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Abstract: Beta-catenin is a component of the intercalated disc in cardiomyocytes, but can also be involved in signalling and activation of gene transcription. We wanted to determine how long-term changes in beta-catenin expression levels would affect mature cardiomyocytes. Conditional transgenic mice that either lacked beta-catenin or that expressed a non-degradable form of beta-catenin in the adult ventricle were created. While mice lacking beta-catenin in the ventricle do not have an overt phenotype, mice expressing a non-degradable form develop dilated cardiomyopathy and do not survive beyond 5 months. A detailed analysis could reveal that this phenotype is correlated with a distinct localisation of beta-catenin in adult cardiomyocytes, which cannot be detected in the nucleus, no matter how much protein is present. Our report is the first study that addresses long-term effects of either the absence of beta-catenin or its stabilisation on ventricular cardiomyocytes and it suggests that beta-catenin's role in the nucleus may be of little significance in the healthy adult heart.

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Stabilised beta-catenin in postnatal ventricular myocardium leads to dilated cardiomyopathy and premature death

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ABSTRACT

Beta-catenin is a component of the intercalated disc in cardiomyocytes, but can also be involved in signalling and activation of gene transcription. We wanted to determine how long-term changes in beta-catenin expression levels would affect mature cardiomyocytes.

Conditional transgenic mice that either lacked beta-catenin or that expressed a non-degradable form of beta-catenin in the adult ventricle were created. While mice lacking beta-catenin in the ventricle do not have an overt phenotype, mice expressing a non-degradable form develop dilated cardiomyopathy and do not survive beyond five months. A detailed analysis could reveal that this phenotype is correlated with a distinct localisation of beta-catenin in adult cardiomyocytes, which cannot be detected in the nucleus, no matter how much protein is present.

Our report is the first study that addresses long-term effects of either the absence of beta-catenin or its stabilisation on ventricular cardiomyocytes and it suggests that beta-catenin's role in the nucleus may be of little significance in the healthy adult heart.

INTRODUCTION

Beta-catenin was initially characterised as an adherens junction protein (for review see e.g. [15]) and subsequently established as the central player of the canonical Wnt signalling pathway, which plays a major role in the development of cancer, particularly colon cancer [46]. In addition, beta-catenin signalling seems to be crucial for the maintenance and expansion of different stem cell niches, not only in the gut but also e.g. in skeletal muscle [34]. During heart development beta-catenin seems to play a dual role: initially it is essential for mesoderm specification [17], however at later stages beta-catenin signalling seems to affect cardiac mesoderm differentiation negatively [27]. Therefore beta-catenin seems to be a molecule that regulates proliferative behaviour rather than differentiation. This is difficult to reconcile with the fact that beta-catenin is highly expressed in adult cardiomyocytes, which are generally thought to be postmitotic. Beta-catenin is a major constituent of the intercalated disc, the specialised type of cell-cell contact in the heart, and its expression has been shown to be upregulated in dilated cardiomyopathy [35].

Since beta-catenin knockout mice die even before the formation of a heart [17], conditional knockout strategies were employed to study beta-catenin function. Several mouse strains have been generated using an inducible strategy in combination with the alpha-myosin heavy chain promoter and under these short-term conditions the lack of beta-catenin is well tolerated, partially due the upregulation of the expression of gamma-catenin (plakoglobin) [2, 5, 51]. However, embryonic lethality was observed when a non inducible alpha-myosin heavy chain promoter was used to drive Cre

expression [49], while heterozygous animals displayed neither a structural nor a functional phenotype at least under base line conditions [37].

Since the turnover of beta-catenin is regulated by phosphorylation by GSK3-beta at its N-terminus, deletion of exon 3, which removes these serine and threonine residues creates a stabilised version of beta-catenin [20]. Inducible expression under the control of the alpha-myosin heavy chain promoter revealed a failure to undergo adaptive cardiac remodelling [2]. This is in contrast to observations by other groups, demonstrating that stabilisation of beta-catenin by different approaches positively affects hypertrophic growth of cardiomyocytes [5, 19]. In addition, it was shown that adenoviral-mediated overexpression of beta-catenin enhances the survival of cardiac cells following myocardial infarction due to the activation of anti-apoptotic pathways [18].

No study exists so far that would examine the effect of a long-term interference with beta-catenin expression levels on the heart. Therefore we created conditional knockout mice that carry a beta-catenin deletion specifically in the ventricular myocardium using the MLC2v-Cre strain [3, 4] and induced consistent over-expression of beta-catenin by crossing mice with floxed beta-catenin exon 3 with the MLC2v Cre strain [4, 20]. The careful phenotyping and subcellular analysis of both mouse strains shows that while the lack of beta-catenin at the intercalated disc is tolerable for heart structure, an excess of beta-catenin is detrimental and leads to dilated cardiomyopathy. Comparison of beta-catenin localisation in cardiac cells at different stages of differentiation showed that nuclear beta-catenin could only be detected in early embryonic cardiomyocytes and in the cardiac cell line HL-1.

We suggest that there is a strict control over the subcellular targeting of beta-catenin in the environment of an adult heart muscle cell and that beta-catenin’s nuclear role may not be relevant in this specific scenario.

MATERIAL AND METHODS

Animal breeding and maintenance

Transgenic knock-in mice expressing Cre recombinase under the control of the myosin light chain 2v promoter [4] were bred with beta-catenin floxed mice [3] to generate animals with a deletion of beta-catenin restricted to the ventricular myocardium (MLC2v$^{Cre/wt}$;beta-catenin $^{flox/flox}$), referred to as cKO animals. In parallel, MLC2v-Cre mice were bred with beta-catenin exon 3 floxed mice [20] to generate animals with a stabilisation of the protein (MLC2v$^{Cre/wt}$;beta-catenin exon 3 $^{flox/flox}$) referred to as cΔex3 mice. Due to different parental lines the animals have a genetically mixed background. Maintenance and animal experimentation were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and followed Swiss Federal Veterinary Office (BVET) guidelines. Genotyping PCR for MLC2v Cre and floxed beta-catenin alleles were performed as described in the original publications [3, 4, 20].

SDS-PAGE and immunoblotting

Preparation of heart samples, SDS-PAGE and immunoblotting were performed as described previously [21].

RNA analysis

Total RNA was extracted from heart tissue with Trizol reagent (Invitrogen, Basel, Switzerland) followed by isopropanol precipitation. RNA was reverse-transcribed with Thermoscript RT-PCR system kit (Invitrogen) according to the manufacturer's instructions. PCR reactions were carried out in 50 μl volumes with a set of gene specific primers. The amplification products were run on standard agarose gels, isolated and sequenced to ascertain accurate gene amplification.

Isolation of adult cardiomyocytes

After cannulation of the aorta, the heart was mounted on a Langendorf setup with a flow rate of 2 ml per minute. Tyrode's solution (137 mM NaCl, 5.4 mM KCl, 20.5 mM MgCl, 1.8 mM $CaCl_2$, 11.8 mM Na-HEPES, 10 mM glucose; pH 7.4) was used for washing out the blood. After this, the heart was perfused with calcium-free Tyrode (130 mM NaCl, 5.4 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 6 mM Na-HEPES; pH 7.2) for about 6 minutes followed by perfusion with 0.1 mg/ml of Blendzyme 3 (Roche Diagnostics, Rotkreuz, Switzerland) dissolved in calcium-free Tyrode. This solution was recirculated for 12 minutes. After washing with calcium-free Tyrode supplemented with 0.18 mM $CaCl_2$ for about 15 minutes, the ventricles were removed, gently minced and agitated in a beaker until a cloudy cell suspension was obtained. Cells were allowed to settle and were washed twice with the same solution. After the isolation, 100 μlitres of cell suspension were immobilised on

gelatinised microscope slides by centrifugation at low speed with a Cytospin apparatus (Shandon Southern Inc, Pittsburgh, USA). Z-stacks of isolated immunostained cardiomyocytes were taken by confocal microscopy and volume rendering and volume calculation was made using the Surpass® module of Imaris (Bitplane AG).

