Caveolin-3 Knock-out Mice Develop a Progressive Cardiomyopathy and Show Hyper-activation of the p42/44 MAP kinase cascade

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Running Title:
Cardiomyopathy in Caveolin-3 Null Mice
Abstract

A growing body of evidence suggests that muscle cell caveolae may function as specialized membrane micro-domains in which the dystrophin-glycoprotein complex and cellular signaling molecules reside. Caveolin-3 (Cav-3) is the only caveolin family member expressed in striated muscle cell types (cardiac and skeletal). Interestingly, skeletal muscle fibers from Cav-3 (-/-) knock-out mice show a number of myopathic changes, consistent with a mild-to-moderate muscular dystrophy phenotype. However, it remains unknown whether a loss of Cav-3 affects the phenotypic behavior cardiac myocytes in vivo.

Here, we present a detailed characterization of the hearts of Cav-3 knock-out mice. We show that these mice develop a progressive cardiomyopathic phenotype. At four months of age, Cav-3 knock-out hearts display significant hypertrophy, dilation, and reduced fractional shortening, as revealed by gated cardiac MRI and transthoracic echocardiography. Histological analysis reveals marked cardiac myocyte hypertrophy, with accompanying cellular infiltrates and progressive interstitial/peri-vascular fibrosis. Interestingly, loss of Cav-3 expression in the heart does not change the expression or the membrane association of the dystrophin-glycoprotein (DG) complex. However, a marker of the DG complex, α-sarcoglycan, was specifically excluded from lipid rafts domains in the absence of Cav-3. Since activation of the Ras-p42/44 MAP kinase pathway in cardiac myocytes can drive cardiac hypertrophy, we next assessed the activation state of this pathway using a phospho-specific antibody probe. We show that p42/44 MAP kinase (ERK-1/2) is hyper-activated in hearts derived from Cav-3 knock-out mice. These results are consistent with previous in vitro data demonstrating that caveolins may function as negative regulators of the p42/44 MAP kinase cascade. Taken together, our data argue that loss of Cav-3 expression is sufficient to induce a molecular program leading to cardiac myocyte hypertrophy and cardiomyopathy.
Abbreviations

WT, wild-type; Cav-1, Caveolin-1; Cav-2, Caveolin-2; Cav-3, Caveolin-3; KO, Knock-out; DG, dystrophin-glycoprotein; MRI, magnetic resonance imaging; LGMD-1C, Limb Girdle Muscular Dystrophy 1C; T-tubule, transverse tubule; MAP, mitogen-activated protein
Caveolae are plasma membrane invaginations that are enriched in cholesterol, sphingolipids, GPI-anchored proteins, and the marker protein, caveolin (1). Three caveolin genes have now been identified (Cav-1, -2, -3) (2-5). Cav-1 and Cav-2 are abundantly co-expressed in a variety of cell types, e.g., adipocytes, endothelial cells, fibroblasts, smooth muscle cells, and Type-I pneumocytes. In contrast, caveolin-3 is muscle-specific, being expressed selectively in all muscle types (cardiac, skeletal, and smooth muscle) (6).

Interestingly, Cav-3 expression is necessary for caveolae formation in skeletal muscle fibers (7). Individual Cav-3 molecules homo-oligomerize to form high molecular mass multimers (∼ 14-16 monomers per oligomer), both in vitro and in vivo (2). This self-assembly is thought to drive the invagination of the plasma membrane through the interaction of caveolin oligomers with cholesterol, sphingolipids, and other membrane protein components.

Cav-3 expression is detectable at embryonic day 10 in mouse heart (8) and Cav-3 has been shown to associate with the developing T-tubule system in skeletal myoblasts (9). In addition, Cav-3 (-/-) knock-out mice demonstrate dilated and longitudinally oriented T-tubules in their skeletal muscle fibers (7). Likewise, the skeletal muscles of patients with mutations in the human Cav-3 gene (LGMD-1C) also show a disorganized T-tubule network (10).

Members of the dystrophin-glycoprotein (DG) complex have been shown to localize to muscle caveolae (6,7). Although not an integral member of the DG complex (11), i) Cav-3 can directly interact with β-dystroglycan, ii) Cav-3 is necessary for the localization of some DG complex members to lipid raft domains/ caveolae in skeletal muscle fibers, and iii) Cav-3 expression increases with the loss of dystrophin, as in mdx mice and Duchenne Muscular Dystrophy (7,12-14). Thus, Cav-3 appears to dynamically interact with the DG complex. As a consequence, it is not surprising that mutations in many dystrophin-associated proteins, such as Cav-3, lead to similar forms of muscular dystrophy (15-17).
Caveolae have also been shown to function as "pre-assembled" signaling complexes through the compartmentalization of signaling molecules that interact with the caveolin proteins and/or “liquid-ordered” caveolar lipids (18). In the heart, a variety of signaling molecules co-fractionate with cardiac caveolae and their residence in caveolae, or movement out of caveolae, is important for their function (19-27). Furthermore, multiple studies have now shown that Cav-3 expression is dramatically decreased in different models of cardiac hypertrophy (25,26,28). This suggests that reduction of Cav-3 expression may be a pivotal event in the ensuing hypertrophic program, perhaps by allowing hypertrophy-inducing signaling cascades to remain constitutively activated.

