systems Inc).

**Analysis of mitochondrial membrane potential.** MEF cells were plated at 10^5/well in 6 well plates and grown overnight. After 30min incubation at 37°C with TMRM (50nM), cells were trypsinized, washed in PBS and the percentage of cells exhibiting low level of TMRM uptake, which reflects loss of mitochondrial membrane potential (ΔΨm), was determined by flow cytometry. In primary hepatocytes mitochondrial membrane potential was determined as the relative fluorescence intensity of living cells analysed by laser confocal imaging. Hepatocytes were plated on coverslips and ΔΨm was analyzed after incubation with TMRM (100nM) 30min at 37°C.

**Glucose oxidation.** Glucose oxidation into CO₂ was measured in MEFs cells grown in 6-well culture plates. Cells were washed in Krebs-Ringer bicarbonate HEPES (KRBH) buffer (135mM NaCl, 3.6mM KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 1.5mM CaCl₂, 2mM NaHCO₃ and 10mM HEPES, pH 7.4), and incubated with 450μl of KRBH and 1μCi/ml of ^14^[C]-glucose 25mM. Plates were sealed at the beginning of the incubation with parafilm drilled in each well
and CO₂ was trapped in a piece of Whatman paper (1cm-diameter circle) saturated with 0.1N KOH. After 3h of incubation at 37°C, 50μl of perchloric acid (40% vol/vol) was injected into each well via a needle through the parafilm to acidify the medium and liberate the CO₂. After 1h of equilibration at 37°C, papers were removed and ¹⁴CO₂ was measured by liquid scintillation.

**Cholesterol, GSH and ROS determination.** Cholesterol in cells and subcellular fractions was measured by HPLC as described [7]. Briefly, free cholesterol was assayed by directly injecting 2-propanol-extracted lipids in a HPLC, as described [9]. HPLC analyses were made using a Waters µBondapak C18 10-μm reversed-phase column (30cm x 4mm inner diameter), the mobile phase was 2-propanol/acetonitrile (50:50, v/v) at a flow rate of 1 ml/min. Cellular GSH was determined by HPLC as previously described [10]. Intracellular ROS in hepatocytes was determined using H₂DCFDA at a concentration of 2μM in the incubation media. After 20 min incubation, hepatocytes were washed, trypsinized and resuspended in PBS for fluorimetric determination of dichlorofluorescein fluorescence (λex: 503nm, λem: 529nm). ROS levels in MEFs cells were determined by incubating cells with 15μM of H₂DCFDA during 30min followed by measurement of fluorescence using Synergy 2 Multi-Mode Microplate Reader (BioTek, Vermont, USA) with a 485/20 excitation and a 528/20 emission filter pair. The values were referred to protein concentration measured by crystal violet staining. Generation of ROS was also determined by dihydroethidium (DHE, Molecular Probes), which upon reaction with superoxide anions forms a red fluorescent product (ethidium) which intercalates with DNA. MEF cells were plated at 15x10⁵/well in 6 well plates and grown overnight. After incubation with 50mM DCA for 5h, dihydroethidium (5uM) was added to each well for 30 min. Cells were trypsinized, resuspend in PBS and fluorescence was detected by flow cytometry (λex: 500-530 nm; λem:590-620 nm).

**Cytochrome C release.** Release of cytochrome C in MEFs was determined as described previously [11]. Briefly, 10⁷ cells were treated with TNFα (6mM). After 24 hours, cells were harvested, pelleted by centrifugation and resuspended in 250mM sucrose, 20mM HEPES-KOH pH 7.4, 10mM KCl, 1,5mM Na-EGTA, 1,5mM Na-EDTA, 1mM MgCl₂, 1mM dithiothreitol (DTT) and a cocktail of protease inhibitors. Cells were then disrupted by 30 strokes in a glass Dounce at 4°C and the homogenates were centrifuged at 800g for 10min at 4°C. Supernatants were further centrifuged at 22,000g for 15min at 4°C. The resulting supernatants were saved as cytosolic extracts and analyzed by Western blotting to measure cytochrome C.
**Purification and analysis of liver mitochondria.** Mitochondria from wild type and CAV1<sup>−/−</sup> mice livers [3] were prepared as described previously [12]. Briefly, the liver was homogenized in 210mM mannitol, 60mM Sucrose, 10mM KCl, 10mM sodium succinate, 1mM ADP, 0.25mM DTT, 0.1mM EGTA, 10mM HEPES, pH 7.4. The homogenates were centrifuged at 600g for 10 min, with the resulting supernatant being centrifuged at 10.300g for 15min. The resulting pellet (crude membrane fraction, cM) was resuspended in 2ml, loaded onto 8ml of 30% (v/v) Percoll gradient and centrifuged at 95.000g for 30min. The mitochondrial pellet was then rinsed twice by centrifuging 15min at 7.000g. Functional mitochondria from wt and CAV1<sup>−/−</sup> MEFs (120×10<sup>6</sup> cells) were isolated by differential centrifugation as described [13]. The extraction of cholesterol from the mitochondrial membranes was achieved by incubating cM with methyl-α-cyclodextrin (4mM) for 5min at RT and subjected to ultracentrifugation to recover intact mitochondria [14]. Mitochondrial cholesterol loading using a cholesterol-albumin complex and cholesterol determination were performed as detailed previously [15]. Cholesterol-BSA complex was made by dissolving 50mg of cholesterol in 5ml of absolute ethanol as described [16]. To the white solution obtained after addition of 5ml of double distilled water, 2g of BSA was added adjusting the pH to 7.3 and then centrifuged at 12,000 × g at 4 °C for 10min. Ten to fifty μl of the cholesterol/BSA mixture was incubated at 4 °C with 50mg of mitochondria protein for 1min. Mitochondria were diluted 20 times with cold solution of 0.25M sucrose, 1mm EDTA, pH 7.3, and immediately recovered by centrifugation and washed three times, to eliminate excess cholesterol. Parallel control experiments were performed using only BSA. This procedure resulted in cholesterol loading from about 25 to 35% over basal levels.