Cell culture and immunofluorescence

HL-1 cells were a kind gift by Dr Katja Gehmlich (University College London) and were grown in Claycomb's medium (Sigma) that was supplemented with 10% fetal cal serum (Sigma), 2mM glutamine, 100 units/μg/ml penicillin/streptomycin and 100 μM noradrenalin (Sigma). Neonatal rat cardiomyocytes were isolated using the Neonatal Cardiomyocyte Isolation System from Worthington (Lakewood, NJ, USA) and maintained as mentioned previously (19). Embryonic mouse heart whole mount preparations and cryosections were prepared as described previously [21]. Isolated cardiomyocytes were fixed for 10 minutes with 4% PFA, permeabilised by incubation with 0.2% Triton X-100 for 10 min and immunofluorescence reactions, mounting of specimens and confocal microscopy using a 63x/1.32NA oil immersion lens were performed as described previously [21].

Subcellular fractionation

Subcellular fractionation was performed on cultured cells and on mouse ventricles as described [23]. Sodium butyrate (SB) was added at a

concentration of 5mM for 12 hours to the cultured cells, while LiCl (10 mM and 20 mM) was added 30 minutes before harvesting the cells. Confluent cells and heart ventricles were washed twice with ice-cold PBS and then disrupted using a Polytron apparatus in 5 ml of buffer B (10 mM HEPES [pH 7.4], 5 mM EDTA), supplemented with 0.32 M sucrose and a protease inhibitor cocktail (Roche Diagnostics). Whole heart homogenate was filtered through 4 layers of cheesecloth to remove large cellular debris. Samples were then centrifuged at 3'800 g for 20 minutes. The supernatant fraction (S1) was clarified by centrifugation at 10'000 g for 20 minutes before centrifugation at 100'000 g for 1 hour. The resulting pellet (P2 fraction) containing the membrane fraction was resuspended in 200 μl of buffer B. The initial 3'800 g pellet (P1) was resuspended in 4 ml of buffer B containing 2.4M sucrose. Nuclei were sedimented by centrifugation at 50'000 g for 90 minutes. The nuclei containing pellet was resuspended in 1 ml of buffer B with 0.32 M sucrose and centrifuged at 3'800 g. The final pellet was resuspended in 50 μl of buffer B. The membrane and nuclear fraction were then mixed 1:1 with SDS sample buffer and separated on SDS-PAGE as described above.

Antibodies

The monoclonal mouse (mM) anti-sarcomeric alpha-actinin (clone E53), anti-vinculin (hVin-1), the polyclonal rabbit (pR) anti-pan-cadherin, anti-beta-catenin, anti-alphaE-catenin, anti-laminin and anti-all actin antibodies were from Sigma. The mM anti beta-catenin (c14), anti-beta1-integrin (c18), anti-plakoglobin (c15) antibodies were purchased from Pharmingen (BD

Biosciences, Allschwil, Switzerland). The mM anti-desmin (D33) was from DAKO (Glostrup, Denmark). The mM anti-alpha-cardiac actin (Ac1-20.4.2) was from Progen (Progen Biotechnik, Heidelberg, Germany). The pR anti-desmoplakin antibody was purchased from Serotec (Oxford, UK). The pR anti-connexin 43 was obtained from Zymed (P.H Stehlin & Co. AG, Basel, Switzerland). The polyclonal goat anti LaminA was from Santa Cruz Biotechnology (Santa Cruz, USA). The pR anti N-RAP was a kind gift from Prof. R. Horowits (National Institutes of Health, Bethesda, USA) The pR anti-MyBP-C antibody was a kind gift from Prof. M. Gautel (King's College, London, UK).

For immunofluorescence stainings, combination of FITC/Cy2, Cy3 and Cy5 conjugated secondary antibodies were used as described previously (19) together with DAPI and Alexa 488/633 phalloidin (Invitrogen) for the staining of nuclei and actin filaments. HRP-conjugated anti-mouse Igs (DAKO, Zug, Switzerland) and anti-rabbit Igs (Calbiochem, Lucerne, Switzerland) were used for immunoblotting.

Statistical analysis

All statistical analyses and tests were carried out using Excel software (Microsoft, Redmond, USA). Data are given as mean +/- standard deviation. Bars in graphs represent standard errors and significance was assed by two-tailed T test with a P value below 0.05.

RESULTS

Generation of ventricle specific deletion and stabilisation of beta-catenin

We used the Cre-lox technology to inactivate or stabilise the beta-catenin gene specifically in ventricular cardiomyocytes. Deletion of beta-catenin (referred to as cKO) was achieved by mating the MLC2v-Cre heterozygous strain [4] with beta-catenin$^{flox/flox}$ mice [3], while stabilisation (referred to as cΔex3) was by crossing heterozygous MLC2v-Cre with beta-catenin exon 3$^{flox/flox}$ animals, which removes 76 amino acids from the N-terminus that contain the four phosphorylation sites, which target beta-catenin for degradation [20]. In both cases offspring with the expected Mendelian frequencies were observed at birth. However, while the cKO animals have a normal life span, all cΔex3 mice die of heart failure between 2.5 and 4.5 months. Determination of the cardiac index revealed that cΔex3 animals developed dramatic cardiac hypertrophy at 2 and 4 months (Figure 1, black columns), whereas cKO animals had a similar HW/BW ratio as controls (Figure 1; see Table 1 for details).

In order to find out, how fast and efficient the deletion of beta-catenin happens in ventricular cardiomyocytes, we stained frozen sections from cKO animals at postnatal day 3 (P3), 1, 2, 6 and 15 months with antibodies against beta-catenin (Supplementary Figure 1). At P3 and 1 month no dramatic difference between WT and cKO animals was observed in the intensity and localisation of the beta-catenin signal. By 2 months the signal for beta-catenin disappeared in the ventricles, indicating that MLC2v Cre leads to efficient

recombination only postnatally and that beta-catenin, once it is integrated at the intercalated disc, may have a remarkable long half-life.

Characterisation of the histological and cellular phenotype of cKO and cΔex3 hearts

To characterise the phenotype of beta-catenin cKO and cΔex3 ventricles, we initially performed hematoxilin-eosin stainings on whole heart cross-sections taken from comparable levels at 4 months of age (Figure 2 A-C). The hearts of cKO animals look similar to WT, however, the hearts from cΔex3 animals display a phenotype of dilated cardiomyopathy. Immunohistochemistry on frozen sections at two months, using two different antibodies against beta-catenin, showed the expected reduction in signal in the cKO and a more intensive staining of the intercalated discs in the cΔex3 hearts. No nuclear signal could be observed with either beta-catenin antibody as demonstrated by the lack of colocalisation with DAPI (Figure 2F and data not shown). The general cytoarchitecture appears to be little affected in either beta-catenin strain since the myofibrils appear normal, when visualised with alpha-actinin staining for the Z-discs and F-actin staining with phalloidin. However, longitudinally cut cardiomyocytes in cΔex3 ventricles appear to have an increased diameter compared to WT or cKO, as delineated by the lateral laminin staining (green signal in Figure 2G, H, I). The overall structural alignment of the myocytes does not seem to be changed though, unlike in other cases of ventricular dilation for example after myocardial infarction [11].

To investigate effects on cell size and structure further, we prepared freshly isolated cardiomyocytes from cKO, WT and cΔex3 mice for immunostainings and volume measurements (Figure 3). The organisation of different components of the sarcomere is indistinguishable between the different beta-catenin animals when we stained for MyBP-C, alpha-cardiac actin and desmin, despite the clear lack of beta-catenin in intercalated discs of cKO cardiomyocytes and the increased signal compared to controls in cΔex3 cardiomyocytes (green signal in Figure 3 A-F). Volume projections revealed that cKO cardiomyocytes have a slightly more irregular shape than WT at 4 months, while the majority of cΔex3 cardiomyocytes are dramatically larger. Cell volume measurements showed that while WT and cKO cardiomyocytes display a narrow Gaussian distribution of their volumes, while cΔex3 cardiomyocytes show a wide distribution of sizes (Figure 3G; for detailed size measurements see Supplementary Table 1). The sizes are in good agreement with previously determined cell sizes in adult mouse hearts and a lack of strict size control was also seen in a well-known mouse model for DCM, the MLP knockout mouse [26]. Comparison of cardiomyocyte populations from cΔex3 animals revealed that the dramatic increase in size always goes hand in hand with increased beta-catenin signal at the intercalated disc, indicating successful Cre lox recombination. A subset of cardiomyocytes display normal sizes and normal intensities of beta-catenin signal, probably representing untargeted cells (left cell in Figure 3H). Again no beta-catenin signal could be detected in the nucleus of cΔex3 cardiomyocytes (Figure 3H).

Is intercalated disc composition affected in cKO or cΔex3 ventricles?

The lack of phenotype in inducible conditional beta-catenin knockout mice may be explained by an upregulation of gamma-catenin (plakoglobin) [51]. To check for alterations in the expression levels of different intercalated disk proteins in our stable conditionally beta-catenin deleted or overexpressing animals, we performed immunoblots on ventricular samples from 2 months (WT, cKO and cΔex3) and 6 months old mice (WT, cKO; Figure 4). The downregulation of beta-catenin expression in ventricular samples of cKO mice compared to controls is already clearly visible at 2 months. A slight upregulation in expression could be seen in the case of alphaE-catenin and plakoglobin in cKO animals, but no changes were apparent for desmoplakin, beta1-integrin, cadherins, vinculin or connexin-43 (Figure 4A, for densitometric quantifications see Supplementary Table 2). Confocal microscopy of intercalated disc structure on immunostained frozen sections also showed no alterations in the localisation of cadherin, desmoplakin or connexin-43 in cKO compared to WT (Supplementary Figure 2). Analysis of cΔex3 ventricles revealed a downregulation of the wildtype form of beta-catenin with a concomitant expression of the truncated, stabilised from (Figure 4B). The signal for the residual wildtype beta-catenin is derived from non-cardiomyocytes, since whole ventricular muscle extracts were used and from the few cardiomyocytes in which Cre recombination has not yet happened. No changes in expression levels were seen for desmoplakin, cadherins, alphaE-catenin, plakoglobin or connexin-43. A dramatic increase was observed for N-RAP, a LIM domain containing protein of the intercalated disc in samples from

cΔex3 mice. This probably reflects the DCM phenotype of these animals, since the upregulation of N-RAP was shown previously to be an early marker for DCM [8].

To characterise the phenotype of ventricles lacking or stably expressing beta-catenin more closely, we analysed the expression of well-known markers of hypertrophy by RT-PCR (Figure 5). In samples from cΔex3 ventricles all markers such as ANF, BNP, alpha-skeletal actin and beta-myosin heavy chain displayed upregulation of expression. No significant upregulation was found for cKO samples. Beta-catenin stabilisation had no effect on the expression of genes that are known downstream targets of Wnt/beta-catenin signalling, since no significant change could be observed in connexin-43, cyclin D1 and c-myc expression in cΔex3 ventricles (Figure 5 and data not shown). The slight increase in fibronectin is due to increased fibrosis in the cΔex3 hearts as demonstrated by immunofluorescence on heart sections (data not shown and increased signal for laminin e.g. in Figure 2I top right). This again suggests that nuclear beta-catenin signalling is not activated in the cΔex3 cardiomyocytes.

Can beta-catenin ever be detected in the nucleus of a cardiomyocyte?

To unequivocally demonstrate the lack of nuclear beta-catenin in cΔex3 ventricular cardiomyocytes we performed subcellular fractionation experiments (Figure 6). Membrane and nuclear fractions were tested for cross-contamination by the presence or absence of cadherin or lamin in the respective fractions by immunoblotting. In both cKO and cΔex3 membrane

samples a downregulation of wildtype beta-catenin is apparent, which is accompanied by the appearance of the smaller stabilsed beta-catenin protein in cΔex3 samples (Figure 6A). No beta-catenin could be observed in any of the nuclear ventricle fractions (Figure 6B). As a positive control, we used LiCl stimulated mouse embryonic fibroblasts (MEFs) and HCT116 cells, where a clear band for beta-catenin could be observed in the nuclear fractions. Sodium butyrate (SB) treatment stabilises beta-catenin at the cell membrane and therefore no nuclear beta-catenin signal is found [47].

We were concerned that the lack of nuclear signal in cΔex3 ventricular cardiomyocytes could be due to a staining artefact, since we would have expected some nuclear beta-catenin at least in a subset of the cells by immunofluorescence. The detection of several cytoskeletal proteins that translocate to the nucleus can require specific fixation conditions [32]. Confocal microscopy revealed that we can detect nuclear beta-catenin with our fixation and staining conditions, but only in HL-1 cells [6] (Figure 7a). HL-1 cells represent a much more immature cardiac phenotype as demonstrated by the staining for the Z-disc protein alpha-actinin, which is only found in premyofibril-like arrangements [38] in these cells and not in proper cross-striated myofibrils like in neonatal rat cardiomyocytes (NRC; Figure 7a). The observed signal is not due to nuclear background since no nuclear signal was detected with cadherin or alpha-catenin antibodies in either cell type and also nuclei in NRC were negative for beta-catenin. Also attempts to enrich for nuclear beta-catenin using lithium chloride stimulation and treatment with leptomycin B to prevent nuclear export failed to reveal significant nuclear signal in NRC (Supplementary Figure 3). We conclude that nuclear beta-

catenin is a phenomenon that is only observed in embryonic or less well differentiated cardiomyocytes such as the HL-1 cells. Comparison of the relative expression levels of cadherin, alpha-catenin and beta-catenin in NRC and HL-1 cells suggest that the dramatically increased expression of alpha-catenin may be responsible for the rapid targeting of beta-catenin to the cell-cell contacts in mature cardiomyocytes (Figure 7b). It was shown previously that the availability of alpha-catenin affects whether beta-catenin is in a molecular form that can be targeted to the nucleus or not [14].

Since these results suggest a developmental and differentiation stage specific nuclear targeting of beta-catenin, we also looked at the localisation of beta-catenin in embryonic mouse whole mount heart preparations obtained at embryonic day 9 (Figure 7c). While beta-catenin targets to nuclear subregions at this developmental stage in cardiomyocytes (arrows in Figure 7c), no nuclear signal is seen for its close relative plakoglobin at the same developmental stage. However, when neonatal mouse cardiomyocytes were analysed for beta-catenin in the nucleus, absolutely no signal could be detected outside of the intercalated disc [35].

In conclusion, while the long-term absence of ventricular beta-catenin is well tolerated in the heart, its stabilisation and concentration at the intercalated disc leads to a dramatic DCM phenotype with the expression of hypertrophic markers and lethality by 5 months. However, this phenotype is not due to increased nuclear beta-catenin in the cardiomyocytes at adult stages and must be caused by the activation of distinct signalling pathways.

DISCUSSION

Aim of our study was to investigate the effects of long-term absence or stabilisation of beta-catenin in the heart. Postnatal deletion of beta-catenin in the ventricle has little effect on cardiac structure and function, while long-term expression of a stabilised form of beta-catenin leads to a phenotype of dilated cardiomyopathy with premature death, despite a lack of beta-catenin in the nucleus. No activation of gene expression of well-known beta-catenin target genes such as connexin-43 or cyclin D1 is detected. These data suggest that the observed phenotype is not due to the untimely activation of beta-catenin signalling, at least not nuclear signalling in the mature heart. This is in good agreement with published data on Wnt reporter animals, where the left ventricle tends to be negative [7, 22, 30, 33]. Wnt signalling is detected in cardiomyocyte populations that arose from the secondary heart field and also in cardiac precursor/stem cells [28]. These observations suggest that canonical Wnt signalling via beta-catenin plays no or little role in the healthy ventricular myocardium. Nuclear beta-catenin in adult cardiomyocytes was only convincingly demonstrated following adenovirus mediated gene transfer of a constitutively active, non-degradable beta-catenin into the heart [18]. In most other cases only indirect readouts of the presence of beta-catenin in the nucleus were taken by fractionation studies of entire heart tissue or by analysis of the activation of downstream target genes, again not using a purified cardiomyocyte population [2, 5]. Recent work by the Engelhardt laboratory has once more pointed out that deductions from whole heart tissue

can be slightly misleading since it may actually be signalling events in cardiac fibroblasts that lead to a particular readout in the entire tissue [44].

