MUSCLE CREATINE KINASE DEFICIENCY TRIGGERS BOTH ACTIN DEPOLYMERIZATION AND DESMIN DISORGANIZATION BY ADVANCED GLYcation END-PRODUCTS IN DILATED CARDIOMYOPATHY

Nicolas Diguet1, Youssef Mallat1, Romain Ladouce1, Gilles Clodic2, Alexandre Prola3, Eva Tritsch1, Jocelyne Blanc1, Jean-Christophe Larcher3, Claude Delcayre5, Jane-Lise Samuel5, Bertrand Friguet5, Gérard Bolbach2, Zhenlin Li1, Mathias Mericskay1

From 1Department of Aging, Stress and Inflammation, 2IFR83 Mass Spectrometry Facility, 3CNRS UMR7622 Developmental Biology, UPtMC Univ Paris 6, Paris, France; 4Inserm UMR-S 769, University Paris-Sud 11, Châtenay-Malabry, France and 5Inserm U942 Biomarkers and Heart Diseases Université Denis Diderot Paris 7, Paris, France.

Address Correspondence to: Mathias Mericskay, PhD - Université Pierre et Marie Curie Paris 6 - UR4 Vieillissement, Stress, Inflammation - 7, quai Saint Bernard - BP 256, 75005 Paris - France Tel: 33 1 42 27 26 45 - Fax: 33 1 44 27 21 35 - e-mail: mathias.mericskay@upmc.fr

Alterations in the balance of cytoskeleton as well as energetic proteins are involved in the cardiac remodeling occurring in dilated cardiomyopathy (DCM). We used 2D-DIGE proteomics as a discovery approach to identify key molecular changes taking place in a temporally controlled model of DCM triggered by cardiomyocyte-specific Serum Response Factor KO in mice. We identified muscle creatine kinase (MCK) as the primary down-regulated protein followed by α-actin and α-tropomyosin down-regulation leading to a decrease of polymerized F-actin. The early response to these defects was an increase in the amount of desmin intermediate filaments and phosphorylation of the αB-crystallin chaperone. We found that αB-crystallin and desmin progressively lose their striated pattern and accumulate at the intercalated disk and the sarcolemma, respectively. We further show that desmin is a preferential target of advanced glycation end-products (AGE) in mouse and human DCM. Inhibition of CK in cultured cardiomyocytes is sufficient to recapitulate both the actin depolymerization defect and the modification of desmin by AGE. Treatment with either cytochalasin D or glyoxal, a cellular AGE, indicated that both actin depolymerization and AGE contribute to desmin disorganization. Heat-shock induced phosphorylation of αB-crystallin provides a transient protection of desmin against glyoxal in a p38-MAPK-dependent manner. Our results show that the strong down-regulation of MCK activity contributes to F-actin instability and induces post-translational modification of αB-crystallin and desmin. Our results suggest that AGE play an important role in DCM because they alter the organization of desmin filaments that normally support stress response and mitochondrial functions in cardiomyocytes.

INTRODUCTION

In the heart, ventricular chambers dilation is a common response to disorders that produce volume overload, such as what occurs following myocardial infarction. This distinct cardiac morphology also occurs in dilated cardiomyopathy (DCM), a cardiomyopathy showing an important genetic contribution (1). Gene mutations that were identified in DCM occur essentially in extra-sarcomeric cytoskeleton proteins like lamin, desmin and cytoskeletal linkers like vinculin or muscle lim protein, but also in sarcomeric proteins like actin (1), and in proteins involved in calcium signaling (2). One of the striking features of both acquired and genetic DCM is the eccentric remodeling of cardiomyocyte, i.e. an increase in the long axis without concomitant increase in the wide axis, and the stretching of the intercalated disks (ID), which connect cardiomyocytes in series and allow force transmission. These cytoarchitectural modifications are paralleled by a reorganization of the major filamentous cytoskeleton networks, including actin microfilaments, microtubules and desmin intermediate filaments, each network being closely interconnected to the other, to the sarcolemma and to organelles of the cardiomyocytes. These cytoskeletal networks are also intimately coupled to the bioenergetic structures, like mitochondria and the systems of energy transfer in the muscle cells like the phosphocreatine shuttle system (3).
The MADS box transcription factor Serum Response Factor (SRF) is a major regulator of genes encoding contractile proteins, notably all muscle actin isoforms, but also of genes involved in energy transfer like muscle creatine kinase (MCK) or in calcium homeostasis like NCX1 (4). SRF is activated by cytokines, growth factors and G-coupled receptors, which are known to play a major role in cardiac remodeling. SRF is also involved in regulatory feedback loops controlled by actin and titin cytoskeleton linked signals that adapt muscle gene expression to the work demand (5,6). A few studies reported the presence of truncated forms of SRF in human heart failure (7,8) and we showed that Cre-LoxP-mediated total or even partial inactivation of the SRF gene in the adult mouse heart leads to actin polymerization defects, cardiac chambers dilation and heart failure (9,10). Altogether, these studies strongly suggest that alterations of SRF activity could be involved in the pathogenesis of both genetic and acquired DCM and heart failure.

The inducible SRF heart knock-out (SRF1KO) mice develop a progressive DCM within an experimentally controlled time frame (9). We took advantage of this unique experimental setting to adopt a proteomic discovery approach in order to identify the key markers of disease progression that could play a role in the pathogenesis of DCM. Here we characterize a temporal cascade of molecular events going from early down-regulation of muscle creatine kinase (MCK) to actin polymerization defects, triggering the recruitment of phosphorylated αB-crystallin at sites of deficient F-actin at the detriment of another major αB-crystallin client protein, the desmin intermediate filament. Finally, we show that desmin is a preferential target of advanced glycation end-products (AGE) that lead to the disorganization of this essential cytoskeletal lattice connecting myofibrils to mitochondria and sarcolemma.

**EXPERIMENTAL PROCEDURE**

**SRF1KO mouse model**

SRF1KO mice homozygous for SRF-floxed allele (Sf) bearing one copy of the tamoxifen-inducible α-MHC-MerCreMer transgene have been described previously (9). The α-MHC-MerCreMer transgenic mice were a kind gift of Dr J. Molkentin (University of Cincinnati, USA (11). The mice used for the present study have been backcrossed with C57BL/6J mice for at least 20 generations. Groups of 2-month-old (α-MHC-MerCreMer:Sf/Sf) and control (Sf/Sf) male mice were given daily intraperitoneal tamoxifen (20 mg/kg/day; Sigma) injections on 3 consecutive days, with the day of the first injection counted as day 0. This study conformed to institutional guidelines for the use of animals in research.

**Patients**

Studies were performed with left ventricular myocardium (LV) from controls (n=3) and end-stage failing hearts from patients with idiopathic dilated cardiomyopathies (n=7). Patients clinical data have been described previously (12). Briefly, patients presented increases in LV mass and decreased LV ejection fraction (14 ± 5%). This study was approved by the Consultative Committee for the Protection of Human Subjects in Biomedical Research (Paris, France).