Although a role for Cav-3 in multiple skeletal muscle processes has now been investigated, the functional role of Cav-3 in the heart remains unknown. Here, we present a thorough characterization of the hearts of Cav-3 (-/-) knock-out mice. Interestingly, we show that Cav-3 knock-out mice develop a progressive, mild-to-moderate, cardiomyopathic phenotype--characterized by myocyte hypertrophy. Thus, loss of Cav-3 expression and cardiac myocyte caveolae is sufficient to induce the activation of a hypertrophic program in cardiac myocytes.
**Materials & Methods**

*Materials.* Caveolin-1, -2, and -3 mAbs were the generous gift of Dr. Roberto Campos-Gonzalez (BD Transduction Laboratories, Inc.) (6,29,30). Other antibodies were purchased as follows: actin mAb clone AC40 (Sigma); dystrophin mAb DYS3, α-β-δ-γ-sarcoglycan mAbs, and β-dystroglycan mAb (Novocasta); p42/44 and phospho-p42/44 pAbs (NEB/Cell Signaling). All reagents were of the highest purity grade and were obtained from the usual commercial sources.

*Animal Studies.* Mice were housed and maintained in a barrier facility at the Institute for Animal Studies, Albert Einstein College of Medicine. The generation of Cav-3 KO mice was we previously described (7).

*Immunoblot Analysis.* WT or Cav-3 KO mice were sacrificed and their hearts harvested and homogenized in lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 60 mM octyl glucoside), containing protease inhibitors (Boehringer Mannheim). Tissue lysates were then centrifuged at 12,000 x g for 10 min to remove insoluble debris. Protein concentrations were analyzed using the BCA reagent (Pierce) and the volume required for 10 µg of protein was determined. Samples were then separated by SDS-PAGE (12.5% acrylamide) and transferred to nitrocellulose. The nitrocellulose membranes were stained with Ponceau S (to visualize protein bands), followed by immunoblot analysis. All subsequent wash buffers contained 10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20 (1X-TBS-Tween), which was supplemented with 1% bovine serum albumin (BSA) and 2% nonfat dry milk (Carnation) for the blocking solution and 1% BSA for the antibody diluent. Primary antibodies were used at the following dilutions: caveolin-1, -2, -3 mAbs (1:500), actin mAb (1:5000), α-sarcoglycan mAb (1:200). Horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution, Pierce for anti-mouse IgG and BD Transduction Labs for anti-rabbit IgG) were used to visualize bound primary antibodies with the Supersignal chemiluminescence substrate (Pierce).

*Preparation of Caveolae-enriched Membrane Fractions.* WT or Cav-3 KO hearts were harvested, minced with a razor-blade, and homogenized for 30 sec in 2 ml of MES-buffered saline with 1% (v/v) Triton-X100, at 4°C. Samples were centrifuged (1000 x g for 5 min at 4°C, and the
supernatant was adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose in Mes buffered saline. A 5-30% linear sucrose gradient was formed above the homogenate and centrifuged at 39,000 rpm for 16 hrs in a SW41 rotor (Beckman Instruments). A light scattering band in the ~15-20% sucrose region was observed. Twelve 1 ml fractions were collected, starting at the top of the gradient. For SDS-PAGE/Western blotting, an equal amount of total protein from each fraction (25 µg) was analyzed.

**Immunofluorescence Microscopy.** WT or Cav-3 KO mice were sacrificed, their hearts harvested, quickly washed in ice-cold PBS and frozen in liquid-nitrogen cooled isopentane. Unfixed frozen sections were incubated in blocking solution (10% horse serum, 1% BSA, 0.1% Triton-X100, 1X-TBS-Tween; no horse serum was used for α-sarcoglycan or dystrophin) for 30 min at room temperature. Primary antibodies were used at the following dilutions: dystrophin NCL3 (1:50); α-sarcoglycan (1:100); β-sarcoglycan (1:100); δ-sarcoglycan (1:00); and γ-sarcoglycan (1:100); and β-dystroglycan (1:50), in TBS-tween /1% BSA for 1 hour at room temperature. Rhodamine Red-X-Conjugated AffiniPure F(ab’)2 Fragment Goat anti-mouse secondary IgG (1:150) in 1% BSA TBS-tween was applied for 30 minutes at room temperature. Slides were mounted with Slow-Fade anti-fade reagent (Molecular Probes, Inc.) and observed under a Bio-Red MR 600 confocal microscope.