One potential explanation for the lack of nuclear beta-catenin in the cardiomyocytes in our cΔex3 animals could be that the deletion of the N-terminus in the stabilised version also removes ubiquitinylation sites that may be relevant for subcellular targeting as shown for e.g. PTEN [45]. However, beta-catenin deletion studies showed that nuclear targeting is mediated by the C-terminus [25]. Nuclear import of beta-catenin requires the interaction with nuclear pore complexes [9] and recently beta-catenin was shown to interact with the nuclear lamina protein emerin [31], which can also be located at the outer nuclear membrane [39]. Emerin and beta-catenin also interact in the heart, where they colocalise at the intercalated disc [48]. Our results show that nuclear beta-catenin is a differentiation stage specific phenomenon in cardiomyocytes and not seen in the healthy adult myocardium, no matter how much beta-catenin would be available in the cells. This specific targeting can be partially explained by the high expression levels of alphaE-catenin in cardiomyocytes, which is decisive for the subcellular targeting of beta-catenin in other cell types [14]. Recently it was shown that beta-catenin turnover is also regulated by PP2A, which rapidly dephosphorylates the protein and prevents proteasomal degradation [43]. PP2A is highly expressed in the heart and can also be found at the intercalated disc in cardiomyocytes [42]. This presents another explanation for the extraordinary stability of beta-catenin in this cell type, taking two months to be cleared from cKO ventricles.

In a previously published inducible beta-catenin targeted mouse strain the lack of cardiac phenotype was explained by an upregulation in expression

levels of plakoglobin (gamma-catenin; [51]). Plakoglobin was shown to act as a counterplayer of nuclear beta-catenin and to suppress canonical Wnt signalling in arrhythmogenic right ventricular cardiomyopathy [12]. However, again this activity seems to be more restricted to islet-positive cardiac progenitor cells originating from the second heart field [29]. Neither the group of Gerdes nor we were able to see nuclear plakoglobin in any of our beta-catenin strains ([51], data not shown).

Mice homozygous for a conditional deletion of beta-catenin using the alpha-myosin heavy chain promoter to drive Cre expression do not survive beyond embryonic day 14.5 [37]. This is in contrast to our cKO mice, where the MLC2v promoter was used and where a normal life-span was observed. The most likely explanation for this difference is that since the MLC2v Cre only leads to postnatal recombination as shown previously in other mouse strains [40, 41, 50], the cardiac cytoarchitecture is well enough established to tolerate the deletion of beta-catenin by then. Interestingly the beta-catenin cKO mice are one of the few mouse strains that bear a conditional deletion of an intercalated disc protein that do not develop dilated cardiomyopathy. For example conditional knockout mice for beta1-integrin, vinculin and alphaE-catenin all display severe DCM by about six months [40, 41, 50]. The deletions are often accompanied by changes in the expression levels of other intercalated disc proteins, with beta1-integrin and cadherin being downregulated in conditional vinculin knockout mice and vinculin downregulated and beta-catenin upregulated in conditional alphaE-catenin knockout mice [41, 50]. This upregulation of beta-catenin expression, which is accompanied by a higher degree of membrane convolution is also typical for

other mouse models of DCM [8]. It is therefore striking that the elevated expression levels of beta-catenin in our cΔex3 animals is sufficient to lead to a dramatic phenotype of DCM. The only other intercalated disc protein that is changed in its expression level in the cΔex3 animals is N-RAP, a LIM domain containing protein at the intercalated disc, which we have previously identified as one of the first marker proteins to become upregulated during the establishment of DCM [8]. Taken together these results suggest that the fine balance of protein composition at the intercalated disc, which takes until adolescence to establish [1, 21] only tolerates interference with expression levels to a certain extent and responds with a DCM phenotype if the balance is perturbed [35]. The extremely convoluted plasma membranes at the intercalated disc that accompany the DCM phenotype are reminiscent of intercalated discs in aged animals [10] and may indicate an inability to cope with mechanical stress, which is also increased due to the alterations in cell shape that are seen. Currently the signalling pathways that relay mechanical stress in cardiomyocytes and result in an altered cellular phenotype are far from well defined. MLP, which has been implicated to sense mechanical stress at the Z-disc [24] may be less a stress sensor rather than a stress transmitter due to its diffuse cytoplasmic localisation, which also includes the intercalated disc [13]. The severity of symptoms of DCM in patients is highly variable even in the case of hereditary DCM with mutations in the same gene (e.g. laminA/C [36]). Since also an extremely heterogeneous onset of the DCM phenotype is observed in human patients with hereditary DCM [16], our cΔex3 animals with their extremely reproducible case history may provide an

ideal tool to investigate the signalling pathways that lead to the establishment of a DCM phenotype more closely.

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Figure Legends

Figure 1: Comparison of the cardiac index of cKO and cΔex3 mice during postnatal development. While at one month the cardiac index shows no significant differences between different strains, it is increased to 8.9 and 9.2 at two and four months in cΔex3 mice. At six months no viable cΔex3 animals remained. *) P< 0.01; n.d. not done; n.a. not available.

Figure 2: Histological analysis at four months and immunohistochemistry at two months show heart dilation and increased thickness of cardiomyocytes in cΔex3 animals. Cryosections of cKO (A, D, G), WT controls (B, E, H) and cΔex3 (C, F, I) hearts were stained with Hematoxilin-Eosin (A, B, C). Immunolabeling with two different antibodies against beta-catenin, a rabbit polyclonal (green signal in D, E, F) and a mouse monoclonal (red signal in G, H, I) showed lack of beta-catenin expression in the case of the cKO ventricles and increased signal at the intercalated discs in cΔex3 mice. Sections were counterstained with antibodies against alpha-actinin (red signal in D, E, F) and DAPI (blue signal in D, E, F) or with

antibodies against laminin (green signal in G, H, I) and phalloidin to visualise F-actin (blue signal in G, H, I). Bar in A equals 1 mm, bar in D equals 30 micrometres.

Figure 3: Analysis of freshly isolated cardiomyocytes demonstrates the dramatic volume increase in a subset of cΔex3 cardiomyocytes. A-F) Freshly isolated cardiomyocytes of cKO (A-A'', B), WT (C-C'', D) and cΔex3 (E-E'', F, H) mice were stained for beta-catenin (green signal) together with myosin binding protein-C (red signal in A, C, E), alpha-cardiac actin (αcA; red signal in A', C', E'), desmin (A'', C'', E'') or F-actin (B, D, F, H), the nuclei in H were visualised with DAPI (blue signal). 3D reconstructions of representative cardiomyocytes (B, D, F; left cell at 2 months, right cell at 6 months in B and D, F is at 2 months; xy and xz projections, respectively) show a slight alteration in cell shape in cKO compared to WT, which is even more pronounced in the cΔex3.

G) cKO and WT cells have a Gaussian distribution around a mean volume of 25,890 and 21,840 micrometres^3. In comparison, cΔex3 cells show a much wider, randomised distribution around a mean of 50,520 micrometres^3. H) Two cells from a single cΔex3 animal with comparatively low levels of beta-catenin (left cell) and a normal volume or a dramatic increase in beta-catenin combined with increased volume but still lacking nuclear beta-catenin (right cell). At least 30 cardiomyocytes were analysed per animal. Bar in A'' and B equals 20 micrometers, bar in H equals 30 micrometres.

Figure 4: Expression of different intercalated disc associated proteins in cKO versus cΔex3 mice shown by immunoblotting of ventricular samples. A) Immunoblots of intercalated disc proteins in cKO and WT animals at two and six months (3 animals each). While beta-catenin levels are decreased to an equal extent at both stages, alphaE-catenin and plakoglobin show a slight upregulation only at six months. The levels of desmoplakin, connexin-43, beta1-integrin, cadherin and vinculin are unaffected. B) The stabilisation of beta-catenin after removal of exon 3 is clearly visible at two months (lower band) while at the same time the WT isoform levels are decreased (upper band). Cadherins, alphaE-catenin and plakoglobin as well as desmoplakin and connexin-43 show unchanged expression levels, N-RAP is dramatically increased. Actin (all isoforms) was used as a loading control in both cases.

Figure 5: The increased cardiac index in cΔex3 mice is accompanied by the expression of hypertrophy markers. Semi-quantitative RT-PCR analysis of total RNA isolated from WT, cΔex3, cKO and MLP knockout mice show that cΔex3 hearts but not cKO show an upregulation of hypertrophy markers such as ANF, BNP, alpha-skeletal actin and beta-myosin heavy chain similar to the positive control, the MLP knockout mouse. Analysis of Wnt/beta-catenin responding genes such as fibronectin and connexin-43 did not show increased expression in the cΔex3 ventricles. Expression of alpha-cardiac actin and GAPDH was used as internal control, the last row (-RT) is the negative control.

Figure 6: Fractionation experiments demonstrate the absence of nuclear beta-catenin in the cΔex3 ventricles. Subcellular fractionation was performed with mouse embryonic fibroblasts (MEF) under different growth conditions, HCT116 (colon carcinoma) cells and ventricles of WT, cKO and cΔex3 mice and followed by immunoblotting. A) Membrane fraction shows decreased beta-catenin (WT) levels in both the cKO and the cΔex3 ventricles and the stabilised shorter beta-catenin form. B) Nuclear fraction shows beta-catenin in MEF cells following LiCl treatment and in HCT116 cells but no signal in cΔex3 samples. Cadherin and lamin antibodies demonstrate the absence of cross-contamination. The two lanes to the right show total lysates to indicate the expected molecular weight of the proteins that were analysed.

Figure 7: Nuclear targeting of beta-catenin in cardiomyocytes is differentiation stage specific. a) Confocal micrographs of neonatal rat cardiomyocytes (NRC, A-F) and HL-1 cells (A'-F') stained with antibodies against cadherin (B, B'), alpha-catenin (D, D') and beta-catenin (F, F') and alpha-actinin (A, C, E, A', C', E'). While the adherens junction proteins are exclusively found at intercalated disc-like structures in NRC, beta-catenin additionally shows nuclear localisation in HL-1 cells. b) An immunoblot shows increased expression of alpha-catenin in NRC compared to HL-1 cells, while cadherin and beta-catenin seem to be expressed at equal levels. c) Emryonic day 9 mouse whole mount heart preparations stained for alpha-actinin (first column, beta-catenin and plakoglobin, respectively (second column), DAPI to visualise the nuclei (third column); the fourth column shows the overlay. Beta-catenin targets to nuclear subregions at this stage (arrows), but is absent from

condensed chromosomes in dividing cardiomyocytes. Plakoglobin is almost exclusively localised to the cell-cell contacts with only faint cytoplasmic but never any nuclear signal.

Supplementary Figure 1: **Beta-catenin protein only becomes fully absent in the ventricle after two months.** Confocal micrographs of frozen sections stained with monoclonal mouse anti beta-catenin from WT (left column) and cKO (right column) mice at postnatal day 3 (P3), one month, two months, six months and fifteen months of age. The beta-catenin signal at P3 and one month is comparable between WT and cKO and only disappears at two months, indicating a slow turnover of the protein at the intercalated disc. Bar in A equals 10 micometres, bar in C equals 20 micrometres.

Supplementary Figure 2: **Intercalated disc organisation is hardly affected by the absence of beta-catenin.** Confocal micrographs of frozen sections of two months old WT (A, B, E, F, I, J) and cKO ventricles (C, D, G, H, K, L) immunostained for cadherins (A, C), beta-catenin (B, D, F, H, J, L), desmoplakin (E, G) and connexin-43 (I, K). Cadherin staining is slightly more pronounced in the cKO (compare C with A), however neither desmosomes nor gap junctions seem to be altered. Occasional residual beta-catenin containing intercalated discs indicate a lack of recombination (arrow in H). Bar equals 20 micrometres.

Supplementary Figure 3: **Nuclear accumulation of beta-catenin following lithium chloride stimulation and inhibition of nuclear export by leptomycin B can be detected in HL-1 cells but not in NRC.** Confocal micrographs of HL-1 cells (top two rows) and NRC (bottom two rows) stained

for beta-catenin and with DAPI to visualise nuclei. Cells were grown either in control medium, in medium supplemented with lithium chloride (LiCl), leptomycin B (LMB) or both. Bar equals 10 micrometres.

Figure 1

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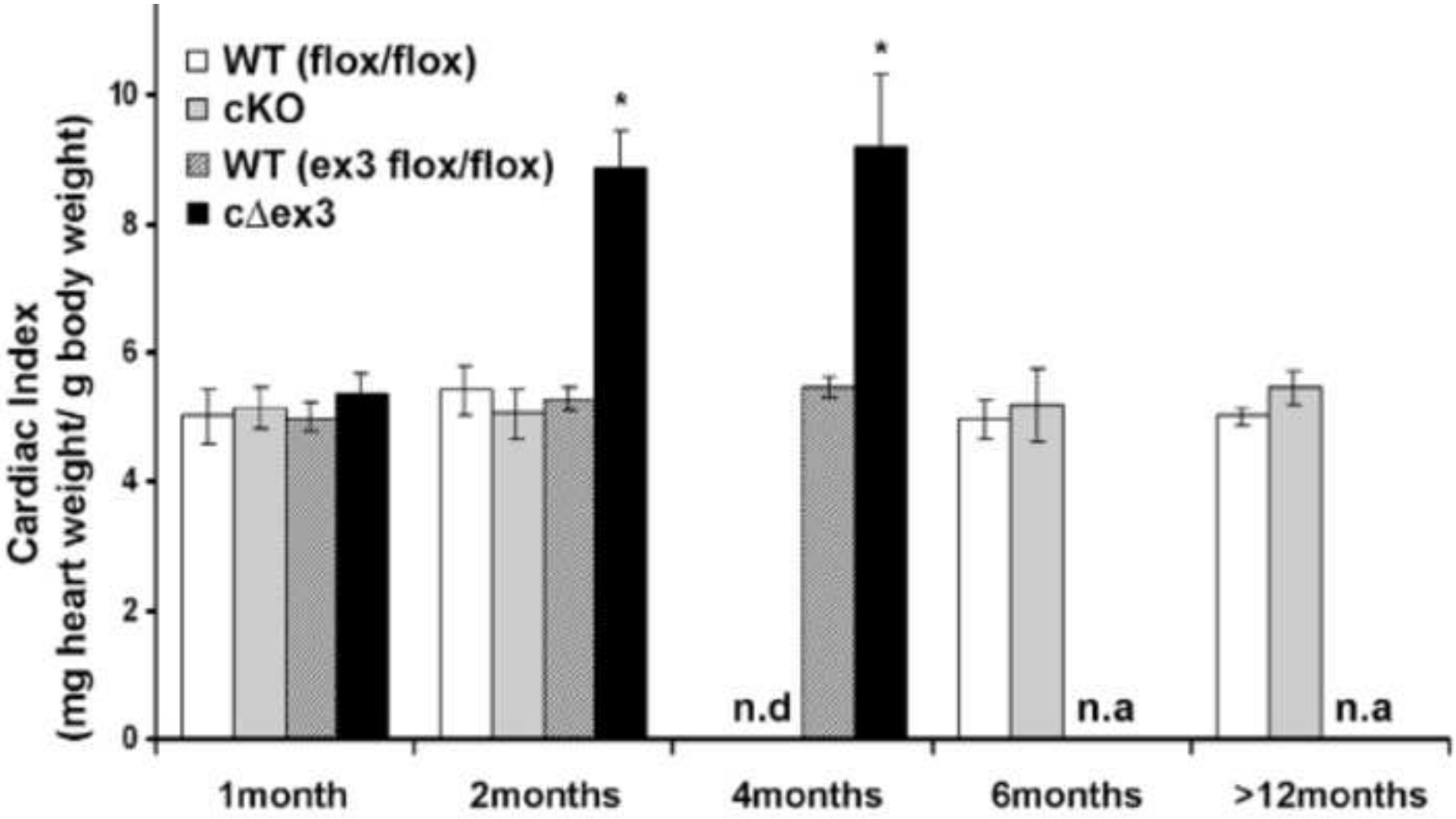

