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Calcineurin Protects the Heart in a Murine Model of Dilated Cardiomyopathy

Joerg Heineke^{*,†}, Kai C. Wollert[†], Hanna Osinska^{*}, Michelle A. Sargent^{*}, Allen J. York^{*}, Jeffrey Robbins^{*}, and Jeffery D. Molkentin^{*}

^{*} Department of Pediatrics, University of Cincinnati, Cincinnati Children's Hospital Medical Center, Howard Hughes Medical Institute, 240 Albert Sabin Way, Cincinnati, OH 45229, USA

[†] Medizinische Hochschule Hannover, Klinik für Kardiologie und Angiologie, Excellence Cluster Rebirth, Carl-Neuberg-Str.1, 30625 Hannover, Germany

Abstract

Dilated cardiomyopathy (DCM) is a relatively common disease with a poor prognosis. Given that the only meaningful treatment for DCM is cardiac transplantation, investigators have explored the underlying molecular mechanisms of this disease in the hopes of identifying novel therapeutic targets. One such target is the serine-threonine phosphatase calcineurin, a Ca²⁺-activated signaling factor that is known to regulate the cardiac hypertrophic program, although its role in DCM is currently unknown.

In order to address this issue, we crossed muscle lim protein (MLP) knock-out mice - a murine model of DCM - with calcineurin A β ko mice, which lack the stress responsive isoform of calcineurin that critically regulates the cardiac hypertrophic response. Interestingly, the majority (73%) of the MLP/calcineurin A β double knock-out mice died within 20 days of birth with signs of cardiomyopathy. Ultrastructural examination revealed enhanced cardiomyocyte apoptosis and necrosis in the postnatal myocardium of these mice. The MLP/calcineurin A β double knock-out mice that survived until adulthood showed reduced left ventricular function, enhanced apoptotic and necrotic cardiomyocyte death and augmented myocardial fibrosis compared to various control groups. Antithetically, mild overexpression of activated calcineurin in the mouse heart improved function and adverse remodeling in MLP knock-out mice.

Collectively, these results reveal an important and previously unrecognized protective function of endogenous myocardial calcineurin in a mouse model of dilated cardiomyopathy.

Keywords

Dilated Cardiomyopathy; Calcineurin; Muscle Lim Protein; Apoptosis; Necrosis

Address for Correspondence: Joerg Heineke, MD, Medizinische Hochschule Hannover, Klinik für Kardiologie und Angiologie, Excellence Cluster Rebirth, Carl-Neuberg-Str.1, 30625 Hannover, Germany. Phone: ++49-511-532-3079. Fax: ++49-511-532-5412. Heineke.Joerg@Mh-Hannover.de.

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INTRODUCTION

Dilated cardiomyopathy (DCM) is a myocardial disorder that is characterized by a dilated left ventricular chamber with systolic left ventricular dysfunction. DCM is the third most common cause for the development of heart failure after coronary artery disease and hypertension, and it is the most common reason for cardiac transplantation, which – despite recent progress in the treatment of heart failure – is still the only curative treatment option [1].

DCM can develop as a consequence of certain inflammatory, metabolic or toxic insults. However, about 25–35% of all cases of DCM are caused by genetic mutations [2]. Affected genes encode proteins that function in the calcium regulatory system, the contractile/cytoskeletal apparatus or that reside at the sarcomeric Z-disc. One of the Z-disc proteins that has been implicated in DCM etiology is the muscle lim protein (MLP) [3–5]. Genetic ablation of MLP in mice leads to an adult onset DCM, which closely resembles the human disease, hence MLP knock-out (ko) mice represent a commonly employed murine DCM model system [4,6]. Interestingly, a W4R mutation has been identified in the MLP encoding gene *CSRP3* and was associated with DCM in humans [5]. Perhaps equally interesting is the fact that MLP was shown to be downregulated in the myocardium by about 50% in more common forms of heart failure like ischemic cardiomyopathy [7]. Beside its role in supporting Z-disc structure, MLP was implicated as a stress sensor molecule, which participates in the transduction and translation of mechanical extracellular stimuli into biochemical signals within the cell [5,8,9]. Indeed, we previously demonstrated that MLP interacts with and supports activation of the prohypertrophic serine-threonine phosphatase calcineurin [9].

Calcineurin is a heterodimer composed of two subunits: calcineurin A (CnA), which contains the catalytic site, and the small regulatory calcium binding calcineurin B (CNB) subunit [10,11]. The A α and A β isoforms of the calcineurin A subunit are expressed in the myocardium, of which the A β subunit was shown to be the stress responsive isoform [12,13]. In the heart, enhanced activation of calcineurin has been shown in cardiac hypertrophic disease, but also during cardiac dilation and even in human dilated cardiomyopathy [10,14,15]. Genetic deletion of calcineurin A β protects the heart from hypertrophy after transverse aortic constriction [12]. Consequently, inhibition of calcineurin has been suggested as a treatment option for hypertrophic heart disease, given that studies with the calcineurin inhibitory agent cyclosporine A have mostly been successful in rodents [10,11,16]. Whether inhibition of calcineurin is also beneficial in DCM is currently unknown.