**Transmission Electron Microscopy.** Heart tissue samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed with OsO4, and stained with uranyl acetate and lead citrate. Microtome sections were examined under a JEOL 1200 EX transmission electron microscope and photographed at a magnification of 15,000x. Caveolae were identified by their characteristic flask-shape, size (50-100 nm), and location at or near the plasma membrane.

**Preparation of Heart Paraffin Sections.** Mice were sacrificed and their hearts were removed and placed in buffered-formalin (10%). The tissue was fixed for ~ 24 hr, washed in PBS for 20 min, dehydrated through a graded series of ethanol washes, treated with xylene for 40 min, and then embedded in paraffin for 1 h at 55 °C. Paraffin-embedded 5-µm thick sections were then prepared using a Microm (Baxter Scientific) microtome and placed on super-frost plus slides (Fisher). Slides were then stained with hematoxylin and eosin (H & E) or Trichrome, according
to standard laboratory protocols. Samples were examined by an experienced cardiac pathologist (Dr. Stephen M. Factor)

**Non-invasive Cardiac Imaging.**

*a) Gated cardiac magnetic resonance imaging.* MRI experiments were performed using a GE Omega 9.4T vertical bore MR system equipped with a micro-imaging accessory and custom-built coils designed specifically for mice, as described previously (31). Just prior to each image acquisition, the heart rate was determined from the ECG, and the spectrometer gating delay was set to acquire data in diastole and systole. Multi-slice spin-echo imaging with an echo time of 18 ms and a repetition time of approximately 100-200 ms was performed. A 35 mm field of view (with a 256 x 256 pixel image matrix) was used. Short and long axis images of the heart were acquired and MRI data were processed off-line with MATLAB-based custom-designed software.

*b) Transthoracic Echocardiography.* Transthoracic echocardiography was performed as described previously (32). Echocardiography was performed with mice in supine position on a heating pad set at 38°C. Light anesthesia was achieved using isoflurane inhalation. Continuous, standard electrocardiograms were taken from electrodes placed on the extremities. Echocardiographic images were obtained using an annular array, broadband, 10/5 MHz transducer attached to an HDI 5000 CV ultrasound system (Advanced Technology Laboratories, Bothel, WA). A small gel standoff was placed between the probe and chest. Two-dimensional and M-mode images of the heart were obtained from the basal short axis view of the heart and stored on 3/4" SVHS video tapes for off-line measurements using the Nova-Microsonic (Kodak) Imagevue DCR workstation (Indianapolis, IN). All measurements were made in three to six consecutive cardiac cycles and the averaged values were used for analysis. Left ventricular end-diastolic and end-systolic diameters, as well as diastolic ventricular septal and posterior wall thickness were measured from M-mode tracings. Diastolic measurements were performed at the point of greatest cavity dimension, and systolic measurements were made at the point of minimal cavity dimension, using the leading edge method of the American Society of Echocardiography (33). Additionally, the following parameters were calculated using the above-mentioned measurements: left ventricular diastolic wall thickness as the average of ventricular septal and left ventricular posterior wall thickness; left ventricular percent fractional shortening as \[100 \times \frac{\text{end-diastolic}}{	ext{end-systolic}}\]
diameter minus end-systolic diameter)/end-diastolic diameter); and relative wall thickness as (2 x left ventricular diastolic wall thickness)/end-diastolic diameter.

Note that differences between the "absolute" wall thickness' measured using MRI and echocardiography are commonly observed and are likely due to technical factors, such as differences in the time of gating; echocardiography may underestimate these values, while MRI may overestimate these values. Most importantly, however, the relative changes measured in left ventricular wall thickness using MRI and echocardiography are in agreement.

Immunoblotting with Phospho-Specific Abs. For the analysis of phospho-proteins, WT and Cav-3 KO hearts were harvested, quickly rinsed in PBS (1X), and immediately frozen in liquid nitrogen. Frozen hearts were then homogenized in 3 ml of boiling lysis buffer (1% SDS, 1.0 mM sodium ortho-vanadate, 10 mM Tris pH 7.4), microwaved for 15 seconds, and centrifuged for 5 min. at 16,000 x g to pellet any insoluble material. The supernatant was transferred to a new tube and aliquots (1:10 dilution) were prepared for protein concentration analysis. Twenty micrograms of protein was run on SDS-PAGE gels and analyzed by Western-blotting.

Blood Pressures. Blood pressure measurements were taken on both WT and Cav-3 KO mice by placing them in a mouse restrainer (RTBP007, Kent Scientific, Inc.) and applying a mouse occlusion cuff (RTBP050, Kent Scientific, Inc.) and mouse plethysmographic cuff (XBP051, Kent Scientific, Inc.) to the tail of the mouse. A heat lamp was used to warm the mice. Systolic and diastolic blood pressure measurements were taken using the XBP1000 apparatus (Kent Scientific, Inc.) connected to a data acquisition system. The occlusion pressure corresponding to the minimal and maximal plethysmographic signals were taken to be the systolic and diastolic pressures, respectively.
Results

Cav-3 KO mice do not express caveolin-3 in the heart and lack cardiac myocyte caveolae.