Figure 1

Figure 2

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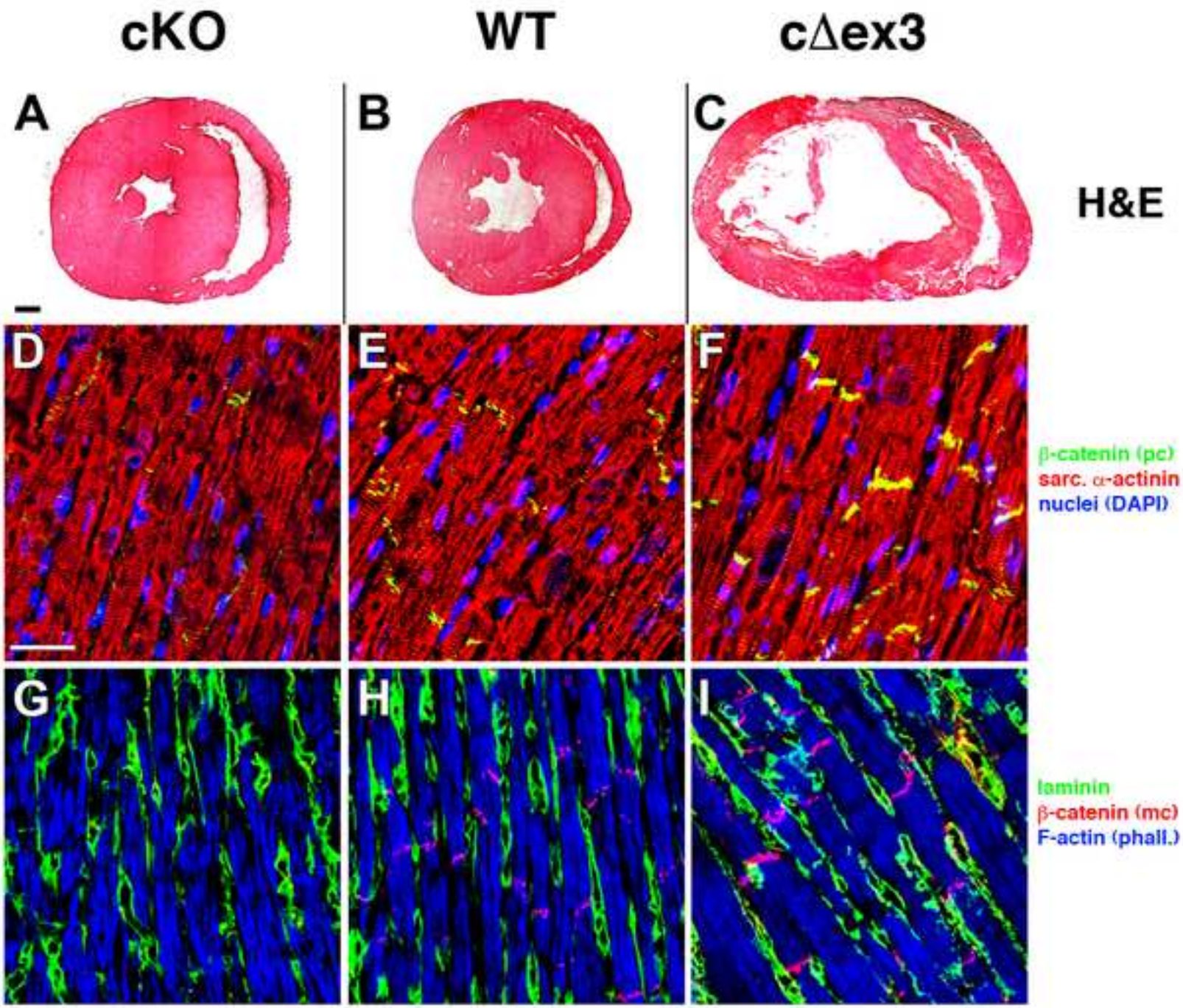

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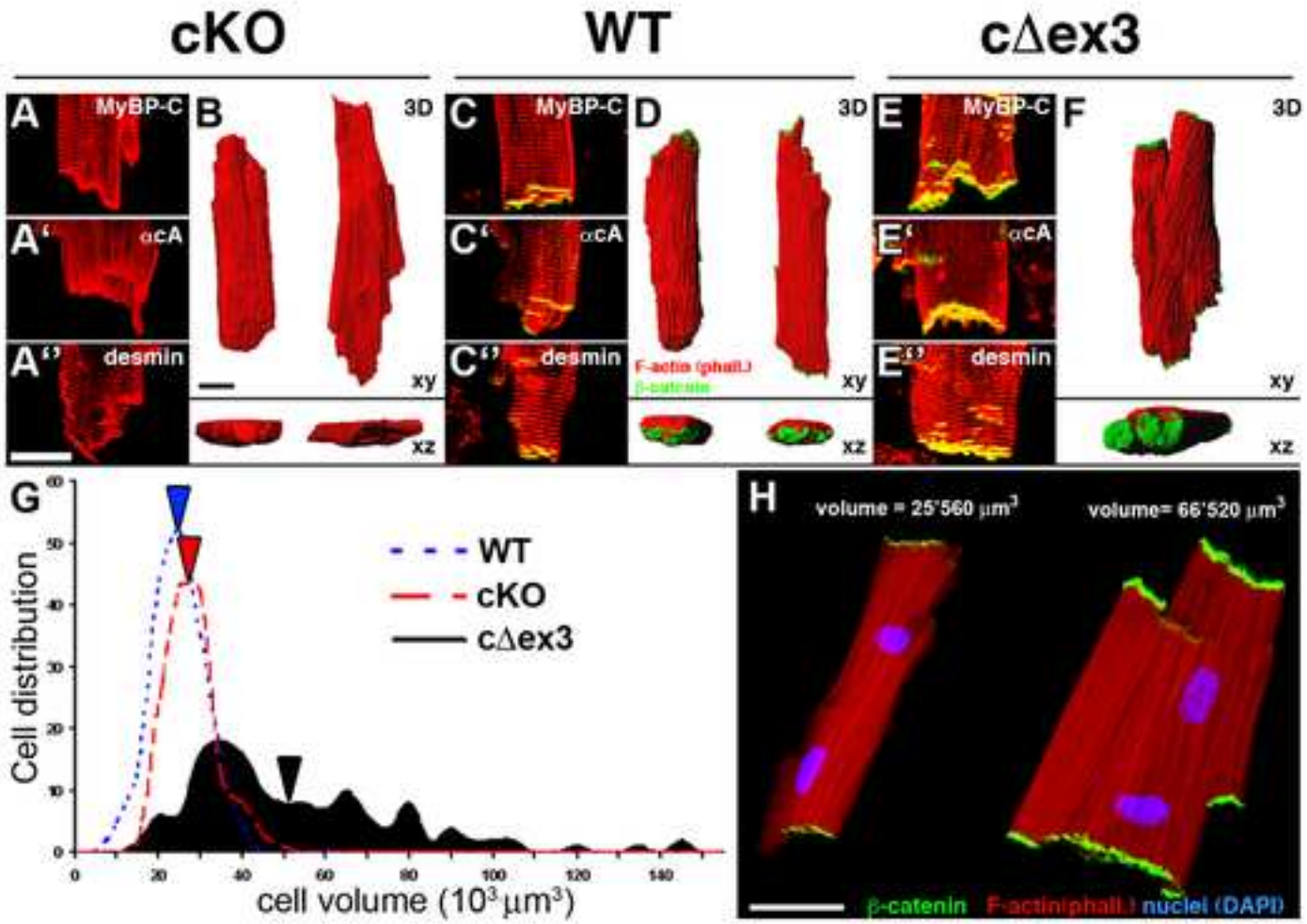

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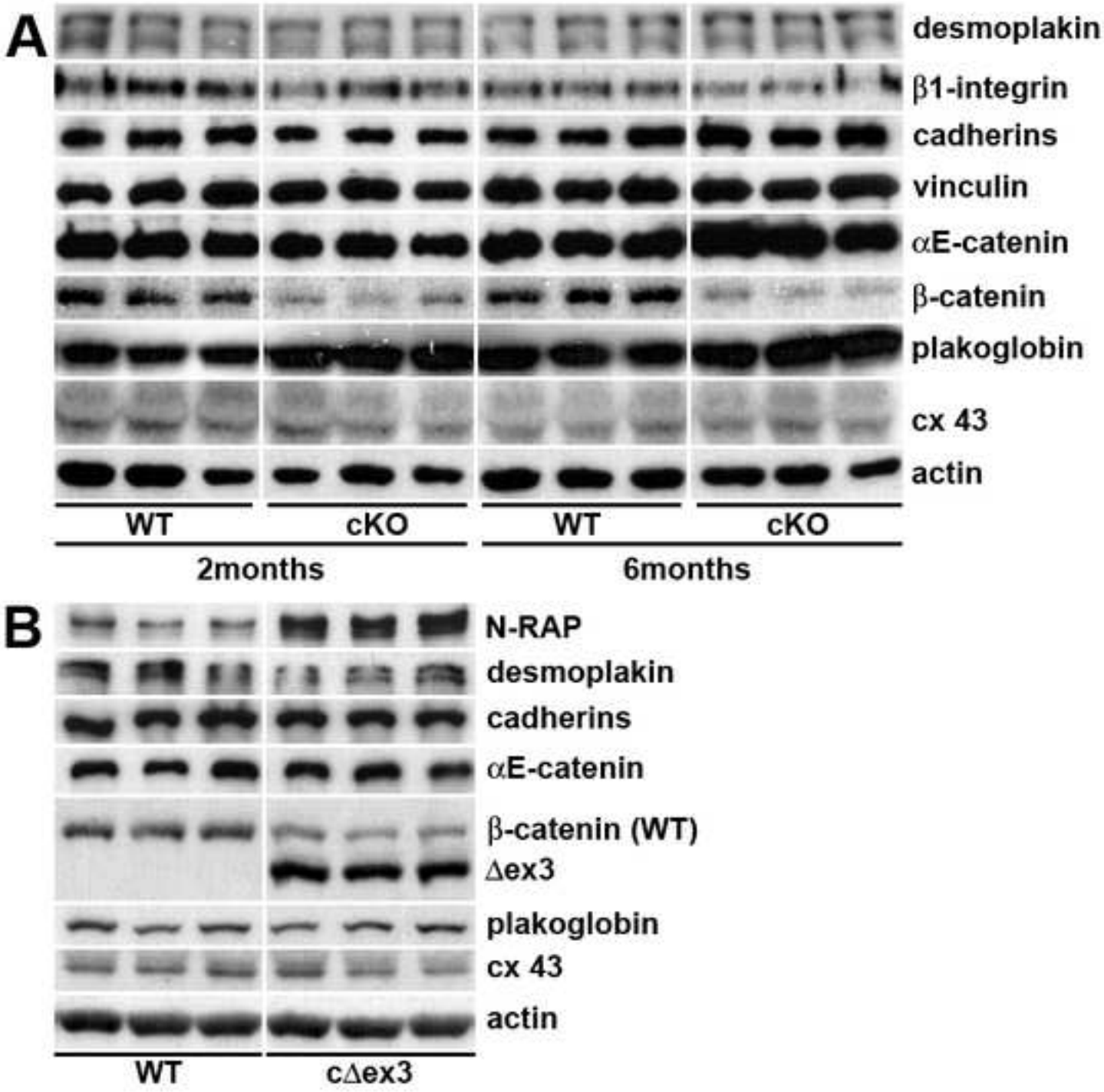

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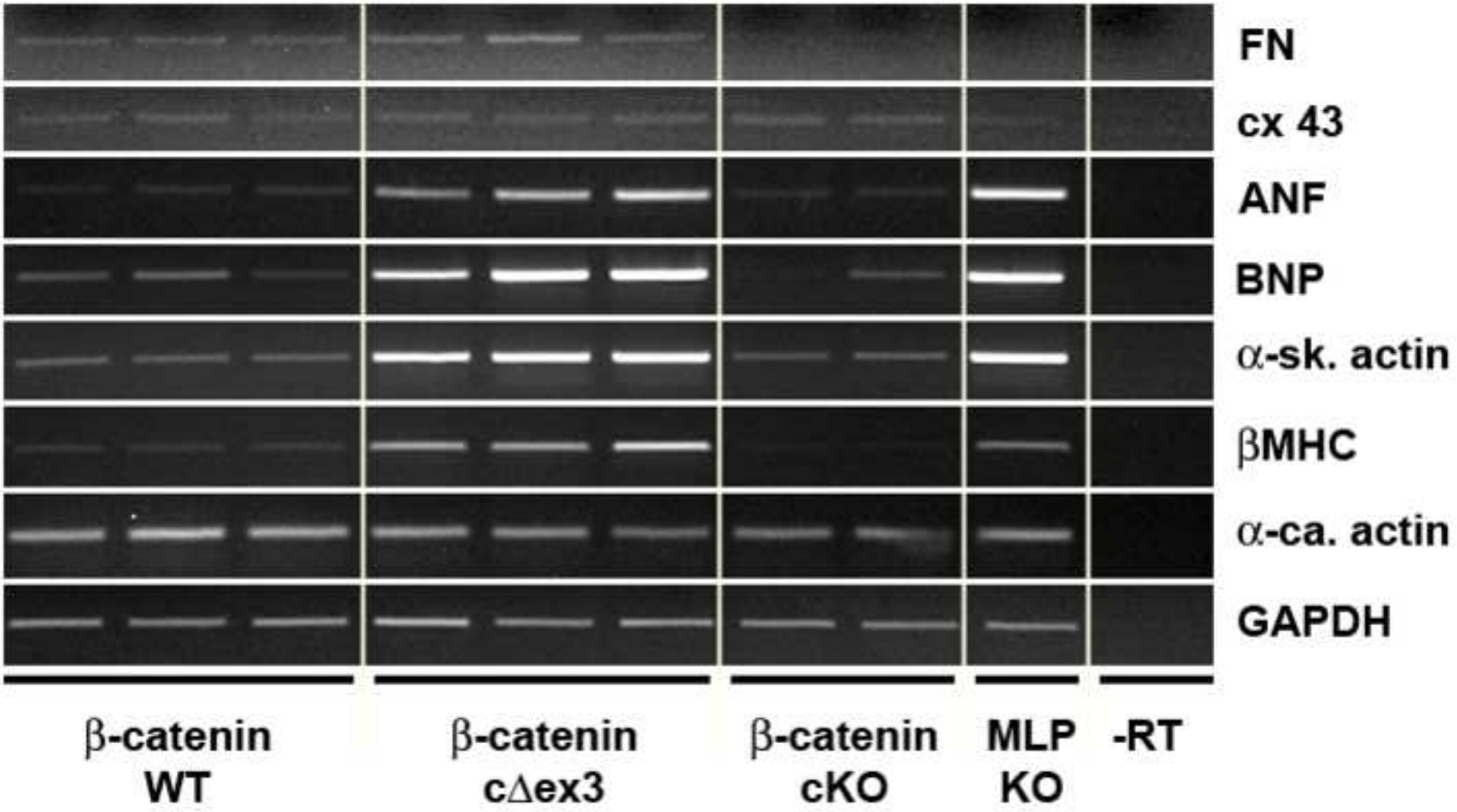