**Left ventricular tissue preparation for use in proteomic and biochemical analyses**

For all proteomic and biochemical experiments, 2 month-old α-MHC-MerCreMer: Sf/Sf and control (Sf/Sf) were sacrificed at 25 (D25) or 45 (D45) days after tamoxifen injection. Male mice were used to eliminate gender effect when comparing the protein profiles of SRF mutant and control hearts. Directly following euthanasia, the LV was excised, blotted, and frozen in liquid nitrogen. Frozen tissue was subsequently powdered in a percussion mortar cooled in liquid nitrogen.

**2D gel electrophoresis, gel staining, image acquisition**

Powdered LV tissue was dissolved in 15 volumes (µl/mg of tissue powder) of lysis UTC buffer (8 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT). The crude extract was homogenized using a syringe and needle (21G) and centrifuged at 20,000 g for 30 min at 15 °C, and the supernatant was precipitated using 2D clean-up kit (GE Healthcare) following manufacturer recommendations. Protein pellets were dissolved in the lysis buffer without DTT. Protein concentration was determined using Bradford assay (Biorad). To screen for protein amount differences and obtain quantitative protein ratios, 2D Differential In Gel Electrophoresis (DIGE) was performed on four paired samples (4 SRF mutant and 4 WT) for each experiment. Control and mutant heart protein extracts (50 µg) were
labeled with the CyDye DIGE Fluor minimal labeling kit (GE Healthcare) following the manufacturers recommendations. An internal standard was made by mixing equal amounts of proteins from each sample and labeled with Cy2. For analytical gels, 18 cm Immobiline DryStrip gels (pH 3–11NL (non linear)) were loaded with a total protein amount of 150 µg (50 µg of each control and mutant extract and 50 µg of internal standard). For preparative gels, we used 450 µg of pooled control or mutant unlabelled protein extracts. Strips were rehydrated overnight in presence of proteins in a volume of 350 µl of rehydration solution (8M urea, 2M thiourea, 2% CHAPS, 1.2% DeStreak Reagent, 0.5% IPG- Buffer pH 3–11NL. Isoelectric focusing was performed with an Etten IPGphor2 apparatus (GE Healthcare) at 150 V for 6 h, followed by stepwise application of 200 V, 1000 V, and 8000 V for a total of 35,000 V.h. After focusing and equilibration, strips were applied to 8-18% gradient SDS PAGE gels and sealed with 1% agarose, containing bromophenol blue. Electrophoresis was performed at 20 °C in an Etten DALT-6 tank with electrophoresis buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 0.2% SDS) for a total of 2000 V.h. For each experiment, 4 analytical and 2 preparative gels were run in parallel. Analytical gels were imaged using an Etten DIGE imager (GE Healthcare) scanner. Data were analyzed with the DeCyder 7.0 software (GE Healthcare) using the batch process mode. An unpaired Student t-test was used to determine the statistical difference of the mean ratio between control and mutant groups. Unlabeled preparative gels were fixed and stained with Coomassie Brilliant Blue G-250 for 5 days and scanned with the Etten DIGE imager using the Cy5 channel. Matching of protein spots of interest between analytical and preparative gels was performed manually.

MS and MS MS analysis
In-gel digests were purified with home-made microcolumns packed with Poros reversed-phase 20 R2 resin (Applied Biosystems) using GEloader tips (Eppendorf) as described previously (13,14). The Poros 20 R2 column was washed with 20 µl of 0.1% TFA, and the bound peptides were eluted directly onto the MALDI target using 0.5 µl of a matrix solution consisting of CHCA (5 mg/mL) in 50% acetonitril, 0.1% TFA. Typically, 1-10 µl of digest were purified. Matrix-Assisted Laser/Desorption Ionization–Time-Of-Flight (MALDI-TOF) mass spectrometry was performed on a 4700 Proteomics Analyzer (Applied-Biosystems).

MALDI-TOF (MS) positive ion mass spectra of the samples were performed in reflector mode and delayed extraction using a focus mass set at 2100 u, near the threshold fluence. MALDI- TOF-TOF (MSMS) experiments were carried with and without gas for the study of fragmentation through metastable decomposition and CID with gas (N2, 2×10⁻⁷ Torr) with collision energy of 1 keV, respectively. For MALDI-TOF-TOF experiments, the precursor ion window was adjusted to be certain of the correct selection. MS and MSMS spectra were recorded by averaging a few hundreds to a few thousands of laser shots allowing a significant isotopic pattern of peptides and fragments ions. MALDI-TOF and MALDI-TOF-TOF spectra were calibrated using classical peptides standard (Applied-Biosystems). Data Explorer 4.6 software was used to analyze the MS and MSMS spectra and to generate the peak lists (MS or MS and MSMS data) which were submitted to the database search program MASCOT (Matrix Science, UK). Parameters for protein identification by MS included: a mass tolerance of 75 ppm, a maximum of two missed trypsin cleavages per peptide and the acceptance of carbamidomethylation of cysteines and oxidation of methionines. For protein identification with MSMS data, the mass tolerance was set to 0.1 Da and the charge state +1 using SwissProt as the main data bank. Positive identifications depended on a MOWSE score (molecular weight search algorithm) of around 40, the location of missed cleavages and whether significant peaks in the MS spectra were those used for the analysis. Finally, both the molecular weight (MW) and isoelectric point (pI) of each database matched protein were compared to those of the sample by referring to its position on the 2D gels.

Measurement of CK activity and ATP
Frozen tissue samples were weighed, homogenized in ice-cold buffer (50 mg wet weight per ml) containing: HEPES 5 mM (pH 8.7), ethyleneglycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA) 1 mM, dithiothreitol 1 mM, and Triton X-100 (0.1%) and incubated for 60 min at 4°C for complete enzyme extraction. CK activity was measured by spectrophotometry with coupled-enzyme system using hexokinase and G6PDH in the presence of phosphocreatine (10mM) and ADP (1.2 mM).
Appearance of NADPH was followed at 340 nm. Isoforms are separated by non denaturing electrophoresis and revealed by enzymatic activity to measure the percentage of each isoform. ATP and ADP/ATP ratio was measured using the bioluminescent ADP/ATP ratio assay kit from Abcam as indicated by the manufacturer with minor modifications. Briefly, cardiac tissue was frozen in liquid nitrogen and powdered with a mortar. Tissue powder was suspended in the provided lysis buffer (10 µl/mg of tissue powder) for 5 minutes exactly at room temperature, followed by centrifugation at 10 000 rpm for 1 min to pellet insoluble material. We used 50 µl of supernatant per assay in a final volume of 100 µl. Luciferase units were converted to pmoles amount of ATP by plotting against a standard curve with serial dilution of ATP. Data were normalized by the amount of protein present in the supernatant as measured by the Bradford assay.

**Western Blot Analysis**

1D-PAGE and 2-D PAGE were carried out as described previously (15,16). Proteins were transferred onto nitrocellulose membranes (Hybond C, Amersham Pharmacia Biotech) and the blots were saturated in TBS-T buffer (20 mMTris, pH 7.5, 136.8 mM NaCl, and 0.1% v/v Tween 20) containing 5% (w/v) low fat milk. The following primary antibodies were used for Western blot: mouse monoclonal anti-sarcomeric α-actin (1:400, Sigma, recognize both cardiac and skeletal α-actin); mouse monoclonal anti-α-tropomyosin; rabbit polyclonal anti-GAPDH (1:400, Santa-Cruz), rabbit polyclonal anti-Histone-3 (1:400, Sigma), mouse monoclonal anti-α-B-crystallin (1:200, Abcam), anti phosphorylated serines 19, 45 and 59 on α-B-crystallin (1:500, StressGen). Polyclonal anti-AGE antibody was a kind gift of Dr. Bakala and has been previously characterized (17,18). It recognizes a variety of AGE products such as carboxymethyl-lysine and carboxymethyl-lysine. Polyclonal anti-HNE recognizes HNE-adducts with lysine, cysteine and histidine amino acids within proteins and were generated as originally described (19). Protein Oxidation Detection Kit, OxyBlot, was purchased from Chemicon (Millipore).