Hearts were harvested from WT and Cav-3 KO mice and examined by Western blot analysis (Figure 1A). Note that genetic ablation of the Cav-3 gene results in the complete loss of the Cav-3 protein from the heart. However, the expression levels of Cav-1 and Cav-2 are unaffected by a lack of Cav-3 expression. Immunoblotting with anti-actin IgG was also performed as a control for equal protein loading.

By immunofluorescent microscopic analysis it is clear that Cav-3 localizes to the plasma membrane of WT cardiac myocytes, while Cav-1 and Cav-2 are found exclusively in the endocardium and the endothelium of the heart (Figure 1B, panels a-c). As expected, Cav-3 expression is not detectable in sections of Cav-3 KO hearts; most importantly, Cav-1 and Cav-2 expression remains properly restricted to the endocardium and endothelium in Cav-3 KO hearts, indicating that there is no compensatory upregulation of Cav-1 and Cav-2 in Cav-3 null cardiac myocytes (Figure 1B, panels d-f). Longitudinal sections demonstrate that Cav-3 co-localizes with the Z-line patterning of the myocardium, as well as the plasma membrane (Figure 1C). This is consistent with an association between Cav-3 and the T-tubule system in cardiac myocytes, as the T-tubule system is in register with the Z-lines in the heart, as opposed to the A-I bands within skeletal muscle.

Due to their “liquid-ordered” and “buoyant” properties, caveolae can be isolated by tissue solubilization in the detergent Triton-X 100 at 4°C, followed by sucrose density-gradient centrifugation (34). In wild-type heart, note that caveolin-1 and caveolin-3 are localized to the "light" buoyant density area of the gradient that contains lipid rafts/ caveolae (fractions 5-6) (Figure 1D). In Cav-3 KO heart, caveolin-1 remains localized to the lipid raft/ caveolae fractions as expected since caveolin-1 is expressed in the endothelium and endocardium of the heart.

Transmission electron microscopy reveals that caveolae are found in both the endothelium and myocardium of wild-type mouse hearts (Figure 1E). However, disruption of the Cav-3 gene
results in the complete loss of caveolae only in the cardiac myocytes, thus demonstrating at the structural level the necessity of Cav-3 expression for the formation of cardiac myocyte caveolae. However, the morphology and number of Cav-1-generated caveolae within the endothelium is unaffected.

Cav-3 KO mice show left ventricular wall thickening, as assessed by gated cardiac MRI

Wild-type and Cav-3 KO hearts were next analyzed using magnetic resonance imaging (MRI) (31). Measurements of left ventricular wall thickness were obtained for hearts in both the systolic and diastolic phases of the cardiac cycle.

Figure 2A shows representative short axis (transverse) images at the mid-level of wild-type and Cav-3 KO mice during diastole. At 2 months of age, Cav-3 KO hearts show a moderate increase (~10%) in left ventricular wall thickness, as compared with age-matched wild-type control mice (Fig 2B and Table I). However, by 4 months of age, the Cav-3 KO hearts show an even more dramatic increase in left ventricular wall thickness (~20%), indicating that this is a progressive cardiomyopathic phenotype. Interestingly, the increase in left ventricular wall diameter is uniform, suggesting concentric hypertrophy.

Cav-3 KO mice demonstrate left ventricular wall thickening, chamber dilation, and reduced systolic function, as assessed by transthoracic echocardiography

Wild-type and Cav-3 KO hearts were further analyzed using transthoracic echocardiography (32). Importantly, the heart rates of all animals tested are not statistically different, thereby allowing for meaningful comparisons. Multiple measurements of chamber size and wall thickness were made during both diastole and systole. At 4 months of age, Cav-3 KO hearts showed a significant increase (~20%) in left ventricular chamber diameter during diastole, as compared with age-matched wild-type control mice. During systole, the increase in Cav-3 KO left ventricular chamber diameter is even more pronounced (~50%) (Table II). Interestingly, the 4 month old Cav-3 KO hearts show a marked increase in left ventricular chamber diameter during both diastole and systole, as compared with Cav-3 KO hearts at 2 months of age [diastole: 3.08 ±
0.07 → 3.33 ± 0.12; systole: 1.63 ± 0.04 → 1.91 ± 0.15 mm]. Thus, 2-4 months is an important
time-frame in the development of this cardiomyopathic phenotype.

Echocardiography was also utilized to determine other parameters of cardiac structure and
function, such as interventricular septum, as well as the posterior and anterior wall thickness. For each of these areas, the measured wall thickness is ~ 20% greater in the Cav-3 KO when
compared to wild-type hearts (Table II). As the increase in thickness is uniform for all the wall
thickness' measured, these data are consistent with a concentric hypertrophy profile.

Functionally, Cav-3 KO hearts results in an ~20% decrease in fractional shortening, consistent with the observed chamber dilation and increases in wall thickness. In addition, our
blood pressure measurements show that Cav-3 KO mice have normal diastolic and systolic blood
pressures, thus ruling out the possibility of pressure-overload induced cardiac hypertrophy
(Table III).