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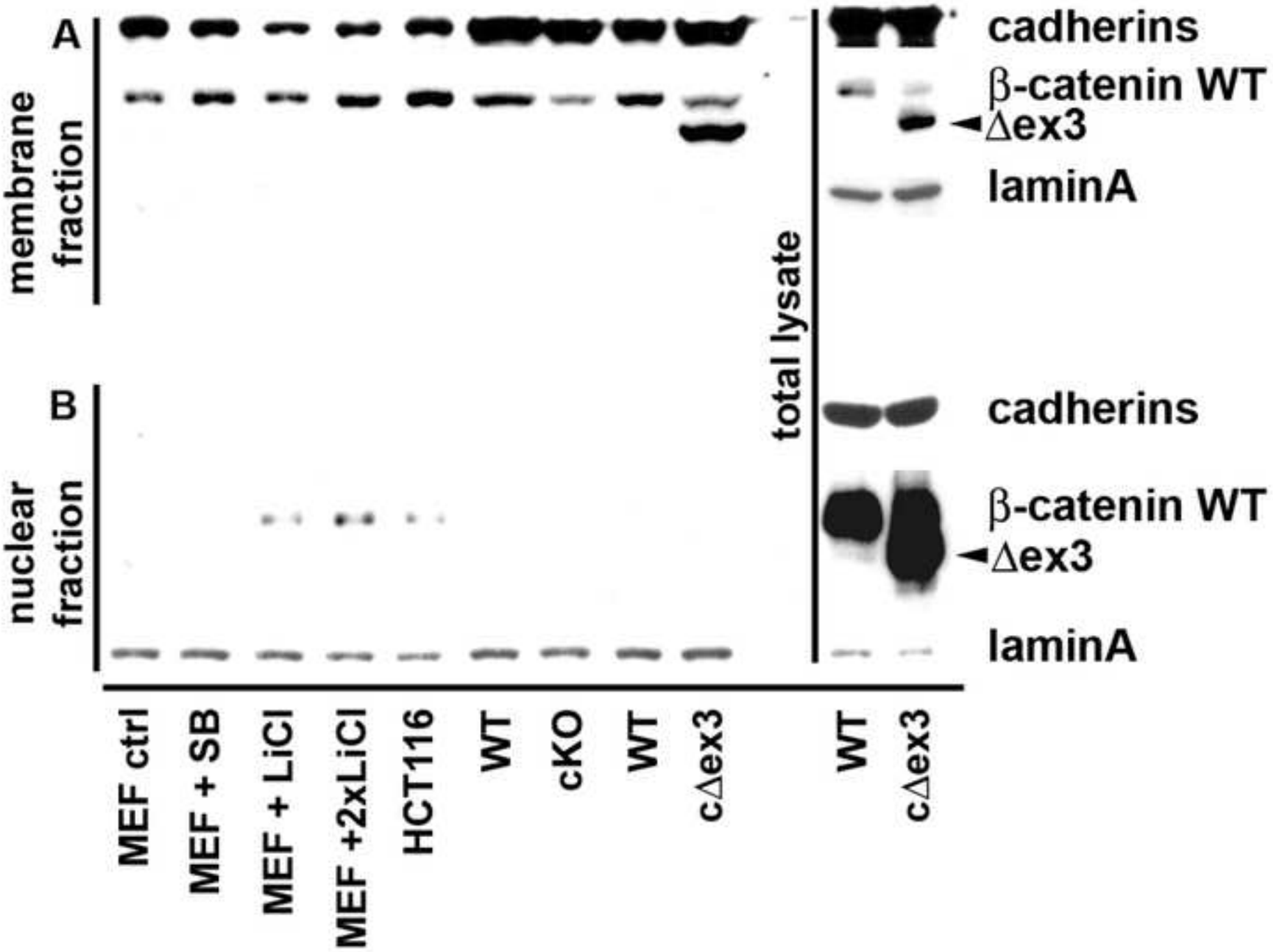

Figure 7

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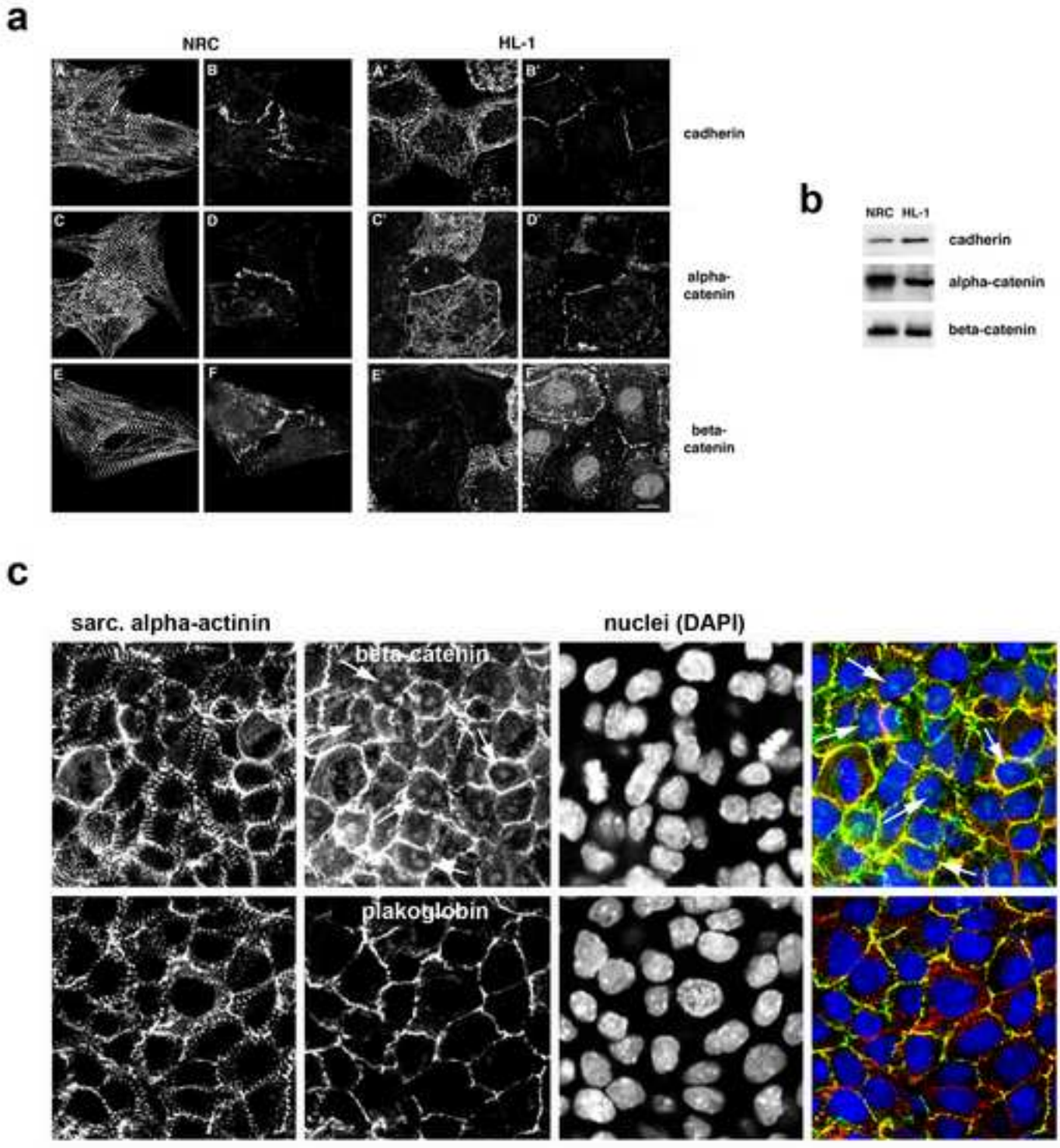

Table 1

Table 1: Echocardiographic data and cardiac index

	cKO measurements		cΔex3 measurements	
	WT §	β-catenin cKO §	WT §	β-catenin cΔex3 §
LVAWD, mm	1.46±0.10	1.45±0.13	1.52±0.11	1.08±0.11*
LVAWS, mm	1.68±0.06	1.77±0.23	1.95±0.10	1.22±0.10*
LVD, mm	3.07±0.11	3.04±0.17	3.38±±0.11	5.43±0.25*
LVS, mm	1.64±0.24	1.60±0.25	1.52±0.06	4.58±0.12*
IVSD, mm	0.28±0.05	0.31±0.03	0.30±0.00	0.28±0.20
IVSS, mm	0.35±0.05	0.39±0.07	0.47±0.07	0.32±0.20
Fractional shortening, %	46.8±6.3	47.4±7.2	55.2±1.1	15.1±5.7*
Cardiac index, mg heart/g body weight	4.90±0.34	4.61±0.42	4.77±0.20	8.75±0.55*

* P< 0.05 compared with WT mice

§) n=8 mice (9-10 weeks old at the beginning of the experiment) in each group with the exception of β-catenin cΔex3 where n=6.

LVAW(D/S): left ventricle anterior wall thickness (at diastole/systole)
LV(D/S): left ventricle (diastolic/systolic) diameter
IVS(D/S): intra-ventricular septum thickness (at diastole/systole)

Fractional shortening: [(LVD-LVS)/LVD]x100
Values are mean ±SD

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