Here, we demonstrate that ablation of calcineurin A β in MLP ko-induced DCM in mice leads to increased mortality, worsening of cardiac dysfunction, increased cardiomyocyte death and fibrosis. Consistent with these results, modest calcineurin overexpression improved cardiac function and reduced fibrosis in MLP ko mice.

METHODS

Animals and animal procedures

MLP ko mice, CnA β ko mice, NFAT-luciferase reporter mice, and mice, which express the activated form of calcineurin (Δ CnA) under the control of the non inducible α -myosin heavy chain promoter (α -MHC) were previously reported [4,12,17,18]. MLP ko and CnA β ko mice were crossed to generate MLP/CnA β double ko, MLP and CnA β single ko and wild-type mice (C57/Bl6-SV129 hybrid background). A tetracycline responsive, binary α -MHC transgene system was employed to allow temporally defined overexpression of Δ CnA in the

adult mouse heart [19]. The cDNA encoding Δ CnA was subcloned into the SalI – Hind III restriction sites of the tetracycline/Doxycycline (Dox) responsive α -MHC promoter (responder mice). Founder mice were interbred with transgenic mice, which expressed the tetracycline-controlled transactivator transgene (tTA) under the control of the constitutive α -MHC promoter (driver mice). Doxycycline was provided in the food (625 mg/kg food) to the breeder pairs and was withdrawn at weaning at about 1 month of age. Δ CnA overexpressing mice were backcrossed into the MLP ko background (C57/B16-SV129 hybrid).

During echocardiography, mice were anesthetized with 2% isoflurane and the heart was visualized with a Hewlett Packard Sonos 5500 instrument and a 15 MHz transducer. Left ventricular dimensions were taken on M mode in triplicate for each mouse. All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

Histological Analyses

Hearts were collected at the indicated time points and fixed in 10% formalin in PBS. Subsequently, they were embedded in paraffin and serial sections were cut at a thickness of 9 μ m. The slides were stained with Masson's Trichrome and the fibrotic content of each heart section was determined using MetaMorph 6.1 software (Universal Imaging Corp.) as described previously in detail [20]. For transmission electron microscopy the hearts were fixed by perfusion with 3.5% glutaraldehyde in cardioplegic solution, followed by 3.5% glutaraldehyde and 1% OsO₄ in cacodylate buffer. After embedding in a Poly/Bed 812 resin mixture, sections were cut and counterstained with uranyl acetate and lead citrate [21]. A detailed electron microscopic analysis of the left ventricular free wall, the septum and the apex was performed in a blinded fashion by H.O..

Assessment of Cell Death

Heart cryosections were used for the quantification of cardiomyocyte death. The TUNEL assay (Roche) was performed according to the manufacturer's instructions. Briefly, the slides were fixed in 4 % paraformaldehyde and permeabilized with 0.2% Triton, 0.1% sodium citrate before the TUNEL reaction mixture (containing the TdT enzyme) was applied to each slide and then counterstaining with wheat germ agglutinin and DAPI was performed.

In situ Oligo Ligation (ISOL, Chemicon) was conducted following the protocol suggested by the manufacturer. In brief, the tissue was fixed in 1% paraformaldehyde, post-fixed in ethanol:acetic acid (2:1) and the endogenous peroxidase was quenched in 0.5% hydrogen peroxide. Then the working strength Oligo B/T4 DNA Ligase mixture (for the detection of blunt end DNA strand breaks) was applied to each slide and incubated overnight. After application of streptavidin peroxidase, color was developed with peroxidase substrate and the slides were counterstained with hematoxylin.

Molecular Analyses

Western blotting was performed with the use of the following antibodies: Collagen 1 (Santa Cruz), calcineurin A (Pan-CnA), ANF (both Chemicon), phospho-Akt (serine 473), Akt, phospho-ERK, ERK (all Cell Signaling) and Gapdh (Fitzgerald Industries International). Luciferase Assays from whole hearts were performed as previously described [18].

Statistics

All values are presented as mean $\pm$ SEM. The log rank test was used to analyze survival after birth. 1-way ANOVA followed by Student-Newman-Keuls post-hoc test was used to

analyze differences between 3 or more groups; the unpaired student's t-test was used to evaluate differences between 2 groups. A 2-tailed P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Calcineurin prevents mortality and cardiomyocyte death in MLP deficient mice

In order to assess the function of myocardial calcineurin in DCM, and also to examine the feasibility of its inhibition for therapeutic purposes in this disease, we crossed mice with genetic deletion of CnA β (CnA β ko, which do not exert a phenotype at baseline) with mice that lack MLP (MLP ko, which normally develop adult onset DCM). Strikingly - and somewhat unexpectedly - the majority (73%) of MLP/CnA β double ko mice died within the first 20 days after birth, while neither MLP ko nor CnA β ko (not shown), nor wild-type mice (not shown) displayed any early mortality (Figure 1A). Before their early postnatal death, the MLP/CnA β double ko mice appeared dystrophic and sick. Because MLP is selectively expressed in skeletal and cardiac muscle, we suspected the reason for the enhanced mortality in these mice was due to defects in one of these tissue compartments. While skeletal muscle weight (normalized to body weight) and histology was normal (not shown), the hearts were enlarged and appeared dilated (Figure 1B). Transmission electron microscopy was performed to assess the ultrastructural features of the cardiac disease in the MLP/CnA β double ko mice. While the known cytoskeletal abnormalities in the MLP ko mice (i.e. broadened Z- discs, disorganized sarcomeres) were unchanged in the absence of calcineurin A β [4], the double ko mice displayed significantly more (necrotic and apoptotic) cell death upon semi-quantitative evaluation (Figure 1C).

Combined calcineurin/MLP deficiency leads to cardiac dysfunction and cardiomyocyte death in adult mice

27 percent of the MLP/CnA β double ko mice survived until adulthood. Left ventricular function was measured as fractional shortening (FS) in these mice and control mice with echocardiography. As shown in Figure 2A, MLP/CnA β double ko mice showed a markedly reduced cardiac function compared to wild-type, CnA β ko or MLP ko mice. Although only a tendency towards enhanced ventricular dilation was evident by echocardiography in the double ko mice (Table 1), higher heart weight/body weight ratios in MLP single ko mice (due to ventricular dilation) that were significantly enhanced in the MLP/CnA β double ko mice, point towards aggravated cardiac enlargement in those mice (Table 1). Since enhanced cell death was found in the double ko mice after birth, we assessed apoptotic and necrotic cell death in the adult myocardium with two quantitative cell death detection assays. It has been suggested, that the TUNEL assay preferentially detects apoptotic cells, while the blunt-end DNA ligation assay detects necrotic cells [22–24]. Importantly, significantly higher rates of apoptotic and necrotic cardiomyocyte death were detected in MLP/CnA β double ko mice compared to wild-type and MLP ko mice (Figure 2B and C). Dying cardiomyocytes are typically replaced by fibrotic tissue in the heart. Masson's Trichrome staining and quantification of the fibrotic (blue) area revealed significantly enhanced myocardial fibrosis in MLP single ko mice, which was dramatically enhanced in the double ko mice (Figure 2D and E). These results were supported by higher Pro-collagen 1 levels, which were detected in the myocardium of the MLP/CnA β double ko mice by Western blotting (Figure 2F). Higher ANF protein levels in the double ko myocardium further suggested aggravated cardiac dysfunction in these mice compared with controls (Figure 2G). As demonstrated in Figure 2H, calcineurin A (the α and β isoforms were both detected by the Pan-CnA antibody) expression was reduced by about 50% in CnA β ko mice in the wild-type background, which is in line with previously published data [12]. Interestingly, however, calcineurin A expression was downregulated to a much greater extent (by about 80%) in

CnA β ko mice in the MLP knock-out background. This is most likely due to the fact that CnA β is upregulated and therefore the predominant calcineurin A isoform during cardiac stress [12, 13]. In addition to calcineurin, protection from cardiomyocyte cell death is mediated by activation of the signaling kinases Akt and ERK [25–27]. While ERK activation showed a mildly reduced activation (higher total ERK levels and reduced phospho-ERK levels) in MLP ko mice (irrespective of the presence or absence of CnA β), Akt activation (serine 473 phosphorylation) was enhanced in CnA β ko mice but was completely abrogated in MLP/CnA β double ko mice (Figure 2H).

In summary, in DCM induced by MLP deletion, calcineurin deficiency leads to early mortality, enhanced cardiomyocyte death and cardiac fibrosis, with more severe ventricular dysfunction and downregulation of intracellular survival signaling.

Expression of activated calcineurin improves myocardial dysfunction and remodeling in MLP ko mice

Since calcineurin A β was required for cardiac compensation in the MLP ko mice, we asked whether enhancement of calcineurin activity in the myocardium of these mice could improve cardiac function. In order to answer this question, we crossed mice with cardiomyocyte specific, constitutive overexpression of the activated form of calcineurin A (Δ CnA, CnA-Tg) with MLP ko mice. As displayed in Figure 3A, high level overexpression of Δ CnA (40kDa; full length, endogenous CnA is detected at 60kDa) was achieved in the MLP ko background. Interestingly, echocardiography revealed a significant improvement of left ventricular function in the MLP ko/CnA-Tg mice compared to MLP ko mice without the transgene (MLP ko/non-transgenic, NTG, Figure 3B). However, it has previously been shown that constitutive, high level overexpression of Δ CnA leads to hypertrophic heart disease [17]. Indeed, heart weight analysis revealed a dramatic form of cardiac hypertrophy in the CnA-Tg mice, both in the wild-type as well as in the MLP ko background (Figure 3C).

While activation of calcineurin appeared to be beneficial in MLP ko mice, the high level constitutive expression of Δ CnA leads to hypertrophic heart disease by itself. Given this confounding issue, we generated novel Δ CnA transgenic mice using an inducible system [19]. The modified α -myosin heavy chain (α MHC_{ind}) promoter was employed as the responder transgene to drive cardiac specific expression, which is regulated by the administration/withdrawal of doxycycline (Dox) in the presence of a second α -MHC promoter-driven tTA transgene (α -MHC-tTA, Figure 4A). Two α MHC_{ind}- Δ CnA transgenic lines were extensively characterized (lines 2.12 and 12.3). α MHC_{ind}- Δ CnA $\times$ α MHC-tTA double transgenic (DTG) mice were maintained on Dox until 1 month of age, which prevented transgene expression (not shown) and any developmental effect (Figure 4B). 1 month after Dox withdrawal, a modest Δ CnA (40kDa) overexpression was observed in DTG mice of both transgenic lines (Figure 4C). DTG mice had a normal appearance, and heart weight analysis demonstrated the development of a mild, but significant, cardiac hypertrophy in line 2.12, but not in line 12.3 (Figure 4D). Control mice consisted of non-transgenic and α -MHC-tTA single transgenic mice. We bred DTG mice of line 12.3 with NFAT luciferase reporter mice [18]. As shown in Figure 4E, DTG mice had a significantly higher NFAT activation than control mice.

The inducible Δ CnA transgenic mice were crossed with MLP ko mice to evaluate the effects of mild, adult onset myocardial expression of Δ CnA on the phenotype of DCM. As demonstrated in Figure 5A (and quantified in Figure 5B), modest Δ CnA (40kDa) expression was achieved in DTG mice of lines 12.3 and 2.12 in the MLP ko background. Interestingly, mild Δ CnA expression markedly augmented cardiac function in MLP ko mice (Figure 5C and D) and the dilation of the left ventricle was mildly, but significantly reduced (Table 2).

Masson's Trichrome staining and its quantification revealed significantly less fibrosis in MLP ko mice with Δ CnA overexpression (Figure 5E, F). The reduced deposition of collagen 1 was consistent with reduced myocardial pro-collagen 1 levels in MLP ko/DTG mice (Figure 5G). In line with the reduction in left ventricular dysfunction, myocardial ANF expression was diminished in MLP ko with Δ CnA overexpression. These results demonstrate that mild enhancement of calcineurin activity promotes compensation in a MLP deficiency model of DCM.

DISCUSSION

Here, we have identified a previously unknown protective effect of myocardial calcineurin in DCM. In previous studies, high levels of calcineurin/NFAT activation were demonstrated in the myocardium of wild-type mice especially around birth, when the heart is adapting to the postnatal systemic circulation [18]. Another (although much smaller) peak of calcineurin activation was demonstrated in adult mice during compensated cardiac stress associated with pressure overload or myocardial infarction, which is even more pronounced in mice with decompensated heart failure [18]. Importantly, myocardial calcineurin activation was also demonstrated in human hypertrophic and dilated cardiomyopathy [14,15]. Although neither genetic deletion of calcineurin A β nor MLP alone during the postnatal time period disturbs normal cardiac development or animal growth, concomitant loss of both proteins leads to cardiomyopathy, sickness, and death in the majority of the calcineurin/MLP double ko mice in the first 20 days after birth. Furthermore, the DCM phenotype in calcineurin/MLP double ko mice that survived the postnatal period was markedly aggravated compared to MLP single ko mice. This indicates that calcineurin can play a protective role in the myocardium, especially shortly after birth, which might become important in the presence of certain disease triggering stimuli (such as the loss of the cytoskeletal protein MLP).

What is the mechanism of calcineurin induced cardioprotection in DCM? Semiquantitative electron-microscopic studies in the neonatal myocardium and quantitative DNA strand-break detection assays in the adult heart revealed elevated levels of cardiomyocyte apoptotic and necrotic death in double ko mice. Cardiomyocyte apoptosis (0.002%–0.25% of cardiomyocytes), and necrosis (0.06% – 1% of cardiomyocytes) have been found in end stage human heart failure and DCM, but are almost absent in control hearts [24,28,29]. Importantly, the induction of either apoptosis or necrosis appears to be sufficient to induce DCM in mice. For example, cardiac restricted overexpression of a procaspase 8 fusion protein that produced chronically low levels of apoptotic cardiomyocytes at rates comparable with those in human heart failure (0.023%) resulted in the development of cardiac dilation and dysfunction [30]. Similarly, transgenic mice with enhanced sarcolemmal L-type Ca²⁺ channel (LTCC) activity developed progressive myocardial necrosis associated with cardiac dysfunction and premature death [31]. Interestingly, both forms of cardiomyocyte death were followed by replacement fibrosis in the myocardium, which we also observed in the MLP/CnA β double ko mice [30,31]. Loss of protective signaling molecules –such as gp130 or Mek1/Erk - has been previously shown to predispose the myocardium to apoptosis when hemodynamic stress is imposed [26,32]. This is comparable to the absence of calcineurin A β , which does not result in a phenotype at baseline, but predisposes the myocardium to enhanced rates of cell death and adverse remodeling in DCM.

The anti-apoptotic properties of calcineurin in the heart have been demonstrated before. For example, calcineurin A β ko mice have higher levels of apoptosis after ischemia/reperfusion injury [33], and calcineurin A transgenic mice were protected from ischemia/reperfusion induced apoptosis [34]. The transcription factor NFAT has been identified as a downstream mediator of the protective effects of calcineurin in cardiomyocytes [35]. In addition to the

reduction in protective calcineurin/NFAT signaling, the activation of the pro-survival kinase Akt was also downregulated in MLP/CnA β double ko mice (versus CnA β single ko mice), creating an intracellular milieu with markedly reduced survival signaling and a strong predisposition to cell death.

In our study, calcineurin overexpression improved cardiac function in MLP ko mice and mild enhancement of calcineurin activity reduced cell death, as suggested by a significantly reduced degree of myocardial fibrosis. Interestingly, the protective effects of calcineurin overexpression appeared to be independent of its prohypertrophic effects, since the improvement in cardiac function and histology were observed with both inducible transgenic lines, of which hypertrophy was only evident in line 2.12. in the MLP wild-type background, although in the MLP ko background mild overexpression of Δ CnA did not increase HW/BW ratio in either of the 2 lines (Table 2 and data not shown). It should be noted, however, that the rescue of MLP deficiency induced cardiac dysfunction by Δ CnA was not complete, most likely because the cytoskeletal abnormalities were still present in MLP ko hearts.

One important question remaining is whether our data are also relevant to other forms of DCM. It is interesting to note in this regard that in ischemic cardiomyopathy and idiopathic DCM, a 50% downregulation of MLP was reported, implying MLP deficiency as a common pathophysiological mechanism in advanced heart failure [7]. As a consequence, calcineurin inhibition could worsen heart failure, if MLP is reduced. Therefore, while calcineurin inhibition in animal models of hypertrophic heart disease is most likely beneficial [10,12,16], care should be taken in DCM and possibly even end stage heart failure of other etiology, when endogenous calcineurin activation may represent a protective mechanism in the myocardium. Indeed, we have previously shown that calcineurin and MLP interact in vivo, potentially generating a unique signaling complex that might signal cardioprotection during pathological conditions associated with highly increased wall stress, such as in DCM [9]. Clearly, more studies in different animal models of DCM and end stage heart failure are needed to further examine the extent to which calcineurin inhibition might be deleterious or beneficial.

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References

1. Towbin JA, Lowe AM, Colan SD, Sleeper LA, Orav EJ, Clunie S, et al. Incidence, causes, and outcomes of dilated cardiomyopathy in children. *JAMA* 2006 Oct 18;296(15):1867–76. [PubMed: 17047217]
2. Grunig E, Tasman JA, Kucherer H, Franz W, Kubler W, Katus HA. Frequency and phenotypes of familial dilated cardiomyopathy. *J Am Coll Cardiol* 1998 Jan;31(1):186–94. [PubMed: 9426039]
3. Arber S, Halder G, Caroni P. Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. *Cell* 1994 Oct 21;79(2):221–31. [PubMed: 7954791]
4. Arber S, Hunter JJ, Ross J Jr, Hongo M, Sansig G, Borg J, et al. MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. *Cell* 1997 Feb 7;88(3):393–403. [PubMed: 9039266]
5. Knoll R, Hoshijima M, Hoffman HM, Person V, Lorenzen-Schmidt I, Bang ML, et al. The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy. *Cell* 2002 Dec 27;111(7):943–55. [PubMed: 12507422]

6. Recchia FA, Lionetti V. Animal models of dilated cardiomyopathy for translational research. *Vet Res Commun* 2007 Aug;31(Suppl 1):35–41. [PubMed: 17682844]
7. Zolk O, Caroni P, Bohm M. Decreased expression of the cardiac LIM domain protein MLP in chronic human heart failure. *Circulation* 2000 Jun 13;101(23):2674–7. [PubMed: 10851202]
8. Heineke J, Kempf T, Kraft T, Hilfiker A, Morawietz H, Scheubel RJ, et al. Downregulation of cytoskeletal muscle LIM protein by nitric oxide: impact on cardiac myocyte hypertrophy. *Circulation* 2003 Mar 18;107(10):1424–32. [PubMed: 12642365]
9. Heineke J, Ruetten H, Willenbockel C, Gross SC, Naguib M, Schaefer A, et al. Attenuation of cardiac remodeling after myocardial infarction by muscle LIM protein-calcineurin signaling at the sarcomeric Z-disc. *Proc Natl Acad Sci U S A* 2005 Feb 1;102(5):1655–60. [PubMed: 15665106]
10. Bueno OF, van Rooij E, Molkentin JD, Doevendans PA, De Windt LJ. Calcineurin and hypertrophic heart disease: novel insights and remaining questions. *Cardiovasc Res* 2002 Mar; 53(4):806–21. [PubMed: 11922891]
11. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* 2006 Aug;7(8):589–600. [PubMed: 16936699]
12. Bueno OF, Wilkins BJ, Tymitz KM, Glascock BJ, Kimball TF, Lorenz JN, et al. Impaired cardiac hypertrophic response in Calcineurin A β 2-deficient mice. *Proc Natl Acad Sci U S A* 2002 Apr 2;99(7):4586–91. [PubMed: 11904392]
13. Taigen T, De Windt LJ, Lim HW, Molkentin JD. Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy. *Proc Natl Acad Sci U S A* 2000 Feb 1;97(3):1196–201. [PubMed: 10655507]
14. Lim HW, Molkentin JD. Calcineurin and human heart failure. *Nat Med* 1999 Mar;5(3):246–7. [PubMed: 10086361]
15. Haq S, Choukroun G, Lim H, Tymitz KM, del Monte F, Gwathmey J, et al. Differential activation of signal transduction pathways in human hearts with hypertrophy versus advanced heart failure. *Circulation* 2001 Feb 6;103(5):670–7. [PubMed: 11156878]
16. Sussman MA, Lim HW, Gude N, Taigen T, Olson EN, Robbins J, et al. Prevention of cardiac hypertrophy in mice by calcineurin inhibition. *Science* 1998 Sep 11;281(5383):1690–3. [PubMed: 9733519]
17. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 1998 Apr 17;93(2):215–28. [PubMed: 9568714]
18. Wilkins BJ, Dai YS, Bueno OF, Parsons SA, Xu J, Plank DM, et al. Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. *Circ Res* 2004 Jan 9;94(1): 110–8. [PubMed: 14656927]
19. Sanbe A, Gulick J, Hanks MC, Liang Q, Osinska H, Robbins J. Reengineering inducible cardiac-specific transgenesis with an attenuated myosin heavy chain promoter. *Circ Res* 2003 Apr 4;92(6): 609–16. [PubMed: 12623879]
20. Parsons SA, Millay DP, Sargent MA, McNally EM, Molkentin JD. Age-dependent effect of myostatin blockade on disease severity in a murine model of limb-girdle muscular dystrophy. *Am J Pathol* 2006 Jun;168(6):1975–85. [PubMed: 16723712]
21. Fewell JG, Osinska H, Klevitsky R, Ng W, Sfyris G, Bahrehmand F, et al. A treadmill exercise regimen for identifying cardiovascular phenotypes in transgenic mice. *Am J Physiol* 1997 Sep; 273(3 Pt 2):H1595–605. [PubMed: 9321854]
22. Gold R, Schmied M, Giegerich G, Breitschopf H, Hartung HP, Toyka KV, et al. Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. *Lab Invest* 1994 Aug;71(2):219–25. [PubMed: 8078301]
23. Al-Lamki RS, Skepper JN, Loke YW, King A, Burton GJ. Apoptosis in the early human placental bed and its discrimination from necrosis using the in-situ DNA ligation technique. *Hum Reprod* 1998 Dec;13(12):3511–9. [PubMed: 9886542]
24. Guerra S, Leri A, Wang X, Finato N, Di Loreto C, Beltrami CA, et al. Myocyte death in the failing human heart is gender dependent. *Circ Res* 1999 Oct 29;85(9):856–66. [PubMed: 10532954]

25. Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation* 2000 Feb 15;101(6):660–7. [PubMed: 10673259]
26. Purcell NH, Wilkins BJ, York A, Saba-El-Leil MK, Meloche S, Robbins J, et al. Genetic inhibition of cardiac ERK1/2 promotes stress-induced apoptosis and heart failure but has no effect on hypertrophy in vivo. *Proc Natl Acad Sci U S A* 2007 Aug 28;104(35):14074–9. [PubMed: 17709754]
27. Shiraishi I, Melendez J, Ahn Y, Skavdahl M, Murphy E, Welch S, et al. Nuclear targeting of Akt enhances kinase activity and survival of cardiomyocytes. *Circ Res* 2004 Apr 16;94(7):884–91. [PubMed: 14988230]
28. Kostin S, Pool L, Elsasser A, Hein S, Drexler HC, Arnon E, et al. Myocytes die by multiple mechanisms in failing human hearts. *Circ Res* 2003 Apr 18;92(7):715–24. [PubMed: 12649263]
29. Foo RS, Mani K, Kitsis RN. Death begets failure in the heart. *J Clin Invest* 2005 Mar;115(3):565–71. [PubMed: 15765138]
30. Wencker D, Chandra M, Nguyen K, Miao W, Garantziotis S, Factor SM, et al. A mechanistic role for cardiac myocyte apoptosis in heart failure. *J Clin Invest* 2003 May;111(10):1497–504. [PubMed: 12750399]
31. Nakayama H, Chen X, Baines CP, Klevitsky R, Zhang X, Zhang H, et al. Ca²⁺- and mitochondrial-dependent cardiomyocyte necrosis as a primary mediator of heart failure. *J Clin Invest* 2007 Sep;117(9):2431–44. [PubMed: 17694179]
32. Hirota H, Chen J, Betz UA, Rajewsky K, Gu Y, Ross J Jr, et al. Loss of a gp130 cardiac muscle cell survival pathway is a critical event in the onset of heart failure during biomechanical stress. *Cell* 1999 Apr 16;97(2):189–98. [PubMed: 10219240]
33. Bueno OF, Lips DJ, Kaiser RA, Wilkins BJ, Dai YS, Glascock BJ, et al. Calcineurin Abeta gene targeting predisposes the myocardium to acute ischemia-induced apoptosis and dysfunction. *Circ Res* 2004 Jan 9;94(1):91–9. [PubMed: 14615291]
34. De Windt LJ, Lim HW, Taigen T, Wencker D, Condorelli G, Dorn GW 2nd, et al. Calcineurin-mediated hypertrophy protects cardiomyocytes from apoptosis in vitro and in vivo: An apoptosis-independent model of dilated heart failure. *Circ Res* 2000 Feb 18;86(3):255–63. [PubMed: 10679475]
35. Pu WT, Ma Q, Izumo S. NFAT transcription factors are critical survival factors that inhibit cardiomyocyte apoptosis during phenylephrine stimulation in vitro. *Circ Res* 2003 Apr 18;92(7):725–31. [PubMed: 12663489]

Figure 1.

Ablation of calcineurin A β leads to early mortality and cardiomyocyte death shortly after birth in MLP knock-out (ko) mice. (A) Kaplan Meier Survival Curve of MLP ko mice in the presence (MLP ko/CnA β wild-type, wt, dotted line, n=12) or in the absence of calcineurin A β (MLP ko/CnA β ko, continuous line, n=11). *p<0.01 vs. MLP ko/CnA β wt. (B) HW/BW (heart weight/body weight) ratio in 10 days old mice. The number of mice per group is indicated within the bar. *p<0.01 vs. other groups (C) Representative electron microscopical (EM) pictures (magnification 20,000 $\times$) of the myocardium at 5 days of age (on the left); semiquantitative assessment of myocyte death from EM analysis (on the right). * necrotic myocyte with sarcolemmal disruption, # apoptotic myocyte with typical marginalization of chromatin.

Figure 2. Combined deficiency of MLP and calcineurin A β leads to enhanced cardiac dysfunction and adverse remodeling in adult mice. (A) Echocardiographic assessment of cardiac function as

fractional shortening (FS) in 2 months old mice. * $p < 0.05$ vs. other groups (B) Assessment of cardiomyocyte apoptosis with the TUNEL Assay. * $p < 0.05$ vs. other groups. (C) Assessment of cardiomyocyte necrosis with Oligo B (blunt end) DNA Ligation Assay. * $p < 0.05$ vs. other groups. (D) Representative Masson's Trichrome staining of myocardial tissue sections (magnification 200 $\times$). (E) Quantitative assessment of fibrotic (blue) area in Masson's Trichrome stained sections. * $p < 0.01$ vs. MLP wild-type (wt) † $p < 0.01$ vs. MLP ko/CNA β wt. (F) Pro-(P.)collagen 1 protein levels normalized to Gapdh protein. * $p < 0.01$ vs. MLP wild-type (wt) † $p < 0.01$ vs. MLP ko/CNA β wt. (G) Western blots for ANF (atrial natriuretic factor) and Gapdh. (H) Western blots for calcineurin A (CnA), phospho(p)-Akt, Akt, p-ERK, ERK and Gapdh. The number of mice per group is indicated within the bar (panels A–C, E, F).

Figure 3.

High level, constitutive overexpression of activated calcineurin improves cardiac function in MLP ko mice, but induces hypertrophic cardiomyopathy. (A) Western blots for CnA and Gapdh. The transgene (activated calcineurin, Δ CnA) is detected at 40kDa. Endogenous, full length CnA is detected at 60kDa. (B) Echocardiographic assessment of cardiac function as fractional shortening (FS). * $p < 0.05$ vs. MLP wild-type (wt) mice, † $p < 0.05$ vs. MLP ko/non-transgenic (NTG). (C) HW/BW (heart weight/body weight) ratio in 2 months old mice. * $p < 0.05$ vs. MLP wt/NTG, † $p < 0.05$ vs. MLP ko/NTG. The number of mice per group is indicated within the bar (panels B, C).

Figure 4.

Modest, adult onset overexpression of activated calcineurin in the heart. (A) Schematic representation of the binary transgenic system. (B) Schematic representation of the time schedule of doxycycline (Dox) administration and transgene expression. (C) Western blots for myocardial calcineurin A (CnA) and Gapdh in mice of lines 2.12 and 12.3. The transgene (activated calcineurin) is detected at 40kDa. Full length CnA is detected at 60kDa. NTG: non-transgenic; tTA: α MHC-tTA single transgenic mice; DTG: α MHC_{ind}- Δ CnA $\times$ α MHC-tTA double transgenic mice. (D) HW/BW (heart weight/body weight) ratio in 2 months old mice. * $p < 0.05$ vs. other groups. (E) NFAT dependent luciferase activity (normalized to total protein concentration) in control or DTG mice (of line 12.3) that carried the NFAT-luciferase reporter transgene. * $p < 0.05$ vs. control. The number of mice per group is indicated within the bar (panels D, E).

Figure 5.

Modest calcineurin overexpression in MLP ko mice improves cardiac function and reduces adverse remodeling. (A) Western blot for calcineurin A (CnA) and Gapdh. The transgene is detected at 40kDa. Full length CnA is detected at 60kDa. DTG: $\alpha\text{MHC}_{\text{ind}} - \Delta\text{CnA} \times \alpha\text{MHC}$ -tTA double transgenic mice of the lines (L) 2.12 or 12.3. (B) Quantification of the Western blot in (A). * $p < 0.05$ vs. control. (C) and (D) Echocardiographic assessment of cardiac function as fractional shortening (FS). * $p < 0.05$ vs. MLP wt/control, † $p < 0.05$ vs. MLP ko/control. (E) Representative Masson's Trichrome staining of myocardial tissue sections (magnification 200 $\times$). (F) Quantitative assessment of fibrotic (blue) area in Masson's Trichrome stained sections. * $p < 0.01$ vs. other groups. (G) and (H) Western blot for Pro-(P.)collagen 1, ANF (atrial natriuretic factor) and Gapdh. The number of mice per group is indicated within the bar (panels B–D, F).

Table 1

Morphometry and echocardiography in MLP, calcineurinA β knock-out mice.

	MLP wt CnA β wt	MLP Wt CnA β ko	MLP ko CnA β wt	MLP ko CnA β ko
Morphometry				
BW (g)	18 $\pm$ 2.8	22.9 $\pm$ 3	22.2 $\pm$ 0.4	21.8 $\pm$ 2.0
HW/BW (mg/g)	5.5 $\pm$ 0.4	5.1 $\pm$ 0.8	6.9 $\pm$ 0.25*	8.4 $\pm$ 0.8 [†]
n	4	6	8	4
Echocardiography				
LVEDD (mm)	3.6 $\pm$ 0.3	3.5 $\pm$ 0.1	4.2 $\pm$ 0.1	4.3 $\pm$ 0.4
LVEDS (mm)	2.4 $\pm$ 0.2	2.3 $\pm$ 0.2	3.0 $\pm$ 0.2	3.4 $\pm$ 0.4
n	4	6	8	5

Wt: wild-type; ko: knock-out; BW: body weight; HW: heart weight; n: number of mice analyzed; LVEDD: left ventricular end-diastolic dimensions; LVEDS: left ventricular end-systolic dimensions.

* p<0.05 vs. MLP wt CnA β wt;

[†] p<0.05 vs. MLP ko CnA β wt

Table 2

Morphometry and echocardiography in MLP knock-out mice with or without Δ CnA overexpression.

	Wt Control	Wt DTG L12.3	MLP ko Control	MLP ko DTG L12.3
Morphometry				
BW (g)	24.5 $\pm$ 1.05	26.3 $\pm$ 0.5	32.2 $\pm$ 2.2	26.6 $\pm$ 1.7
HW/BW (mg/g)	5.0 $\pm$ 0.1	5.0 $\pm$ 0.1	6.2 $\pm$ 0.6	5.4 $\pm$ 0.5
n	10	9	7	7
Echocardiography				
LVEDD (mm)	3.8 $\pm$ 0.1	3.8 $\pm$ 0.1	4.5 $\pm$ 0.1 [*]	4.2 $\pm$ 0.1 ^{*†}
LVEDS (mm)	2.3 $\pm$ 0.2	2.3 $\pm$ 0.1	3.4 $\pm$ 0.1 [*]	2.9 $\pm$ 0.2 ^{*†}
n	7	7	7	8

Wt: wild-type; DTG L12.3: α MHCind- Δ CnA $\times$ α MHC- τ TA double transgenic mice from line 12.3; ko: knock-out; BW: body weight; HW: heart weight; n: number of mice analyzed; LVEDD: left ventricular end-diastolic dimensions; LVEDS: left ventricular end-systolic dimensions;

^{*} p<0.05 vs. MLP wt control;

[†] p<0.05 vs. MLP ko