HRP-conjugated secondary anti-rabbit (1:5000) or anti-mouse (1:5000) antibody (Dako) was used to detect the proteins by ECL. For western blot with anti phospho-ser αB-crystallin antibodies, heart tissue proteins were extracted in anti-phosphatase buffer (20 mM MOPS pH 7.2, 0.5% Nonidet-P40, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 40 mM sodium pyrophosphate, 1 mM NaVO4, protease inhibitor cocktail (Roche), 1 mM PMSF). For all other Western blots, cardiac protein extracts in urea/thiourea/chaps buffer were the same than used for 2D-DIGE. For Western blot analyses of cultured cardiomyocytes, proteins were harvested either in UTC buffer except for cell fractionation. For fractionation experiments, proteins were extracted in RIPA buffer (150 mM NaCl, 5 mM EDTA, 1% Tween-20, 50 mM Tris, pH 7.4, protease inhibitor cocktail [Roche]), incubated for 30 minutes on ice, and then centrifuged for 1 hour at 20 000g. Supernatant form the soluble fraction containing G-actin. Pellets were dissolved in UTC buffer to extract cytoskeletal proteins including F-actin.

**Isolation and treatments of neonatal rat cardiomyocytes**

1 day-old rat pups were killed by decapitation and the cardiac ventricles were harvested and minced into 1 to 2 mm wide cubes with scissors. After washing with Tyrode solution, heart fragments were subjected up to 10 rounds of digestion with 0.05 mg/mL Liberase Blendedyme 4 (Roche) in oxygenated Tyrode under agitation. The supernatant of the first digestion was discarded and the following digestion was stopped in DMEM/FCS 10%. Cultures were enriched in cardiomyocytes by centrifugation in a discontinuous percoll gradient (bottom 58.5%, top 40.5%, 30 min, 3000 rpm). Neonatal cardiomyocytes were seeded at a density 5x10^5 cells/well in 6-well plate on coverslips coated with 10 µg/mL laminin (BD Biosciences) in DMEM/horse serum 10%/ FCS 5% and cultured at 37°C in 1% CO2 atmosphere. For siRNA transfections, Rn_Ckm_2 siRNA (SIO1499190), Rn_Tpm1_1 siRNA (SIO2047507) and Negative Control siRNA (SIO3650325) from Qiagen were transfected at 10 nM final concentration using RNAiFect (Invitrogen) from day 5 to 8. For phosphocreatine shuttle inhibition, cells were cultured in presence of 10 mM β-guanidinopropionic acid (β-GPA) between day 3 to 8 in presence of 20 µM phenylephrine to increase energy expenditure in culture. For heat shock experiments, 6 days cell cultures were put at 42°C for 30 min. Cytochalasin D (20 µM) or glyoxal (10 mM) were added to the medium and
cells were put back at 37°C for 30 min. When the p38 MAPK inhibitor SB202190 (20 μM) was used, it was added to the medium 6 hours before heat shock treatment.

Immunofluorescence
Immunofluorescence staining were done on frozen heart sections or isolated cardiomyocytes with the following primary antibodies: TRITC coupled phalloidin, (1:100, Fluka), mouse monoclonal anti-desmin (1:50, D33, Dako); mouse monoclonal anti-αB crystallin (1:1000, Abcam); rabbit polyclonal anti-phospho-S45 and S59 αB crystallin (1:100, StressGen), followed by Cy3-coupled secondary antibodies (1:400, Jackson Immunoresearch) or Alexa-488 or 633 coupled secondary antibodies (1:400, Invitrogen).

Desmin striation pattern
To estimate in unbiased manner the degree of striation from the pattern of desmin signal, we acquired the desmin signal intensity profile along a line parallel to the alignment of the main axis of phalloidin-stained F-actin filaments in at least 5 cardiomyocytes for each condition, using Image J software. Data were processed using Fast Fourier transform function in Microsoft Excel using the signal along a distance in pixel equal to a power of 2 as requested (128 pixels = 35 μm, in the central region of the cardiomyocyte). We obtained series of spectra giving the sinusoid amplitude (A) as a function of spatial frequency (pixels converted to μm⁻¹ based on the scale of magnification). Data are shown as an average spectrum for each group +/- s.e.m.

Statistical Analysis
The data are expressed as mean ± SEM. An unpaired Student t test was used to determine the statistical difference of means between the control and mutant groups.

RESULTS
Proteomic changes in the SRF deficient diluted heart
Adult mice deficient for SRF in the cardiomyocytes of the heart (SRF<sup>HKO</sup>) develop a progressive reduction of ejection fraction starting around 3 to 4 weeks after SRF gene inactivation followed by a dilatation of all cardiac chambers and overt heart failure and death around D60-65 after gene inactivation (9). So, D25 was established as an early stage in disease progression at the beginning of the alterations of cardiac functions and D45 was established as a model of intermediate stage dilated cardiomyopathy for our subsequent proteomics analyses. To identify differentially expressed proteins, we prepared whole left ventricle proteins from control and mutant hearts at each time point and we performed 2D-DIGE analyses at D25 and D45 (Fig. 1A). Detailed quantitative analyses and protein identification data are provided in the online supplemental data (tables S1 and S2). Whole tissue protein preparations revealed an average of 1900 unique protein spots at each time point. Principal component analysis using all matched spots separated the control and mutant samples at D45 but not at D25 (Fig. S1). The 2D-DIGE analysis identified 16 protein spots that were significantly altered (p ≤ 0.05, Fold Change (FC) ≥ 1.3) at the early stage (D25) and 118 spots at the intermediate stage (D45) using the same criteria. We identified 45 protein spots by MALDI-TOF peptide analysis and validated 29 spots by MALDI-TOF-TOF peptide fragmentation, representing altogether 33 unique proteins (Fig. 1A, Table S1 and S2).

Early down-regulation of MCK and loss of polymerized actin in the SRF<sup>HKO</sup>
At D25, when the heart of SRF<sup>HKO</sup> mice is just starting to show signs of reduced contractility, the proteomic screen indicated that MCK was the most down-regulated protein (FC -2.02, p=3.8E-05, Table S1). RT-qPCR analysis showed a strong down-regulation of MCK mRNA as soon as D15 in agreement with the fact that MCK is a direct target of SRF (Fig. S2). In accordance with the result of the proteomic screen, ventricular muscle of SRF<sup>HKO</sup> mutant at D25 demonstrated a significant decrease of total CK activity that could be attributed exclusively to a 50% loss of MCK MM-dimer activity without changes in mitochondrial CK and brain cytosolic CK activity (Table 1). At D45, we observed a down-regulation of several mitochondrial enzymes involved in fatty acid β-oxidation and of β-enolase, a major enzyme in muscle glycolysis, and further down-regulation of MCK (FC -5.8, p=1.3E-07, Table S1). Measurement of ATP amount in the myocardial tissue at D45 showed a significant reduction in the SRF<sup>HKO</sup> heart (SRF<sup>HKO</sup> 5.5±1.6 vs Controls 15±3.4 pmole/mg of protein; pval= 0.044, N=6 each group). Accordingly ADP/ATP ratio raised
in the SRF^HKO heart (SRF^HKO 2.74±0.62 vs Controls 0.82±0.28; pval= 0.014). Altogether, these results show that energy reserve is altered in the failing SRF-deficient heart as in most models of heart failure (20). Up-regulation of AK1 and ATP-synthase subunit-d likely reflects an attempt of compensatory response to maintain ATP synthesis (Table S1 and S2).