**Purification and analysis of brain mitochondria.** Cerebral cortices were removed and homogenized in 210mM mannitol, 60mM sucrose, 10mM KCl, 10mM sodium succinate, 1mM ADP, 0.25mM DTT, 0.1mM EGTA, 10mM HEPES, pH 7.4. The homogenates were centrifuged at 600g for 10min, with the resulting supernatant being centrifuged at 10.300g for 15min. The resulting pellet (cM) was suspended in 2ml, loaded onto 8ml of 30% (v/v) Percoll gradient and centrifuged at 95.000g for 30min. The mitochondrial pellet was then rinsed twice by centrifuging 15min at 7.000g. The removal of cholesterol from the mitochondria membrane was achieved by incubating pellet cM with methyl-α-cyclodextrin (4mM) for 5min at RT and subjected to ultracentrifugation to purify mitochondria [14]. In some experiments, mitochondria purified from mice brain were exposed to oligomeric human recombinant Aα1-42 (5μM, Bachem, CA, USA) for 45min at 37ºC [12]. Hydrogen peroxide generation was determined spectrofluorometrically using H2DCFDA (2mM). The fluorescent probe was added in the
incubation buffer and at the indicated time fluorescence was recorded at 529nm for emission and 503nm for excitation.

**Lipidomic analysis.** Mass spectrometry lipidomic analyses of glycerophospholipid species was carried out as described [17].

**Oxygen consumption.** Oxygen consumption was measured with an Oxygraph (Hansatech Instruments Ltd, Norfolk, England) equipped with a Clark-type electrode attached to a temperature-controlled reaction vessel. An aliquot of mitochondrial suspension (2 mg protein) was incubated with 1.2 ml of respiration medium (225mM sucrose, 5mM MgCl2, 10mM KH2PO4, 20mM KCl, 10mM Tris, 5mM Hepes, pH 7.4). Succinate (17mM) was added as substrate for complex II. State 3 respiration state was determined by addition of ADP (0.7mM). The rate of oxygen consumption in the presence of ADP over the absence of ADP is defined as the acceptor control ratio (ACR) (state 3/4). Maximal electron transport was determined in the presence m-CCCP (13μM). The ratio of oxygen consumed in the presence of oligomycin (13μg/ml) is defined as the UCR. For measuring oxygen consumption in intact cells, 10⁶ cells were collected in DMEM and respiration measured with the Oroboros Oxygraph. DatLab software (Oroboros Instruments, Innsbruck, Austria) was used for data analysis. The routine respiration is defined as respiration in cell-culture medium without additional substrates and the ratio of routine respiration and maximal ETS capacity (uncoupled, m-CCP) is defined as routine flux control ratio.

**Quantification of mitochondrial membrane condensation.** Mouse liver mitochondria were isolated by differential centrifugation as described previously [13]. 1mg/ml of mitochondria were incubated with di-4-ANEPPDHQ (final concentration 1μg/ml) and/or MitoTracker Deep Red FM (final concentration 250nM) during 10min at 37°C. Di-4-ANEPPDHQ’s fluorescence emission undergoes a spectral blue-shift between the liquid-ordered (cholesterol enriched) and the liquid-disordered (cholesterol poor) phases [5]. Particles corresponding to mitochondria were recognized and selected in a flow cytometer by co-labelling with Deep-Red Mitotracker (excitation at 644 nm and emission at 665 nm). Deep-Red Mitotracker positive mitochondria were then excited at 488 nm and emission intensities were simultaneously recorded at 530/30nm and 670LPnm. Intensities for each particle were finally converted into a general polarization index (GP) following the equation GP = (I_{530/30} − I_{670LP}) / (I_{530/30} + I_{670LP}) [6]. The GP was calculated for 10,000 mitochondria in each condition and all the experiments were repeated at
least 3 times, thus each GP corresponds to the average of at least 30,000 mitochondria. GP values range from -1 (very fluid) to +1 (very condensed). Samples were analyzed on a BD FACS Canto Flow Cytometer. Data analysis was performed with Wasel FACS software. The removal of cholesterol from the mitochondria membrane was achieved by incubating pellet cM with methyl-α-cyclodextrin (4mM) for 5min at RT and subjected to ultracentrifugation to purify mitochondria [14]. Mitochondrial cholesterol loading using a cholesterol-albumin complex and cholesterol determination were performed as detailed previously [15].