Histological examination of Cav-3 KO heart tissue reveals cardiac myocyte hypertrophy, the
presence of cellular infiltrates, and progressive interstitial/peri-vascular fibrosis

H & E stained sections of Cav-3 KO hearts were examined at low and high magnification. No abnormal histo-pathological features are evident in 2.5 week old Cav-3 KO hearts (not
shown). By 2 months of age, however, Cav-3 KO hearts clearly show hypertrophic myocytes
and an increase in the overall number of nuclei per field, as compared to age-matched wild-type
control hearts (Figure 3, panels a,b,d and e). Similar findings were also present at 4 and 11
months of age (not shown).

The identification of fibrosis at the junction of the left and right ventricles, a recognized
point of stress, is common in both aging mouse and human hearts. Trichrome staining shows
greater interstitial/peri-vascular fibrosis at this junction in older Cav-3 KO hearts, as compared to
age-matched wild-type control mice (Figure 3, panels c and f). However, signs of ischemia were
not observed in Cav-3 KO hearts.
α-Sarcoglycan, a maker of the dystrophin-glycoprotein (DG) complex, is specifically excluded from lipid rafts domains in the absence of Cav-3

A significant fraction of the DG complex expressed in myocytes is localized within lipid rafts/ caveolae microdomains (7,22). In addition, mice with null mutations in many of the DG complex proteins demonstrate a cardiomyopathic phenotype. Thus, we next examined the expression and localization DG complex members in Cav-3 KO hearts. However, immunofluorescence analysis clearly demonstrates that the expression levels and membrane localization of each of the DG complex members examined (dystrophin, α-, β-, δ-, and γ-sarcoglycans, and β-dystroglycan) remains unchanged in Cav-3 KO heart tissue sections (Figure 5A).

The DG complexes present within caveolae may function in cellular signaling, as caveolae have been shown to serve as platforms for organizing and integrating a variety of signal transduction processes. In this regard, α-sarcoglycan is the best studied member of the DG complex that has been implicated in signaling (35-38). Thus, we further examined the expression and membrane localization of α-sarcoglycan, as a biochemical marker for the DG complex.

Figure 5B shows that the expression levels of α-sarcoglycan remain unchanged in Cav-3 KO hearts, as seen by Western blot analysis. However, sucrose density gradient fractionation reveals that α-sarcoglycan is specifically excluded from lipid rafts (fractions 5-6) in Cav-3 KO hearts (Figure 5C). These results suggest that Cav-3 expression is normally required for maintaining the localization of the DG complex within cardiac myocyte lipid rafts/ caveolae.

Cav-3 KO hearts show hyper-activation of the p42/44 MAP kinase cascade

We and others have previously demonstrated that both Cav-1 and Cav-3 can function as inhibitors of the Ras-p42/44 MAP kinase cascade (using a variety of in vitro approaches), probably through a direct interaction with MEK or ERK (39-43). As the Ras-p42/44 MAP kinase cascade has been clearly implicated as a mediator of cardiac hypertrophy hypertrophy
(44), we next assessed the activation state of ERK-1/2 in Cav-3 KO hearts, using a phospho-specific antibody probe that selectively recognizes activated ERK-1/2.

Figure 6 shows that ERK-1/2 is hyper-activated in Cav-3 KO hearts, as compared with hearts derived from wild-type control mice. These results provide the first \textit{in vivo} evidence that Cav-3 can function as a negative regulator of the p42/44 MAP kinase cascade. Importantly, immunoblot analysis with a phospho-independent antibody revealed that total levels of ERK-1/2 remain unchanged in Cav-3 KO hearts.
Discussion

Since their discovery in the 1950’s, cardiac myocyte caveolae have been postulated to perform a variety of important functions. However, a molecular understanding of cardiac myocyte caveolae only began recently in the mid 1990’s with the identification of Cav-3, a muscle-specific caveolin related protein (2,4,6). Since the molecular identification and cloning of Cav-3, a wealth of in vitro data has been accumulated demonstrating a role for Cav-3 in cardiac myocyte signaling (19-27). Interestingly, several distinct in vivo animal models of induced cardiac hypertrophy have shown reductions in Cav-3 protein expression within the heart (25,26,28). Taken together, these data argue that Cav-3 may play an important functional role as a negative regulator of hypertrophic signaling in the heart. However, this hypothesis remains untested.

Mutations in Cav-3, as well as mutations in any one of the sarcoglycans (α, β, δ, or γ), result in a Limb-Girdle Muscular Dystrophy (LGMD) phenotype. In addition to skeletal muscle symptoms, many LGMD patients show cardiac involvement (45,46). Analysis of skeletal muscle tissue biopsies from these patients reveals the loss or dramatic reductions in the expression of all the sarcoglycans when there is a disease-related mutation in a single sarcoglycan family member. The inter-dependence of sarcoglycan expression is also evident from studies employing sarcoglycan-null mouse models, although cardiac abnormalities are found in only β-, δ-, and γ- sarcoglycan null mice (47-50). Unlike these sarcoglycan-null mouse models, Cav-3 KO mice show no changes in expression or overall membrane localization of the sarcoglycans. However, using α-sarcoglycan as a marker for the DG complex, we demonstrate that the DG complex is no longer correctly targeted to lipid rafts/caveolae in the hearts of Cav-3 KO mice. Although distinct functional roles for DG complexes that localize to different membrane microdomains of the plasma membrane have not been elucidated, it is possible that specific signaling functions of the DG complex take place within caveolae (51).

Multiple lines of evidence implicate the DG complex in cellular signaling. It has been proposed that α-dystroglycan, as well the β-, δ-, and γ-sarcoglycans may possess a receptor
function, while α-sarcoglycan acts as a down-stream effector (37,52). In addition, β-dystroglycan, as well as α- and γ-sarcoglycans, have been shown to be tyrosine phosphorylated upon stimulation with different ligands, implicating them as signal transducing molecules (35,53). Specifically, α-sarcoglycan may function in bi-directional signaling in concert with the integrin-adhesion system, as well as possess ecto-ATPase activity (35,36).

Interestingly, the DG complex and caveolins share the feature of serving as scaffolds for signaling molecules, the perturbation of which may result in muscle pathology (51). The loss of α-sarcoglycan from detergent-insoluble domains may thus correspond with altered DG complex cell signaling. Animal models of β- and δ-sarcoglycanopathies demonstrate vasculature constriction/ focal narrowing that initiates ischemic events in the cardiac muscle (48,50,54,55). However, signs of ischemia were not observed in Cav-3 KO hearts, suggesting that the Cav-3 KO cardiomyopathy is not due to pathological constriction of the coronary arteries. Vascular constriction in humans with sarcoglycanopathies has not been demonstrated (56); however, a more detailed analysis is needed.

Activated p42/44 MAP kinase (ERK-1/2) has been shown to play an important role as an effector of the cardiac hypertrophic response (57-59). ERK-1/2, as well as known upstream activators MEK1/2 and multiple membrane receptors, have all been shown to co-localize to caveolae (60-62). In vitro data also supports a role for Cav-1 and Cav-3 as negative regulators of p42/44 MAP kinase signaling, as over-expression of Cav-1 and Cav-3 inhibits p42/44 MAP kinase activation, and targeted down-regulation of Cav-1 using an anti-sense approach results in the hyper-activation of the p42/44 MAP kinase cascade in NIH 3T3 fibroblasts (42,43).

Upon examination of Cav-3 KO hearts, we observed hyperactivation of the p42/44 MAP kinase cascade, as predicted. These findings are consistent with the notion that loss of Cav-3 expression results in dys-inhibition of the p42/44 MAP kinase cascade, thereby contributing to the development of a hypertrophic cardiomyopathy. As such, this is the first in vivo demonstration that a loss of Cav-3 causes the activation of a hypertrophic signaling program related to p42/44 MAP kinase activation.
In summary, we have presented the first detailed characterization of the hearts of Cav-3 KO mice. We clearly demonstrate that there are no derangements in the expression or localization of the other caveolin family members within Cav-3 KO hearts. Using a combination of non-invasive techniques (cardiac gated MRI; transthoracic echocardiography) and histological analysis, we showed that Cav-3 KO mice develop a progressive, mild-to-moderate, cardiomyopathic phenotype. As we show that loss of Cav-3 results in the mis-localization of the DG complex and hyper-activation of the p42/44 MAP kinase cascade, these alterations could mechanistically explain the observed cardiac pathology.
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Figure Legends

Figure 1. Cav-3 KO mice do not express caveolin-3 in the heart and lack cardiac myocyte caveolae.

(A) Western blotting. Hearts were harvested from wild-type and Cav-3 KO mice. Tissue lysates were prepared (see Materials & Methods) and subjected to SDS-PAGE/transfer to nitrocellulose. Blots were probed with isoform specific mAb probes that selectively recognize either caveolin-1, caveolin-2, or caveolin-3. Note that there is a complete loss of caveolin-3 in Cav-3 KO mouse hearts, without any changes in the expression levels of caveolin-1 or caveolin-2 as compared to wild-type (WT) control mice. Immunoblotting with anti-actin IgG is shown as a control for equal protein loading.

(B) Immunostaining (cross-sections). Frozen sections of the heart were prepared from wild-type and Cav-3 KO mice and immunostained with antibodies directed against either caveolin-1, caveolin-2, or caveolin-3. Note that caveolin-3 is localized to the plasma membrane (sarcolemma) of wild-type cardiac myocytes (panel a), but is completely absent in heart tissue derived from Cav-3 KO mice (panel d). In contrast, caveolin-1 and caveolin-2 expression is exclusively restricted to the endothelium and endocardium (see white arrowheads) as expected and remains unchanged in Cav-3 KO heart tissue (caveolin-1, panels b,e; caveolin-2, panels c, f). chamber = left ventricle chamber.

(C) Immunostaining (longitudinal-sections). Frozen sections of the heart were prepared from wild-type mice and immunostained with antibodies directed against caveolin-3. The fluorescence image and the corresponding phase image are shown; arrowheads point at the Z-lines. Note that the Z-lines identified in the phase image clearly co-incide with the immunostaining pattern observed for caveolin-3.
(D) *Cell Fractionation.* Hearts were harvested from wild-type and Cav-3 KO mice. Tissue lysates were prepared and subjected to sucrose density gradient analysis. In wild-type heart, note that caveolin-1 and caveolin-3 are localized to the "light" buoyant density area of the gradient that contains lipid rafts/ caveolae (fractions 5-6). In Cav-3 KO heart, caveolin-1 remains localized to the lipid raft/ caveolae fractions as expected since caveolin-1 is expressed in the endothelium and endocardium of the heart.

(E) *Transmission Electron Microscopy.* Heart tissue samples were fixed and embedded as described under Materials & Methods. Caveolae were identified by their characteristic flask-shape, size (50-100 nm), and location at or near the plasma membrane. Arrowheads point at detached caveolae, while arrows are used to indicate caveolae that remain attached to the plasma membrane. Note that in wild-type animals caveolae are present in both the cardiac myocyte and adjacent endothelial cell. In contrast, in Cav-3 KO animals there is a selective loss of muscle caveolae in the cardiac myocyte, while the adjacent endothelial cell retains its non-muscle caveolae. Myo, cardiac myocyte; Endo, endothelial cell; Lumen, blood vessel lumen that may contain RBCs.
Figure 2. Cav-3 KO hearts demonstrate progressive left ventricular wall thickening, as assessed by gated cardiac MRI.

(A) MRI images. Representative short axis (transverse) images at the mid-level of wild-type and Cav-3 KO mice during diastole. Arrows point at the left ventricle. Note that the left ventricle wall thickness is increased significantly in Cav-3 KO mice. LV = left ventricle.

(B) Graphic representation. A bar graph of the left ventricular wall thickness of Cav-3 KO hearts at 2 and 4 months of age is shown and compared with age-matched WT control mice. Note the progressive increases in Cav-3 KO left ventricle wall thickness from 2 to 4 months (n = 5 for each group tested, p < 0.05).
Figure 3. Histological examination of Cav-3KO heart tissue reveals cardiac myocyte hypertrophy, interstitial/peri-vascular fibrosis, and cellular infiltrates.

Representative H & E staining of WT (panels a,b) and Cav-3 KO (panels d,e) heart paraffin-embedded sections is shown. Note the marked hypertrophic cardiac myocytes (arrows) and cellular infiltrates (arrowheads) in the Cav-3 KO sample. Interestingly, Trichrome staining of 11-month old WT (panel c) and Cav-3 KO (panel f) hearts reveals increased interstitial/peri-vascular fibrosis at the junction of the right and left ventricle in Cav-3 KO hearts at this age.
Figure 4. Analysis of the dystrophin-glycoprotein complex in Cav-3 KO mouse hearts: α-Sarcoglycan is no longer properly targeted to lipid rafts in the absence of Cav-3.

(A) Immunostaining (cross-sections). Frozen sections of the heart were prepared from wild-type and Cav-3 KO mice and immunostained with antibodies directed against components of the dystrophin-glycoprotein (DG) complex, including dystrophin, the sarcoglycans (α- β- δ- γ-) and β-dystroglycan. Note that there is no change in the expression levels or the distribution of the DG complex in Cav-3 KO cardiac myocytes.

(B) Western blot analysis. Hearts were harvested from wild-type and Cav-3 KO mice. Tissue lysates were prepared and subjected to SDS-PAGE/transfer to nitrocellulose. Note that the total amount of α-sarcoglycan remains unchanged in Cav-3 KO heart tissue. Immunoblotting with anti-actin IgG is shown as a control for equal protein loading.

(C) Cell Fractionation. Hearts were harvested from wild-type and Cav-3 KO mice. Tissue lysates were prepared and subjected to sucrose density gradient analysis. In wild-type heart, note that a significant portion of total α-sarcoglycan is localized to the light buoyant density area of the gradient that contains lipid rafts/ caveolae (fractions 5-6). In contrast, in Cav-3 KO heart the distribution of α-sarcoglycan is altered; α-sarcoglycan is now excluded from the lipid raft/ caveolae fractions.
Figure 5. Hyper-activation of the p42/44 MAP kinase cascade in Cav-3 KO heart tissue.

Hearts were harvested from wild-type and Cav-3 KO mice. Lysates were prepared and subjected to immunoblot analysis with antibodies directed against phospho-ERK-1/2. Immunoblotting with phospho-independent antibodies to ERK was also performed as a control for equal protein loading. Note that Cav-3 KO mouse hearts show hyper-activation of ERK-1/2 (Upper panel), without any changes in the total cellular levels of ERK-1/2 (Lower panel).
Fig. 1A

A

WT  Cav-3 KO

Caveolin-3
Caveolin-1
Caveolin-2
Actin
B

Caveolin-3

WT

a

d

Caveolin-1

b

e

Caveolin-2

c

f

Caveolin-3 KO

chamber

chamber

chamber
Phase 3

Cav-3
Figure 1D

Cav-3 Expression

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|-----|----|----|
| WT |   |   |   |   |   |   |   |   |     |    |    |
| Cav-3 KO |   |   |   |   |   |   |   |   |     |    |    |

Cav-1 Expression

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|-----|----|----|
| WT |   |   |   |   |   |   |   |   |     |    |    |
| Cav-3 KO |   |   |   |   |   |   |   |   |     |    |    |
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Fig. 1E

WT

Cav-3 KO

Myo

Endo

Lumen

Myo

Endo

Lumen w/RBC
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Figure 2A

A

WT

Cav-3 KO
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**Figure 2B**

B

---

**Left Ventricular Wall Thickness (mm)**

|       | 2 months | 4 months |
|-------|----------|----------|
| WT    |          |          |
| Cav-3 KO | 0.95    | 1.3      |

* indicates significant difference.
H&E: low magnification

H&E: high magnification

Trichrome stain

WT

Cav-3 KO

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Figure 3
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Figure 4A

A

| Protein            | WT          | Cav-3 KO    | WT          | Cav-3 KO    |
|--------------------|-------------|-------------|-------------|-------------|
| Dystrophin         | ![Image]    | ![Image]    | ![Image]    | ![Image]    |
| \(\alpha\)-Sarcoglycan | ![Image]    | ![Image]    | ![Image]    | ![Image]    |
| \(\beta\)-Sarcoglycan | ![Image]    | ![Image]    | ![Image]    | ![Image]    |
| \(\delta\)-Sarcoglycan | ![Image]    | ![Image]    | ![Image]    | ![Image]    |
| \(\gamma\)-Sarcoglycan | ![Image]    | ![Image]    | ![Image]    | ![Image]    |
| \(\beta\)-Dystroglycan | ![Image]    | ![Image]    | ![Image]    | ![Image]    |
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Figure 4B

B

WT  Cav-3 KO

α-Sarcoglycan

Actin
C

α-Sarcoglycan Expression

WT

Cav-3 KO

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Figure 4C
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Figure 5

Phospho ERK-1/2

ERK-1/2
Table I. Magnetic Resonance Imaging Analysis

| Groups     | LV Wall Thickness (mm) |
|------------|------------------------|
| 2 months   |                        |
| WT         | 0.94 ± 0.02            |
| Cav-3 KO   | 1.03 ± 0.01*           |
| 4 months   |                        |
| WT         | 1.01 ± 0.01            |
| Cav-3 KO   | 1.21 ± 0.03*           |

* represents p < 0.05; each group (n=5)
| Group       | Heart rate (bpm) | End diastolic diameter (mm) | End systolic diameter (mm) | Intraventricular septal thickness (mm) | Posterior wall thickness (mm) | Wall thickness diastole (mm) | Relative wall thickness (ratio) | Fractional shortening (%) |
|-------------|------------------|-----------------------------|-----------------------------|----------------------------------------|-------------------------------|-----------------------------|-------------------------------|-----------------------------|
| WT          | 653 ± 25         | 2.80 ± 0.05                 | 1.25 ± 0.10                 | 0.71 ± 0.03                            | 0.71 ± 0.02                   | 0.71 ± 0.02                 | 0.51 ± 0.01                   | 55.70 ± 2.80                |
| Cav-3 KO    | 605 ± 51         | 3.33 ± 0.12*                | 1.91 ± 0.15*                | 0.85 ± 0.01*                           | 0.84 ± 0.01*                  | 0.85 ± 0.01*                | 0.51 ± 0.02                   | 43.03 ± 2.83*               |

* represents p < 0.05; WT (n=5); Cav-3 KO (n=5)
Table III. Blood Pressure Measurements

| Pressure (mmHg)                  | WT        | Cav-3 KO   |
|---------------------------------|-----------|------------|
| Systolic Blood Pressure         | 140.2 ± 1.8 | 135.6 ± 5.8 |
| Diastolic Blood Pressure        | 101.5 ± 2.6 | 99.0 ± 5.0 |
| Mean Arterial Pressure          | 114.0 ± 2.0 | 115.0 ± 5.0 |

Mean Arterial Pressure = \[\frac{(\text{Diastolic Pressure} \times 2) + \text{Systolic Pressure}}{3}\]; WT (n=4); Cav-3 KO (n=5); p > 0.05
Caveolin-3 knock-out mice develop a progressive cardiomyopathy and show hyperactivation of the p42/44 MAP kinase cascade
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