Two sarcomeric proteins, troponin-T and -I were down-regulated at D45 (Table S1). In addition sarcomeric α-actin and α-tropomyosin were down-regulated albeit only by a factor 1.2 at D45 (Table S2). The low FC value for these proteins in 2D-DIGE may be explained by the saturation of the cyanine signal for these abundant proteins. The cardiac and skeletal α-actin genes that encode sarcomeric actins and the Tpm1 gene encoding α-tropomyosin were strongly down-regulated at an early stage as expected from being direct SRF targets (Fig. S2). Western-blot analyses, however, showed a minor reduction of these 2 proteins at D25 and only 50% reduction at D45; highlighting the long half-life of these proteins in vivo (Fig. 2A). Yet, the polymerization state of the actin filament was strikingly already altered at D25 in isolated cardiomyocytes as shown by the 50% decrease in the F-actin specific phalloidin signal accompanying the beginning of eccentric remodeling at this stage (Fig. 2C,D,F,G,I). Interestingly, cytoskeletal β-actin, which has been shown to play an important role in ID structure (21), was up-regulated in the SRF^HKO heart and was localized in subsarcolemal domain as well as connecting the last sarcomere to the stretched ID, in an apparent attempt to cope for sarcomeric actin down-regulation (Fig. 2B,E,H).

**Up-regulation and post-translational modification of αB-crystallin and desmin in the SRF-deficient cardiomyocytes**

In parallel to the reduction in energy metabolism and sarcomeric proteins, there was a significant increase in stress response proteins including αB-crystallin, Heat Shock Proteins HSP90β, HSP70 and HIP. Likewise, cytoskeletal proteins including desmin as well as tubulin β-2C and Csrp3/MLP (Cysteine and glycine-rich protein 3/Muscle Lim Protein) were increased. Among the up-regulated proteins, αB-crystallin and desmin were the only markers to be up-regulated at D25 suggesting they are the first responders to the thin filament and MCK defect (table S1).

This up-regulation occurred at the post-transcriptional level since corresponding mRNAs were not increased in the SRF mutant (Fig. S2). The spots 41 and 42 migrating at different pI but identical apparent MW were identified as αB-crystallin suggesting post-translational modification such as phosphorylation (Fig. 1B). In agreement with this hypothesis, we detected a phosphorylation on serine 45 and 59 of αB crystallin that was significantly increased as soon as D25 (Fig. 3). The level of serine 19 phosphorylation was not modified (data not shown). Increased phosphorylation on serine 45 and 59 has been associated with recruitment on cytoskeletal components (22,23).

For desmin, the DIGE analysis showed the presence of four spots migrating at different pI but identical apparent MW that were all up-regulated with a FC ranging from 2.49 to 2.88 (Fig. 1C, Table S1). The increase of desmin was confirmed by Western-blot analysis (Fig. 4A).

MS and MS/MS analysis revealed the presence of a m/z 2158.08 peptide compatible with a single phosphorylation (+ 80 Da) of the m/z 2078.11 peptide (residues 17-37) in the desmin spots 29, 30 and 31 but not in the basic spot 32. MS/MS fragmentation of m/z 2158.08 peptide revealed that the phosphate group was added to serine 28 (Fig. S3). Sequential 2D Western-blot analysis with anti-phosphoserine residues followed by anti-desmin antibody showed that the acidic desmin spots did contain phosphoserines (Fig. 4B) although the main basic spot equivalent to spot 32 was also labeled, suggesting that serine residues other than serine 28 are also phosphorylated.

**Desmin is modified by advanced glycation end-products in SRF^HKO dilated hearts and human patients with DCM**

We assayed other types of post-translational modification (Fig. S4). Oxidative stress as estimated from oxiblot assay was increased in the SRF^HKO heart at D45 while anti-HNE (4-hydroxy-2-nonenal lipid peroxidation product) did not show major changes. Anti-AGE polyclonal antibody revealed a significant increase in the intensity of a band migrating at an apparent MW of 55-kDa at D45 (Fig. S4 and 4A). This band overlapped with the desmin band in the same blot (Fig. 4A). Sequential 2D Western-blot with anti-desmin and anti-AGE antibodies confirmed that the anti-AGE antibody
labeled the main desmin spot (Fig. 4B and S5). Because the increase in desmin-AGE was rather specific among the bands present on the blot, we hypothesized that human desmin which is conserved from mouse to human could also be targeted by AGE in human DCM. As a first step in identifying potential alterations of desmin in the context of human heart failure, we examined the pattern of AGE modification in samples of human hearts from patients presenting cardiac dilation (12). Sequential Western-blot with the anti-AGE antibody and the anti-desmin antibody revealed an increased band with identical apparent MW than desmin in 6 out of 7 patients (Fig. 4C) in a similar pattern than observed in SRF<sup>HKO</sup> mice (Fig. 4A).

**Recruitment of phosphorylated αB-crystallin at deficient actin filaments is accompanied by a progressive disorganization of the desmin network**

To investigate the sub-cellular localization of αB-crystallin and desmin in the SRF<sup>HKO</sup> mutant, we stained heart sections at D25 and D45 with αB-crystallin and desmin antibodies as well as phalloidin to detect polymerized F-actin. Phalloidin signal displayed a progressive decline in cardiomyocytes from D25 to D45 (Fig. 5A-F) in agreement with the data obtained on isolated cardiomyocytes (Fig. 2B). Anti-total αB-crystallin antibody displayed a punctiform striated pattern of expression at the level of the sarcomeres in control cardiomyocytes and labeled the ID (Fig. 5A). The αB-crystallin staining increased at D25 and D45 in the SRF<sup>HKO</sup> mutant when the phalloidin signal progressively declined and the ID became highly stretched and disorganized (Fig. 5B-C). At D25, the phospho-S59 αB-crystallin increased in the SRF<sup>HKO</sup> at the Z-disk level where it co-distributed with desmin (Fig. 5G-H). Phospho-S59 and –S45 αB-crystallin increased at the ID at D25 and at D45 where it colocalized mainly with the remnant of F-actin linking the ultimate sarcomere to the stretched ID (Fig. 5E-F, inset, arrowhead, Fig. S5) but also around and between aggregates of desmin at the sarcolemma and ID level (Fig. 5H-I, Fig. S5). Anti-desmin antibody labeled the cardiomyocytes at the Z-disks, costameres and ID in the control (Fig. 5G). From D25 to D45, the desmin staining increased progressively in the SRF<sup>HKO</sup> in agreement with the 2D-DIGE data. The organization of desmin, however, was altered with a trend toward accumulation at the sarcolemma and a progressive loss of the striated pattern which culminated at D45 (Fig. 5H-I, arrows, sarcolemma; asterisk, reduced or absent signal inside the cardiomyocyte in right insets). At the level of the ID, the desmin signal appeared fuzzy and less filamentous at D45 (Fig. 5I and Fig. S5F).

**Knock-down of MCK expression in neonatal rat cardiomyocytes recapitulates major aspects of the in vivo SRF<sup>HKO</sup> phenotype**

To understand the molecular mechanisms underlying the cascade of events going from MCK down-regulation to cytoskeleton disorganization, we addressed the role of the primary SRF targets defects in this process using neonatal rat cardiomyocytes (NRC) as an in vitro model.

The 2D-DIGE experiments (Fig. 1 and Table S1) showed that MCK was a labile protein which was more quickly and strongly down-regulated than the stable actin protein (Fig. 2A and Table S2). However, the polymerization of actin was already affected at D25 (Fig. 2B). MCK is structurally coupled to the myofibrils to provide rapid phosphate transfer from mitochondrial CK-derived phosphocreatine to ADP and generate ATP for local consumption by the myosin ATPase. However, ATP is also known to be required for actin polymerization, which in turn could affect desmin. To determine if there is a direct link between the loss of MCK activity and cytoskeleton disorganization, we knocked-down the expression of MCK using a specific siRNA. We validated the down-regulation of the gene at the mRNA level (Fig.6A). The down-regulation was corroborated by a 50% loss of total CK activity in siMCK transfected versus scrambled control siRNA transfected NRC (siMCK, 2157±207 CK UI/g prot, vs scramble siRNA, 3879±129, pval=0.00396), which fit with the proportion of MCK in these neonatal cells.

Knock-down of MCK resulted in the loss of F-actin and decreased F/G actin ratio as measured by cell fractionation (Fig. 6B,C) and phalloidin/DNAse I staining (Fig. 6D-G). At the same time, siMCK triggered an increase of desmin protein that was modified by AGE (Fig. 6H-J). Desmin network was highly disorganized with accumulation at the membrane and loss of striated pattern (Fig 6. compare L to K). We performed fast Fourier transform of the desmin signal along a line parallel to the actin myofibrils to obtain an unbiased estimation of the
periodicity of desmin striations. In control NRC, desmin signal presented a peak with maximal amplitude at a mean frequency of 0.6 μm⁻¹, i.e. every 1.7 μm, which was in good agreement with the average length of a sarcomere in cardiomyocytes (Fig. 6N). This peak disappeared in siMCK transfected cells.

Treatment of NRCs with β-guanidinopropionic acid (β-GPA), an analogue competitor of creatine, which slows down the phosphocreatine shuttle by the CK system, resulted in a similar reduction of polymerized F-actin and increase in formation of AGE adducts on desmin (Sup Fig. S7). Altogether, these results showed that the inhibition of CK activity in vitro is sufficient to recapitulate the major characteristics of the SRFkip phenotype in vivo.

Desmin network anchorage at the Z-disk of the sarcomere is sensitive to both actin depolymerization and AGE modification

SiRNA-mediated knock-down of MCK altered both actin polymerization and desmin modification by AGE raising the question whether actin depolymerization and/or AGE modification contributed to desmin disorganization. Because α-tropomyosin, which is also down-regulated in the SRFkip heart, is known to play a role in thin filament stability but unlike MCK, not for energy transfer, we thought to address the consequence of siRNA-mediated knock-down of Tpm1 on actin polymerization and indirectly on desmin organization. SiTpm1 triggered a loss of F-actin similar to siMCK in NRC (Fig. 6B-G) while it did not affect CK activity (siTpm1 3238±61 CK UI/g prot, NS vs control). SiTpm1 also triggered a severe disorganization of desmin network (Fig. 6M,N). Because α-tropomyosin is not known to interact directly with desmin, we hypothesize that desmin disorganization is secondary to actin depolymerization. In agreement with this hypothesis, the use of cytochalasin D, an actin depolymerizing agent, was sufficient to trigger desmin disorganization in NRC (Fig. 7A bottom panel, B-C).

On the contrary to siMCK, however, siTpm1 induced desmin disorganization did not result in an increase of AGE adduct formation on desmin (Fig.6H-J). The apparition of AGE modification on desmin protein in vivo (Fig. 4) also coincided with the strongest deterioration of desmin filament pattern in vivo at D45 (Fig. 5). To investigate whether the AGE modification of desmin could be directly responsible for disorganization of the desmin filaments, we treated NRCs with glyoxal, a dicarbonyl precursor of AGE derived from oxidized triosephosphates or lipids, which is naturally present in the cells. The addition of glyoxal to the culture medium for 30 minutes was sufficient to alter the striated organization of desmin, which accumulated at the membrane. Interestingly, glyoxal did not affect the striated organization of actin suggesting a rather specific effect toward desmin intermediate filaments (Fig. 7A, left panel). The peak of striated desmin disappeared in glyoxal treated cells. As mentioned previously, cytochalasin D treatment had as strong disorganizing effect on desmin (Fig. 7A, bottom panel). We noticed that glyoxal and cytochalasin D treatment modestly stimulated phosphorylation on serine 59 of αB-crystallin (Fig. 7A, compare S59-CryAB staining in treated versus non treated cells). In order to increase αB-crystallin phosphorylation prior to the addition of the drugs, we decided to prime cells by a 30 minutes heat shock (HS). HS priming increased the signal of phospho-S59 αB-crystallin compared to unshocked cells and did not affect desmin organization per se (Fig. 7A, right panel). HS priming preserved the striated pattern of desmin in glyoxal and cytochalasin D treated cells (Fig. 7A and B). HS priming also protected the striated pattern of actin filaments from the effect of cytochalasin D (Fig. 7A, lower panel). The p38 MAPK has been involved in the phosphorylation of serine 59 on αB-crystallin upon most stress signals including vinblastin, cytochalasin D and HS (22,23). We show that the p38 MAPK inhibitor SB202190 blocked the appearance of phospho-S59 αB-crystallin and prevented the protective effect of heat shock against glyoxal while it had no deleterious effect on desmin organization in basal conditions (Fig. 7A and B). These results suggest that phospho-S59 αB-crystallin is potentially able to maintain desmin filament attachment to the sarcomere. However, a longer period of cytochalasin D treatment led to preferential recruitment of phospho-S59 αB-crystallin on collapsing actin filaments at the exclusion of desmin network that aggregated on the periphery of collapsed actin and at the cell membrane (Fig. 7C). This result explains how a longer period of actin polymerization defect alters the protection of desmin by αB-crystallin.
DISCUSSION

Natural history of DCM following SRF inactivation

Although different etiologies lead to DCM, this type of cardiac remodeling is characterized by major changes in the cardiomyocytes contractile apparatus and energetic metabolism. Taking advantage of the precise temporal control on pathogenesis that is provided by the inducible Cre-LoxP technology, we carefully analyzed the natural history of the DCM disease and we propose the following model. In the basal physiological state, SRF, a transcription factor regulated by important cardiac signaling pathways, maintains a balanced expression of MCK and sarcomeric actin gene expression ensuring the adequacy between phosphocreatine flux and ATP demand by the actomyosin complex (Fig. 8A). Following SRF inactivation, MCK, actin and Tpm1 mRNA are down-regulated but the more labile MCK protein is down-regulated first at the onset of DCM, affecting the ATP-dependent stability of actin filaments which in turns triggers the recruitment of phosphorylated αB-crystallin and an increase of desmin (Fig. 8B). With the progression of the disease, further alterations in the thin filament due to down-regulation of actin, tropomyosin and troponin proteins as well as complete fall of MCK result in a quasi-exclusive recruitment of phosphorylated αB-crystallin at the deficient thin filaments. As a consequence of impaired αB-crystallin protection and increased oxidative stress associated with mitochondrial dysfunctions, desmin is exposed to AGE modifications, which, together with the alteration of actin thin filaments, further disrupt the anchorage of desmin intermediate filaments to the sarcomere (Fig. 8C).

Importance of MCK regulation by SRF for thin filament stability

The failing heart is characterized by a metabolic remodeling that includes a fall in CK activity and a decrease in fatty acid oxidation while the potential increase in glucose uptake and utilization driven by the [ADP] and [AMP] increase is not sufficient to compensate for overall decreases in the capacity for ATP supply (3,20). The reduction of the contractile reserve is also due to alterations in the pool of contractile proteins, through either down-regulation of expression, shift of isoforms or post-translation modifications (1). SRF activity is down-regulated in the failing human heart (7,8) and is repressed by muscle-specific RING Finger ubiquitin E3 ligases Murf1 and Murf2 which are activated in heart failure (6,24). Our study highlights the fact that SRF is an essential co-regulator of MCK gene and genes encoding sarcomeric proteins in the adult heart as predicted by previous in vitro studies (25). Altogether, these studies suggest that deregulation of SRF activity may play an important role in the pathogenesis of heart failure, a hypothesis that will be worth testing in the future on other models of heart failure like experimental infarcts or transverse aortic constriction.

Interestingly, MCK is unique among CK isoforms because it is intimately linked to the myofibrillar compartment and catalyzes the rapid transfer of a phosphate moiety from the mtCK-derived phosphocreatine to ADP, generating ATP right at the site of maximal consumption by the actomyosin complex (26). Here, we show that inhibition of CK-mediated ATP transfer in cultured cardiomyocytes results in a rapid loss of polymerized actin as previously described for other cell types (27,28) suggesting that the early down-regulation of MCK in vivo plays a major role in actin filament instability. Though, we show that at least one other target of SRF, α-tropomyosin, contributes to the loss of polymerized actin and we cannot exclude that other targets of SRF involved in actin polymerization may have not been detected in the 2D-DIGE experiments. The fact that the MCK KO mice display only minor alterations of cardiac parameters in the basal state seems to contradict the apparent importance of MCK down-regulation in the SRFKO heart (29). However, it should be noticed that in the MCK KO, the gene product is missing since the beginning of the embryonic development and major adaptive remodeling takes place in this mutant that allows a direct transfer of mitochondria-derived ATP to the myofibrils, including an increased number of mitochondria and a closer interaction of the mitochondria with myofibrils which are thinner than in control mice (30). In the inducible SRFKO as in the patients experiencing heart failure, the loss of MCK takes place in an adult context in which mitochondrial and myofibrillar networks are already organized in an adult specific.
conformation. This hypothesis is in agreement with the idea that the reduction of PCr/ATP ratio is a primary defect in heart failure and is a cause of contractile dysfunction rather than a consequence (31).

**Role of αB-crystallin chaperone activation in relation with actin and desmin filament organization in DCM**

Our results showed that the loss of MCK activity and the subsequent down-regulation of F-actin, triggered a response characterized by phosphorylation of the αB-crystallin chaperone and a striking increase of one of its client protein, the desmin intermediate filament (Fig. 8B). Desmin filaments interconnect myofibrils and mitochondria in the sarcoplasm and mutations in desmin as well as in αB-crystallin have been directly involved in the development of DCM (32-35). Perturbation of cytoskeletal networks with drugs including cytochalasin D that depolymerizes F-actin, trigger the activation of the p38/MAPKAP2 kinase pathway in a PKA dependent manner and lead to the specific αB-crystallin phosphorylation at serine 59 (22,23). We found that desmin is stabilized at the post-transcriptional level in the SRF\(^{HKO}\) DCM. Phosphorylation of serine 59 on αB-crystallin has been shown to favor actin filaments stabilization in H9C2 rat cardiomyocytes (23). Our in vivo and in vitro analyses are in agreement with a protective role for phosphorylated αB-crystallin for both actin and desmin filament network, at least in the initial stage of the disease when αB-crystallin co-distributes with both proteins (D25). Heat shock priming, which activates αB-crystallin phosphorylation, protected actin from cytochalasin D induced disruption but also desmin. HS priming also protected desmin from glyoxal induced disorganization. When phosphorylation of serine 59 on αB-crystallin was blocked by an inhibitor of the p38 MAPK, which was shown to be upstream of αB-crystallin phosphorylation (22), it suppressed the protective effect of the heat shock. Although p38 MAPK inhibition has certainly a broader impact than simply inhibiting αB-crystallin phosphorylation, we note that it does not impact desmin organization in the basal state. Finally, we showed that upon prolonged cytochalasin D treatment, phosphorylated αB-crystallin was recruited at collapsing F-actin while desmin was excluded from this interaction. Hence, in this specific model of SRF-related DCM, αB-crystallin appears to be able to provide a limited transient protection to desmin network because it is diverted to deficient actin filaments. This observation raises the hypothesis that desmin filament network could present similar defect in other forms of DCM associated to thin filament defect (e.g; nemaline myopathy) because of the preferential recruitment of αB-crystallin at the deficient actin filaments.

**Desmin modification by AGE in DCM**

We discovered that desmin is a preferential target of AGE in the heart in SRF-related mouse model of DCM as well as in human patients. Our hypothesis is that the alteration of the energy transfer due to the early down-regulation of phosphocreatine shuttle, leads to altered mitochondrial functions and increased oxidative stress. Mitochondrial dysfunction and oxidative stress is likely further increased with the down-regulation of mitochondrial enzymes involved in fatty acid β-oxidation and glucose metabolism like the β-enolase at D45. This, in turn, can increase formation of highly reactive dicarboxyls like glyoxal and methylglyoxal coming from triose sugars oxidation or lipids peroxidation and subsequent AGE adduct formation on proteins. The reason why desmin is a preferential target of AGE in the cardiomyocytes remains elusive for now. Partial colocalization of AGE adducts and desmin aggregates was reported in skeletal muscle fibers from patients suffering from desminopathies suggesting that desmin is prone to AGE adducts modifications in the context of muscle disease (36). Interestingly, vimentin, another type III intermediate filament protein closely related to desmin, is also a preferential target of AGE in senescent fibroblast where it participates to aggresome formation (37,38). Desmin and vimentin play an important role in mitochondria distribution in muscle cells and through their interaction with tubulin regulate mitochondrial affinity for adenosine diphosphate (ADP), V\(_{max}\) of oxygen consumption, and creatine kinase coupling with the adenine nucleotide translocator (39-41). The close vicinity of desmin and mitochondria may explain why desmin is targeted by oxidative stress-derived AGE. We demonstrated that AGE formation on desmin is deleterious for desmin filament network organization. AGE modification of desmin occurs at an intermediate stage in the establishment of DCM (D45) and we speculate that it plays a role in the further deterioration of the cardiomyocyte architecture.
that take place in the late stage of the disease between day 50 to 60. AGE induced disorganization of desmin is likely to create a vicious circle in the course of DCM, aggravating myofibrillar disarray and mitochondrial dysfunction and leading to reduced contractile power and energetic reserve. Hence, it would be interesting in the future to analyze the effect on desmin glycation of therapeutic compound that inhibit AGE formation like pyridoxamine, a vitamin B6 derivative, or Alagebrum (ALT-711), an AGE cross-link breaker (sugar/protein crosslink), which has been used in clinical trials (42,43). Succeeding to limit AGE formation on desmin in DCM patients may help to preserve cardiac functions. In addition, we note that desmin was also modified by phosphorylation at serine 28. Phosphorylation of several serines at the N-terminus head domain of chicken desmin has been shown to inhibit the formation of intermediate filaments (44,45) and phosphorylated desmin isoforms increase in human myofibrillar myopathies (46,47). Therefore, the phosphorylation of serine 28 on desmin could be a specific marker of altered desmin filament network in the context of cardiomyopathy.

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Abbreviations list: AGE: advanced glycation end-products; 2D-DIGE: 2D-Differential In Gel Electrophoresis; DCM: dilated cardiomyopathy; FC: fold change; ID: intercalated disk; HNE: 4-hydroxy-2-nonenal lipid peroxidation product; HSP: heat shock proteins; MALDI-TOF, matrix-assisted laser desorption/ionisation-time-of-flight; MCK: muscle creatine kinase; MLP: muscle lim protein; MS: mass spectrometry; m/z: mass-to-charge ratio; SRF: serum response factor; SRFHKO: SRF heart knock-out.
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FIGURE LEGENDS

Fig. 1: 2D-DIGE analysis of cardiac proteins from controls and SRF$^{HKO}$ mice
(A) Coomassie Brilliant Blue stained 2D-gels used for spot identification by MALDI-TOF/TOF. Whole ventricular protein preparations were separated by isoelectric focusing (Immobiline DryStrip gels pH 3–11NL) and apparent molecular weight (nominal range 15,000 to 100,000). Down-regulated spots (green) and up-regulated spots (red) were picked on control (left) and mutant gel (right), respectively, and are listed in supplementary table S1. Additional spots identified by MS only and/or with low FC (black) are listed in table S2. (B-C) Decyder 7.0 visualization of cyanine-labeled spots and 3D spots volume: (B) αB-crystallin spots at day 25. (C) Desmin spots at day 25. Images are representative of n=4 gels.

Fig. 2: Reduced actin level and polymerization in the SRF$^{HKO}$ cardiomyocytes.
(A) Slow progressive reduction of sarcomeric α-actin and α-tropomyosin 1 (α-Tpm1) is detected by Western-blot analysis of cardiac tissue cytoskeletal extracts (5 μg/lane) at D25 and D45. β-actin is increased at D45. Loading controls: Histone-3 and GAPDH. Right: quantification of band intensities normalized on loading controls. Data are expressed as fold change over mean control value ± s.e.m. (B, C, E, F) Immunofluorescent labeling of cardiomyocytes isolated from control (B, C) and SRF$^{HKO}$ hearts at D25 (E, F) with TRITC-phalloidin (red) and SRF antibody (green). C and F: higher magnification of representative cardiomyocyte from each genotype. Note the presence of SRF in nuclei of control cardiomyocytes in C. (D, G) immunofluorescent labeling of controls (D) and SRF$^{HKO}$ hearts at D25 (G) with β-actin in green. (I) Quantification with image J of phalloidin fluorescent signal (integrate intensity), cardiomyocytes length and width (μm), N=30 for each group. Data are given as mean ± s.e.m. □ : control ■ : SRF$^{HKO}$. * p < 0.05, *** : p < 0.001. Scale bar: 50μm in B and D, 15μM in C and E.

Fig. 3: Induction of phosphorylation on αB-crystallin in SRF$^{HKO}$ hearts. Upper panel: Western-blot analysis of control and mutant heart at D25 and D45 with anti-αB-crystallin (CryAB), -phospho-S45-CryAB, -phospho-S59-CryAB and –GAPDH antibodies. Lower panel: quantification of Western-blot (N=3 for each group). Band intensities are normalized on GAPDH as a loading control. Ratios are expressed as fold change over mean control value ± s.e.m. □ : control ■ : SRF$^{HKO}$. * : p ≤ 0.05.

Fig. 4: AGE formation on desmin in SRF$^{HKO}$ induced DCM and human DCM. (A) 1D Western-blot analysis of cardiac protein extracts from SRF$^{HKO}$ with anti-desmin, -AGE and -GAPDH antibodies. (B) 2D Western-blot of anti-desmin, -phospho-serine and -AGE antibodies at D45 after SRF inactivation. (C) Upper panel: Western-blot on human DCM extracts with anti-desmin, -AGE and -GAPDH antibodies. Controls: extracts from normal human ventricles; DCM: heart extracts from patients with dilated cardiomyopathy. Lower panel: quantification of desmin/GAPDH ratios and AGE/desmin ratio of control (n=3) and patient (n=7). □ : control ■ : DCM. * : p ≤ 0.05.

Fig. 5: Changes in αB-crystallin and desmin localization in SRF$^{HKO}$ cardiomyocytes. Immunofluorescent co-staining for total αB-crystallin (CryAB) (A-C) or phospho-S59 CryAB (D-I) in green and F-actin using phalloidin-TRITC (A-F) or desmin (G-I) in red on heart cryosections at D25 and D45. (A, D, G) control, (B, E, H) mutant D25, (C, F, I) mutant D45. Right insets: higher magnification of boxed area with separate channels and merged picture. Total CryAB increases at D25 (B) both on actin filaments in the sarcomeres and at the level of intercalated disks (id) while actin start to depolymerize. At D45 (C) Total CryAB is mainly increased at the ID although signal is still detected at the level of the Z-disk (right inset). (D) Phospho-S59 CryAB signal is weakly detected at the ID in the control (D) but increases strongly in both the sarcomere and ID at D25 (E). Phospho-S59 CryAB is detected only in ID at D45 (F) where it colocalizes with weakly stained actin filaments connecting the last sarcomeres to the ID (inset, white arrowheads). Global desmin signal is increased in mutant at D25 (compare H to G) but desmin trends to accumulate close to the sarcolemma at the
periphery of sarcomeres and ID (white arrows) rather than in the center of the myofibrils (*, inset in H). This phenomenon is amplified at D45 (I, inset). Scale bar: 20μm.

**Fig. 6: Inhibition of creatine kinase leads to desmin network disturbance, actin depolymerization, and AGE formation on desmin:** Neonatal rat cardiomyocytes were transfected at day 5 after plating with siRNA against muscle creatine kinase (MCK), tropomyosin (Tpm1) or scramble sequence (Scr) as a control. Analyses were performed at at day 8 of culture. (A) Analysis of mRNA expression of siRNA target by RT-qPCR. (B) Western-blot analysis with anti-sarcomeric α-actin and GAPDH antibodies in the insoluble versus the soluble fraction and (C) quantification of F/G-actin ratio from western blot. (D-F) Fluorescence staining for F-actin (phalloidin-TRITC), G-actin (DNAse1-Alexa488) and nucleus (Hoechst). (G) Quantification of the phalloidin and DNase1 signal integrate density was measured on individual cardiomyocytes (N>40). (H) Western-blot analysis with anti-desmin, -AGE and –GAPDH antibodies and (I, J) quantification of desmin and AGE ratio. (K-M) Fluorescence staining for desmin (red) and nucleus (Hoechst). (N) Quantification of desmin network striation: Mean fast Fourier transform of desmin signal along a central line in the cardiomyocyte gives mean amplitude A as a function of spatial frequency (μm⁻¹). The major peak is centered around 0.62 μm⁻¹ (i.e. every 1.6 μm). All ratios are expressed as fold change over mean control value ± s.e.m. *: p ≤ 0.05. ***: p≤ 0.001.

**Fig. 7: Pre-activation of αB-crystallin phosphorylation protects desmin network from short-term treatment with glyoxal or cytochalasin-D** (A) Immunofluorescent co-staining for phospho-S59 αB-crystallin (orange), desmin (green) and F-actin (phalloidin-TRITC, red). Neonatal rat cardiomyocyte were pre-treated or not by SB202190 (P38 inhibitor) during 6 hours, then there are treated or not by heat shock during 30 min at 42°C, finally cardiomyocytes are treated by glyoxal or Cytochalasin D. (B) Mean fast Fourier transform of desmin signal along a central line in the cardiomyocyte gives mean amplitude (A) as a function of spatial frequency (μm⁻¹). The major peak is centered around 0.6 μm⁻¹ (every 1.7 μm). (C) Merged immunofluorescent staining for phospho-S59 αB-crystallin (orange) with F-actin (phalloidin-TRITC, red in left panel) and desmin (green in right panel) after 6 hours of cytochalasin D treatment. Arrows point to the aggregate of phospho-S59 αB-crystallin (orange) that colocalizes with collapsed actin (red, inset in left panel) but not desmin (green, inset in right panel).

**Fig. 8: Model for the progressive establishment of DCM and heart failure following SRF inactivation.** (A) Normal molecular cytoarchitecture of a cardiomyocyte in the basal state of heart. SRF maintains the transcription of genes encoding contractile proteins, sarcomeric α-actin and MCK. (B) After 25 days of SRF deletion, cardiac-actin and MCK mRNA are strongly decreased. The more labile MCK protein is decreased and the rapid phosphate transfer from phosphocreatine to ADP is deficient reducing energy delivery to the myofibrillar F-actin. αB-crystallin is phosphorylated and accumulates on actin filaments both in the sarcomere and at the intercalated disk (ID). Desmin protein level is increased but maintains its striated pattern and connection to the sarcolemma and ID. (C) At 45 days, MCK is further down-regulated and enzymes involved fatty acids β-oxidation decrease causing a general energy failure and oxidative stress leading to AGE formation. Desmin is highly sensitive to AGE and the IF network is fragmented with desmin filaments accumulating under the membrane and periphery of the ID. IDE is severely stretched and phosphorylated αB-crystallin is essentially recruited on remnants of F-actin linking the last sarcomere to the ID.
Table 1. CK isoenzyme profile of activity in control and SRF<sup>HKO</sup> mice at D25

|                   | Total CK | MM       | MB      | BB      | sMit CK   |
|-------------------|----------|----------|---------|---------|-----------|
| Control (N=6)     | 1818±124 | 1270±54.4| 62±10.5 | 56±11   | 430±70.2  |
| SRF<sup>HKO</sup> (N=6) | 1122±90* | 593±34.2 | 51±12.6 | 62±14.7 | 416±72.5  |

MM: MCK dimer activity. MB: MCK- BCK heterodimer. BB: BCK homodimer. sMitCK: sum of sMitCK dimer and octamer activity. *: pval = 1.1.E-3. ¶: pval=1.E-6.
Figure 1

A

Control

pH 3

pH 11

Mutant

B

Control

Mutant

C

Control

Mutant
Figure 2
Figure 3
Figure 4

A) 25 days and 45 days

B) 2-D Western Blot on mutant at 45 days

C) Human DCM extracts

Desmin network disorganization by AGE in DCM
Figure 5

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Figure 6

**A** RT-qPCR

**B**

- siScr
- siMCK
- siTpm1

| Protein       | siScr | siMCK | siTpm1 |
|---------------|-------|-------|--------|
| F-actin       |       |       |        |
| G-actin       |       |       |        |
| GAPDH         |       |       |        |

**C** Ratio F-Actin/G-actin

**D** siScr

**E** siMCK

**F** siTpm1

**G** Phalloidin/DNasel

**H**

| Protein          | siScr | siMCK | siTpm1 |
|------------------|-------|-------|--------|
| Desmin          |       |       |        |
| AGE              |       |       |        |
| GAPDH           |       |       |        |

**I** Desmin/GAPDH

**J** AGE/GAPDH

**K** siScr

**L** siMCK

**M** siTpm1

**N** Desmin striation

AGD in DCM 22
Figure 7

A

| Treated Condition | Without Heat Shock | With Heat Shock |
|-------------------|-------------------|-----------------|
|Not treated DMSO | ![Image](image1) | ![Image](image2) |
|SB202190 | ![Image](image3) | ![Image](image4) |
|Glyoxal | ![Image](image5) | ![Image](image6) |
|CytoD | ![Image](image7) | ![Image](image8) |

B

Desmin striation index

| Treated Condition | Without Heat Shock | With Heat Shock |
|-------------------|-------------------|-----------------|
|Not treated DMSO | ![Graph](graph1) | ![Graph](graph2) |
|SB202190 | ![Graph](graph3) | ![Graph](graph4) |
|Glyoxal | ![Graph](graph5) | ![Graph](graph6) |
|CytoD | ![Graph](graph7) | ![Graph](graph8) |

C

Phalloidin/S59-CryAB

Desmin/S59-CryAB
Figure 8

A. D0 Basal state
- Desmin
- α-B-crystallin
- Myofilaments
- Myosin
- Actin
- Actin mRNA
- MCK mRNA
- F-Actin
- ATP
- ADP
- MCK
- Intercalated disk
- Mitochondria

B. D25 onset of DCM
- Desmin
- Phospho-α-B-crystallin
- Myofilaments
- Myosin
- Actin
- Actin mRNA
- MCK mRNA
- F-Actin
- ATP
- ADP
- MCK
- Intercalated disk

C. D45 advanced DCM - overt HF
- Desmin-Dismin
- Stretched intercalated disk
- Protein Glycation
- Oxidative Stress
- Energy failure
Muscle creatine kinase deficiency triggers both actin depolymerization and desmin disorganization by advanced glycation end-products in dilated cardiomyopathy

Nicolas Diguet, Youssef Mallat, Romain Ladouce, Gilles Clodic, Alexandre Prola, Eva Tritsch, Jocelyne Blanc, Jean-Christophe Larcher, Claude Delcayre, Jane-Lise Samuel, Bertrand Friguet, Gerard Bolbach, Zhenlin Li and Mathias Mericskay

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