**Kinetics of mGSH transport.** mGSH transport was performed in uptake buffer (70mM sucrose, 1mM KH2PO4, 5mM sodium succinate, 5mM HEPES pH 7,4, 220mM mannitol, 0,1mM ethylenediaminetetraacetic acid, and 0,1% bovine serum albumin (fatty acid free) at 10mM GSH with 1μCi [3H]-GSH in a final volume of 250μl as described previously [18]. The non-specific binding to mitochondria and filters was estimated by adding ice-cold buffer to mitochondria before adding label that were subtracted from transport measurements.

**Pregnenolone determination in F2-CHO cells.** CAV1 small interference RNA (from published sequences [19]) was carried out as previously described [20] in steroidogenic F2-CHO cells stably transfect with CYP11A1 [21]. After 48 hours cells were plated on 6-well culture plates in phenol red-free, serum-free medium containing trilostane (10μM) to inhibit conversion of pregnenolone to downstream steroids. Concentration of pregnenolone in the culture media was finally measured 24 hours later by an Elisa Kit (Diagnostics Biochem Canada Inc., Ontario, Canada) according to the manufacturer’s instructions. Measured by rtPCR and Western blotting after 72h, cells showed a reduction of more than 90% of RNA and protein corresponding to CAV1.

**Steroid and transaminases determination in mice serum.** Serum parameters were routinely determined by the CORE facility of “Hospital Clínic i Provincial de Barcelona” in an Advia 1650 (Bayer Hispania) using commercial enzymatic based kits from Bayer Hispania (Barcelona).

**Striatal lesions.** Wt and CAV1-/- mice (11 week-old) were anesthetized with pentobarbital (50mg/Kg) and 3-NP (30nmol in PBS) was intrastriatally injected at the following coordinates relative to bregma: AP +0,6mm, ML +2mm and 2,7mm below the dural surface with the incisor bar at 3mm above the interaural line. The contralateral striatum was injected with vehicle (PBS) at the following coordinates relative to bregma: AP +0,6mm, ML -2 mm and 2,7mm below the
dural surface with the incisor bar at 3mm above the interaural line. PBS or 3-NP were injected over 2min and the cannula was left in place for additional 5min. Animals were sacrificed 24 hours after intrastriatal injection for morphological analysis.

**In situ detection of DNA fragmentation in mice striatum.** 24 hours after intrastriatal 3-NP injection mice (n = 5 for each condition) were deeply anesthetized and immediately perfused transcardially with saline followed by 4% paraformaldehyde/phosphate buffer. Brains were removed and post-fixed for 1-2h in the same solution, cryoprotected by immersion in 30% sucrose/PBS and then frozen in dry ice-cooled isopentane. Serial coronal cryostat sections (40µm) through the whole striatum were collected on silane-coated slides. DNA fragmentation was examined using the *in situ* Apoptosis Detection System (Fluorescein, Promega, Madison, WI, USA) as described elsewhere [22]. Stained sections were visualized on a computer, and the border of the lesion was outlined using the Computer-Assisted Stereology Toolbox (CAST) software (Olympus). The volume of the lesion was estimated by multiplying the sum of all the sectional areas (µm²) by the distance between successive sections (320 µm), as described previously [22].

**Western blotting.** Cells were washed twice with cold PBS before being scraped into ice-cold 10mM Tris, pH 7.5, 150mM NaCl, 5mM EDTA, and a mixture of protease inhibitors. Cells were homogenized by passing through a 22-gauge needle 25 times at 4ºC. Western blotting of cells and subcellular fractions was performed as described previously [23]. Protein was quantified by BCA Protein Reagent Assay kit (Pierce Chemical Co., IL, USA). After incubation with primary antibodies, membranes were washed and incubated with peroxidase-conjugated secondary antibodies (1:5000, Amersham-Pharmacia) and detected with ECL (Biological Industries, Ltd. Israel) on Kodak X-OMAT film (Eastman Kodak).

**Statistical analysis.** All data shown in the graphs are the mean ± SD, and the statistical significance of differences were determined using the Student's t test, *P*<0.05, **P**<0.01.
Supplementary references:


