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Early sensitization of myofilaments to Ca^{2+} prevents genetically linked dilated cardiomyopathy in mice

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Background Dilated cardiomoypathies (DCM) are a heterogeneous group of inherited and acquired diseases characterized by decreased contractility and enlargement of cardiac chambers and a major cause of morbidity and mortality. Mice with Glu54Lys mutation in α-tropomyosin (Tm54) demonstrate typical DCM phenotype with reduced myofilament Ca^{2+} sensitivity. We tested the hypothesis that early sensitization of the myofilaments to Ca^{2+} in DCM can prevent the DCM phenotype.

Methods and results To sensitize Tm54 myofilaments, we used a genetic approach and crossbred Tm54 mice with mice expressing slow skeletal troponin I (ssTnI) that sensitizes myofilaments to Ca^{2+}. Four groups of mice were used: non-transgenic (NTG), Tm54, ssTnI and Tm54/ssTnI (DTG). Systolic function was significantly reduced in the Tm54 mice compared to NTG, but restored in DTG mice. Tm54 mice also showed increased diastolic LV dimensions and HW/BW ratios, when compared to NTG, which were improved in the DTG group. β-myosin heavy chain expression was increased in the Tm54 animals compared to NTG and was partially restored in DTG group. Analysis by 2D-DIGE indicated a significant decrease in two phosphorylated spots of cardiac troponin I (cTnI) in the DTG animals compared to NTG and Tm54. Analysis by 2D-DIGE also indicated no significant changes in troponin T, regulatory light chain, myosin binding protein C and tropomyosin phosphorylation.

Conclusion Our data indicate that decreased myofilament Ca^{2+} sensitivity is an essential element in the pathophysiology of thin filament linked DCM. Sensitization of myofilaments to Ca^{2+} in the early stage of DCM may be a useful therapeutic strategy in thin filament linked DCM.

Keywords DCM • Myofilament Ca^{2+} sensitivity • New therapy

1. Introduction

Dilated cardiomyopathy (DCM) is a cause of significant morbidity and mortality and the main indication for cardiac transplant in patients with refractory heart failure. Despite significant research efforts, DCM-related mortality remains high, approaching 50% at 5 years in symptomatic patients. DCM is characterized by chamber dilation, systolic dysfunction and is often associated with arrhythmia and sudden cardiac death (SCD) (for review see Ref. 1). The etiology of DCM varies, but currently it is estimated that about 30–50% of DCM cases have genetic causes mainly with autosomal dominant inheritance and usually associated with mutations in cytoskeletal and sarcomeric proteins.[2,3] Identification of mutations that cause hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM) or DCM have guided both *in vitro* and *in vivo* studies to better understand how the specific mutation alters molecular, cellular and whole heart function leading to development of cardiomyopathy.[4] However, there are relatively few animal models that recapitulate human DCM and studies testing specific

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treatments are limited.[5,6] Moreover, the few reports involving therapies for treatment of DCM mice have significant limitations.[6–9]

At the level of the cardiac sarcomere, most of the mutations in thin filament proteins that are linked to DCM show decreased myofilament sensitivity to Ca^{2+} [5,6,10–14] with only few exceptions.[15–18] Increasing sarcomere activity and Ca^{2+} sensitivity have been demonstrated to have beneficial effects in acute treatment of acquired HF in humans.[19–21] Nevertheless, little is known as to whether early interventions that promote the myofilament response to Ca^{2+} would be beneficial, but also long-lasting in DCM in which a potential decrease in the myofilament response to Ca^{2+} can be predicted in children based on family history and genetic screening. Since the primary defect of DCM in most cases is associated with decreased myofilament sensitivity to Ca^{2+}, the most straightforward therapy would be to sensitize the myofilaments to Ca^{2+}, bringing the sensitivity close to normal physiological levels.

There are several good targets within myofilaments for altering Ca^{2+} sensitivity, including the troponin subunits TnI and TnC, as well as the thick filament protein myosin.[22,23] Our demonstration that expression of the neonatal (slow skeletal) isoform of TnI (ssTnI) in the adult mouse induces a number of beneficial effects including an increase in myofilament responsiveness to Ca^{2+} [24–28] provides the basis for a genetic approach to restoring sarcomere function in DCM. In the current studies, we therefore employed a transgenic mouse model documented to mimic DCM[29] that expresses mutated tropomyosin at position 54 (TmGlu54Lys; Tm54).[5] There are at least 50 sarcomeric mutations that are linked to DCM from which 12 have been identified in Tm (TPM1).[30] As proof of principle that shifting the myofilament sensitivity close to the normal level is therapeutic in DCM, we crossed Tm54 mice with TG mice that express ssTnI within the myocardium. Our data show a long-lasting, protective effect of myofilament Ca^{2+} sensitization and indicate that myofilament sensitization to Ca^{2+} may be a useful preventative therapeutic strategy in sarcomere-linked DCM associated with decreased sensitivity.

2. Methods

For more detailed methods see Supplementary material online.

2.1 Generation of new transgenic (TG) mice

New TG mouse line was generated by crossbreeding existing lines of mice, TG mice with mutated tropomyosin (Tm) at position 54 (TmGlu54Lys)[5] and TG mice expressing skeletal isoform of troponin I (ssTnI).[25] All mice used in this work were in the same mixed genetic background. Four groups of mice were used for experiments: (1) NTG (non-transgenic), which expresses wild-type (WT) Tm and cardiac TnI; (2) ssTnI, which expresses WT Tm and ssTnI; (3) Tm54, which expresses Tm54 and cardiac TnI; (4) Tm54/ssTnI (DTG), which expresses Tm54 and ssTnI.

All animal procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Review Board of the University of Illinois at Chicago.

2.2 pCa-Force relationship in skinned fibre preparation

Measurements of pCa-force relations were performed as previously described.[31]

2.3 Echocardiography

Echocardiography was performed using a Vevo 770 High-Resolution In Vivo Imaging System, RMVTM 707B scan head with a centre frequency of 30 MHz (VisualSonics, Toronto, ON, Canada), and Analytic Software as previously described.[32,33] Echocardiographic studies were performed in each animal at 5 months of age.

2.4 *In situ* hemodynamic

In situ pressure-volume measurements were performed as previously described.[34] A tracheotomy was performed, the right common carotid artery was then isolated and the artery cannulated with an ultra-miniature P-V catheter (1.4F PVR1045, Millar Instruments, Houston, TX, USA). LV pressure (LVP) and volume were continuously monitored and digitally recorded on Chart software (v.5.5, AD Instruments). To record P-V loops in different loading conditions, an abdominal access was obtained to allow transitory vena cava occlusion right below the diaphragm.

2.5 Western blots

The Western blots were performed as previously described[33,35] with slight modifications. For immuno-detection, the membranes were probed with specific primary anti-rabbit antibodies from Cell Signaling, MA, USA: (1) Phospho (T202, Y204 sites of phosphorylation)-p44/42 MAPK (mitogen-activated protein kinase 1) Erk1/2 (extracellular signal-regulated kinase 1 and 2) 1:1000; (2) p44/42 MAPK (Erk1/2) 1:2500; (3) Phospho (Ser21/9) -GSK-3α/β (Glycogen synthase kinase 3 alpha/beta) 1:1000; (4) GSK-3α/β, 1:2000; (5) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1:50,000; (6) Phospho (S473)-Akt (protein-kinase-B)1:2000; (7) Akt 1:2000. Membranes were also probed with antibodies from Abcam Cambridge, MA, USA: (1) Phospho (S105)-GATA4 (GATA binding factor-4) 1:1000; (2) GATA4 1:1000; and anti-mouse Troponin I from Fitzgerald Acton, MA, USA.

2.6 Assessment of β-MHC abundance

The expression level of βMHC was assessed as previously described.[36]

2.7 Assessment of myofilament modifications by 2D-DIGE

2D-DIGE gels were run to determine the post translational modifications of sarcomeric proteins.[35]

2.8 Statistical analysis

All statistical analysis was performed using GraphPad Prism 6. Data in the manuscript are presented as mean ± SE, n = number of samples. Differences among four groups were analysed by one-way ANOVA followed by post-hoc analysis indicated in figure legends or text. Differences were considered significant when $P < 0.05$.

3. Results

Expression of ssTnI prevents Tm54 hearts from pathological remodeling. High resolution echocardiography and gross pathological evaluation demonstrated that Tm54 hearts developed cardiac chamber dilation that was prevented by expression of ssTnI (*Figure 1*). Compared to NTG hearts, Tm54 hearts displayed significantly enlarged left atria (LA) (*Figure 1A*) and severe LV dilation (increased LV internal diastolic dimension (LVIDd) (*Figure 1B*); eccentric hypertrophy, with lower posterior and relative wall thickness (*Figure 1C* and *D*) and higher LV calculated mass

(*Figure 1E*). Heart weight to body weight (HW/BW)) ratio was also increased in Tm54 hearts compared to NTG and ssTnI mice (*Figure 1F*). Most of these altered parameters were restored to normal values in DTG mice (Tm54 hearts expressing ssTnI).

Expression of ssTnI prevents the LV dysfunction observed in Tm54 hearts. Echocardiography and Doppler studies showed that Tm54 mice developed severe systolic and diastolic left ventricular dysfunction (*Figure 2* and see Supplementary material online, *Table S1*). *Figure 2* shows the representative M-mode echo images (panel A), pulsed Doppler (panel D) and tissue Doppler (TDI, panel E) in all groups of mice. *Figure 2B, C, F, G* and see Supplementary material online, *Table S1* summarize the evaluation of the systolic and diastolic left ventricular functions in all four groups of mice. Compared to the NTG group Tm54 mice showed reduced ejection fraction (EF; *Figure 2B*) and fractional shortening (FS; *Figure 2C*), peak myocardial velocity (S_m) and stroke volume (see Supplementary material online, *Table S1*). These parameters, with the exception of S_m, were similar to NTG levels in DTG mice. *Figure 2F–G* and see Supplementary material online, *Table S1* summarize the evaluation of diastolic function by pulsed Doppler and TDI studies. Tm54 mice also showed a restrictive LV filling pattern with higher the E/A ratio (*Figure 2F*), prolonged isovolumic relaxation time (IVRT) (see Supplementary material online, *Table S1*) and lower peak myocardial velocities in the early phase of diastole (E_m) (*Figure 2G*) and after atrial contraction (A_m) (see Supplementary material online, *Table S1*), when compared to NTG mice. The expression of ssTnI in Tm54 mice partially prevented the development of diastolic dysfunction as it is seen by restoration of the E/A ratio to normal level (*Figure 2F*), but prolonged IVRT and lower myocardial velocities E_m and A_m (*Figure 2G* and see Supplementary material online, *Table S1*)

We also used a P-V conductance catheter to perform hemodynamic studies in all four groups of mice (*Figure 3*). The representative P-V loops are presented in *Figure 3A*. Compared to NTG mice Tm54 mice showed reduced values for all assessed contractile parameters: ejection fraction (EF) (*Figure 3B*), rate of pressure development (+dP/dt) (*Figure 3C*) and preload recruited stroke work (PRSW) (*Figure 3D*). The depressed contractile parameters and cardiac output in Tm54 hearts were restored in DTG mice (*Figure 3B–E*). The diastolic function was also impaired in Tm54 mice as assessed by reduced rate of pressure decay (–dP/dt) (*Figure 3F*) and by the load-independent parameter end diastolic pressure volume relation (EDPVR) (*Figure 3G*). Overall, cardiac performance was near normal in DTG mice, shown by the normal values of cardiac output (*Figure 3E*).

Expression of ssTnI in Tm54 mice increases myofilament Ca^{2+} sensitivity and partially restores expression of β-MHC. We have previously reported that myofilaments from ssTnI mouse hearts are more sensitive to Ca^{2+}.[25] To test the degree to which expressing ssTnI in Tm54 hearts restored myofilament Ca^{2+} sensitivity and contributed to restoration of cardiac function in DTG mice, we evaluated the myofilament Ca^{2+} sensitivity in skinned fibres prepared from NTG, ssTnI, Tm54 and DTG mouse hearts (*Figure 4*). The pCa-force relations showed that myofilaments from Tm54 hearts had lower Ca^{2+} sensitivity (pCa_{50} = 5.75 ± 0.004; n = 8) than NTG (pCa_{50} =5.85 ± 0.002, n = 8), while ssTnI hearts had higher Ca^{2+} sensitivity (pCa_{50} = 6.10 ± 0.003, n = 8). The Ca^{2+} sensitivity of fibres from DTG hearts (pCa_{50} = 5.98 ± 0.003, n = 8) was significantly higher than Tm54 and NTG fibres (*Figure 4A* and *B*). The

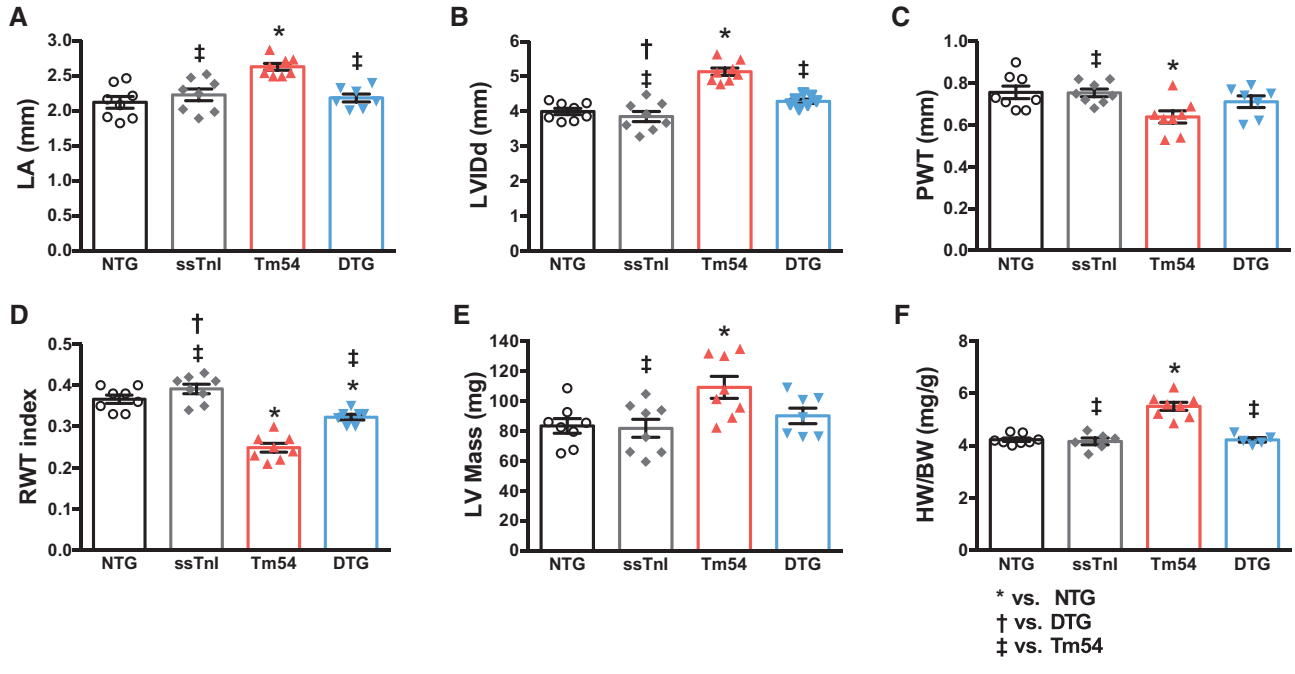

Figure 1 DTG mice show improved cardiac morphology compared to Tm54 mice. Cardiac morphology was evaluated by high resolution echocardiography (panels *A–E*) and whole heart gross morphology (panel *F*). (*A*) Left atrium (LA) size, (*B*) LV internal diastolic dimension (LVIDd), (*C*) posterior wall thickness (PWT), (*D*) relative wall thickness (RWT), (*E*) left ventricular (LV) calculated mass, and (*F*) heart weight to body weight (HW/BW) ratio. Data are presented as mean ± SE. *Significantly different from NTG, †significantly different from DTG, ‡significantly different from Tm54 based on post-hoc multiple comparison analysis (Newman-Keuls test). n = 7–8 per group.

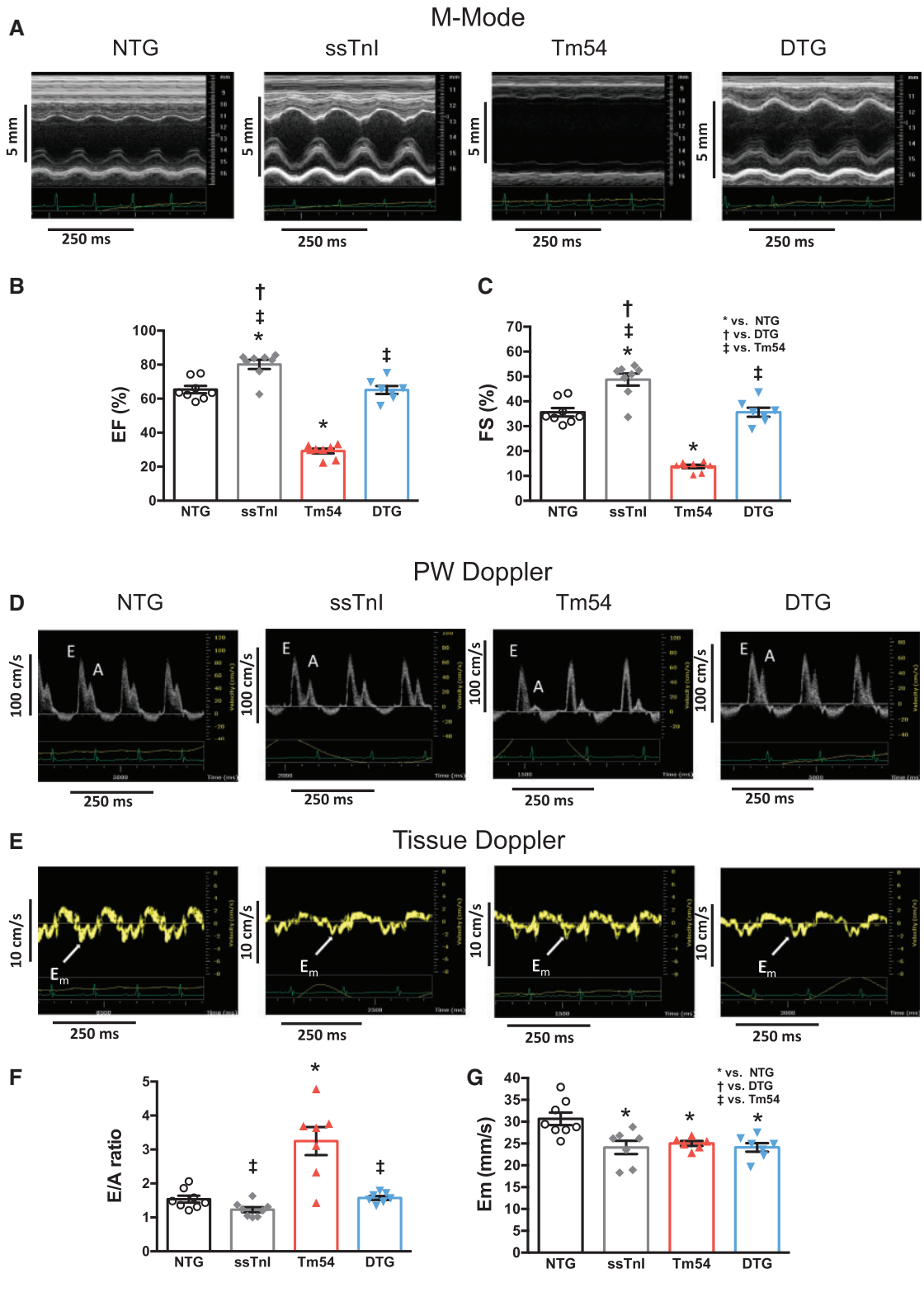

Figure 2 DTG mice show improved cardiac function compared to Tm54 mice. Systolic (panels *A–C*) and diastolic (panels *D–G*) functions were assessed by high resolution echocardiography. (*A*) representative M-mode echo images of all four group of mice, (*B*) Ejection fraction (EF) and (*C*) fractional shortening (FS) calculated from B-mode. (*D*) representative pulsed Doppler and (*E*) tissue Doppler images of four groups of mice, (*F*) peak velocity of mitral blood inflow in early diastole (E) to peak velocity of mitral blood inflow in late diastole (A) (E/A ratio), (*G*) peak myocardial velocity in early diastole (E_m). Data are presented as mean ± SE. *Significantly different from NTG, †significantly different from DTG, ‡significantly different from Tm54 based on post-hoc multiple comparison analysis (Newman-Keuls test). $n = 7–8$ per group.

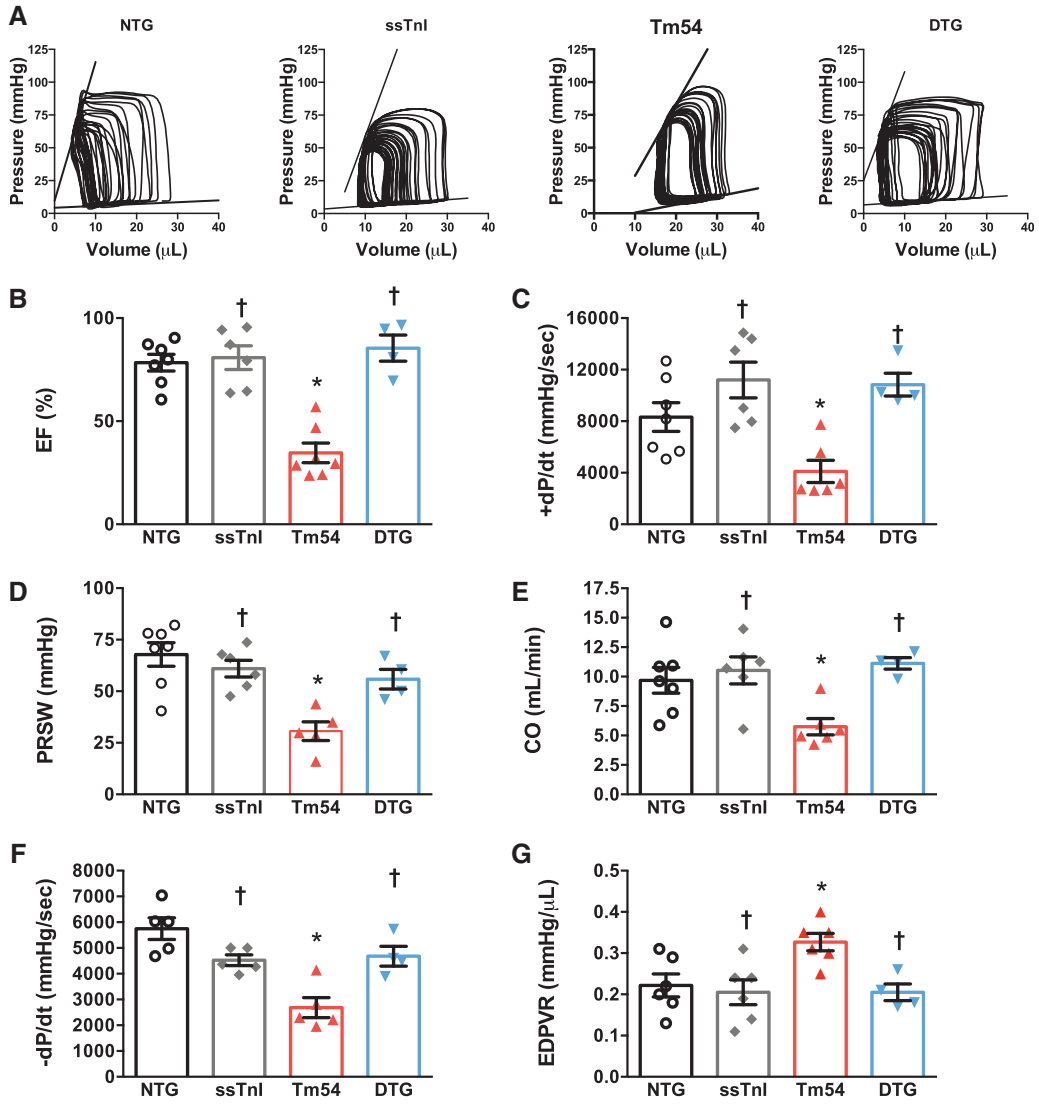

Figure 3 *In situ* cardiac function was improved in DTG compared to Tm54 mice. (*A*) Representative P-V loops in NTG, ssTnI, Tm54 and DTG mice, (*B*) ejection fraction (EF), (*C*) the maximal rate of contraction (+dP/dt), (*D*) Preload recruited stoke work (PRSW), (*E*) the cardiac output (CO), (*F*) maximal rate of relaxation (-dP/dt), and (*G*) end diastolic pressure-volume relation (EDPVR). Data are presented as mean ± SE. $P < 0.05$. *Significantly different from NTG and †significantly different from Tm54 based on post-hoc multiple comparison analysis (Newman-Keuls test). $n = 4$–8 per group.

Hill coefficient, an indicator of cooperativity or steepness of the pCa-force relationship, was lower in ssTnI fibres compared to other groups (*Figure 4C*). There were no significant differences in the maximal generated tensions between groups (*Figure 4D*). Since myofilament functional properties depend on expression of myofilament protein isoforms we assessed the level of expression of myosin heavy chain isoforms (*Figure 5A* and *B*). *Figure 5A* and *B* indicates that expression of β-MHC is significantly increased in Tm54 (38.5 ± 5.4%, $n = 6$) compared to NTG (7.09 ± 0.74%, $n = 6$) hearts and partially restored in DTG (19.2 ± 2.7%, $n = 6$) hearts. The ssTnI hearts expressed a normal level of βMHC (5.98 ± 0.67%, $n = 6$). Since Tm54 and ssTnI were co-expressed in DTG mice, we assessed the levels of expression of Tm54 in Tm54 and DTG hearts. There was no significant difference in Tm54 expression between hearts from Tm54 and DTG mice. The % replacement of WT Tm in the Tm54 and DTG was 43.2 ± 3.7% ($n = 6$) and 43.4 ± 5.4% ($n = 5$), respectively.

Expression of ssTnI in Tm54 mice decreases the overall phosphorylation level of TnI in the DTG group. *Figure 5C* summarizes M1–M4 modification levels of TnI calculated from 2D DIGE gels (*Figure 5D*). 2D-DIGE gels separate protein based on both molecular weight and isoelectric point and are TnI isoform independent, therefore the horizontal shifts are due to charge changes of the proteins. M1 through M4 spots are distinct charge variants of cTnI, the M indicates modified spot. Therefore M2 is more negatively charged than the M1 spot hence M4 spot is the most negatively charged cTnI spot detected. In previous studies it has been shown that the M1–M4 spots are phosphorylated entities based on phosphatase experiments.[37] Mice expressing ssTnI lack modifications M1–M4 due to no detectable phosphorylation in TnI likely due to the missing N-terminal cardiac extension of TnI. Only modifications in M1 and M3 are detected in the DTG group with significantly reduced levels compared to the NTG and Tm54 groups likely due to decreased cardiac

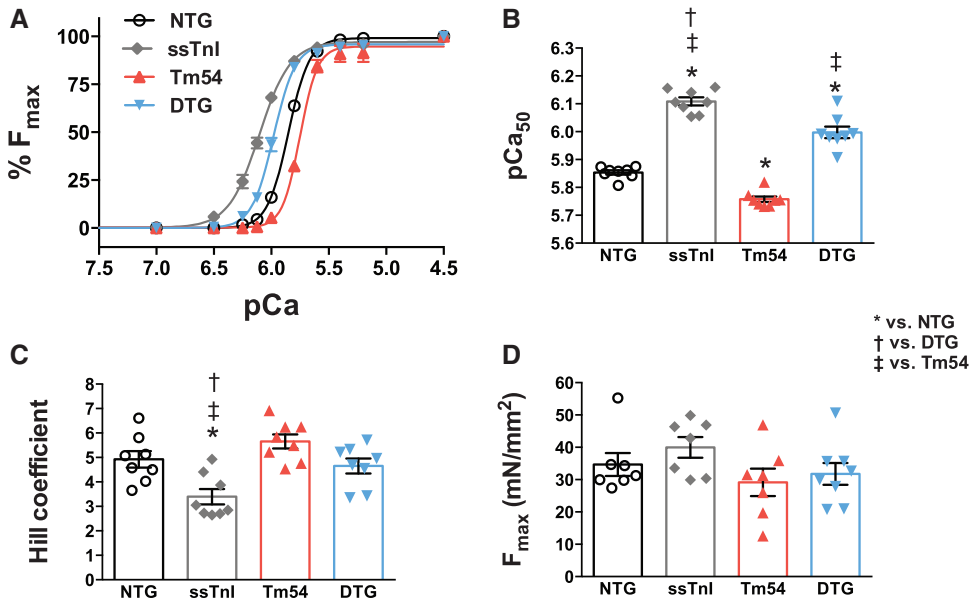

Figure 4 pCa-force relations in skinned fibre bundles indicate significant changes in Ca^{2+} sensitivity. (*A–B*) Expression of ssTnI in the presence of Tm54 mutations (DTG) caused an increase in the myofilament Ca^{2+} (pCa_{50}) sensitivity. (*C*) Hill coefficient and (*D*) Maximal tension (F_{max}). Data are presented as mean ± SE; $P < 0.05$. *Significantly different from NTG, †significantly different from DTG, ‡significantly different from Tm54 based on post-hoc multiple comparison analysis (Newman-Keuls test). $n = 8$ per group.

TnI expression and significantly lower levels of phosphorylation, thus M2 and M4 spots were below the level of detection (*Figure 5E*). In the DTG group the % replacement of cTnI with ssTnI was 78 ± 3% $n = 6$. No significant changes in modifications of TnT (see Supplementary material online, *Figure S1*), Tm (see Supplementary material online, *Figure S2*), MLC_2 (see Supplementary material online, *Figure S3*) and MyBP-C (see Supplementary material online, *Figure S4*) were detected by 2D-DIGE.

Expression of ssTnI in Tm54 hearts results in increased phosphorylation of ERK1 at T202 and Y204. We have previously shown that activity of ERK1/2 and GATA4 is altered in the HCM mouse model which bears a mutation in Tm at position 180,[34] and likely contributes to hypertrophic remodeling. Whether this may also occur in DCM models with eccentric hypertrophy is yet unknown. Therefore, to address this question, and further assess whether signaling may be attenuated in our DTG mice, we assessed both ERK1/2 and GATA4 levels in hearts from all four groups of mice. *Figure 6A* and *C* shows that the level of phosphorylation of ERK1 was higher in ssTnI and DTG hearts compared to NTG group. Although ERK2 phosphorylation trended to increase in these two groups as well, no significant changes were found (*Figure 6B* and *C*). In addition, there were no observed differences in the phosphorylation of GATA4 (Ser105), Akt (Ser473) or GSK3α/β (Ser21/9) between groups (*Figure 7*).

4. Discussion

A major novel finding from experiments reported here is the demonstration that an early intervention restoring myofilament Ca^{2+} sensitivity in familial DCM is able to prevent progression of the disorder. Although our approach involved an intervention with pleiotropic effects, a major aspect of our use of expression of ssTnI in the DCM54 model is an increase in myofilament Ca^{2+} sensitivity. Thus, our data provide proof of principle for the development of therapies more specifically affecting sarcomeres than traditional therapies in current use. These therapies comprise a combination of angiotensin converting enzyme (ACE) inhibitors, angiotensin II (AngII) receptor blockers, β-blockers, aldosterone antagonists and diuretics.[4] Despite a need for new specific treatments for DCM, there are few *in vivo* studies in mouse models with DCM and these studies are limited to one model of DCM with a deletion mutation ΔK210 in TnT.[6,7,8,9,38] Moreover, some of the studies tested the traditional therapies such as β- and angiotensin II receptor (ARB) blockers.[7,9] Zhang *et al.*[7] reported that treatment of TnT ΔK210 DCM mice with β_1-selective β-blocker metoprolol was able to prolong survival, reduce myocardial remodeling and cardiac dysfunction compared to vehicle treated DCM mice, but treated DCM mice were still morphologically and functionally different to a significant degree from age-matched wild type (WT) mice. These beneficial effects were not seen in mice treated with non-specific β-blocker carvedilol and hydrophilic β_1-selective β-blocker atenolol. In another study using ΔK210 TnT mouse model Odagiri *et al.*[9] have shown that candesartan, an ARB blocker also had a beneficial effect on improving survival rate, cardiac histology and preventing some electrical remodeling, but most of the hemodynamic parameters remained maladaptive when compared with WT age matched mice. As a potential new treatment it was recently reported that ghrelin, a growth-hormone releasing peptide has the beneficial effects in the ΔK210 TnT mouse model, however there was only partial restoration of morphological and functional parameters in the treated DCM mice.[38]

Since most of the mutations that are linked to DCMs show decreased sarcomere activation resulting in systolic dysfunction and HF, interventions that increase myofilament sensitivity to Ca^{2+} should be studied in

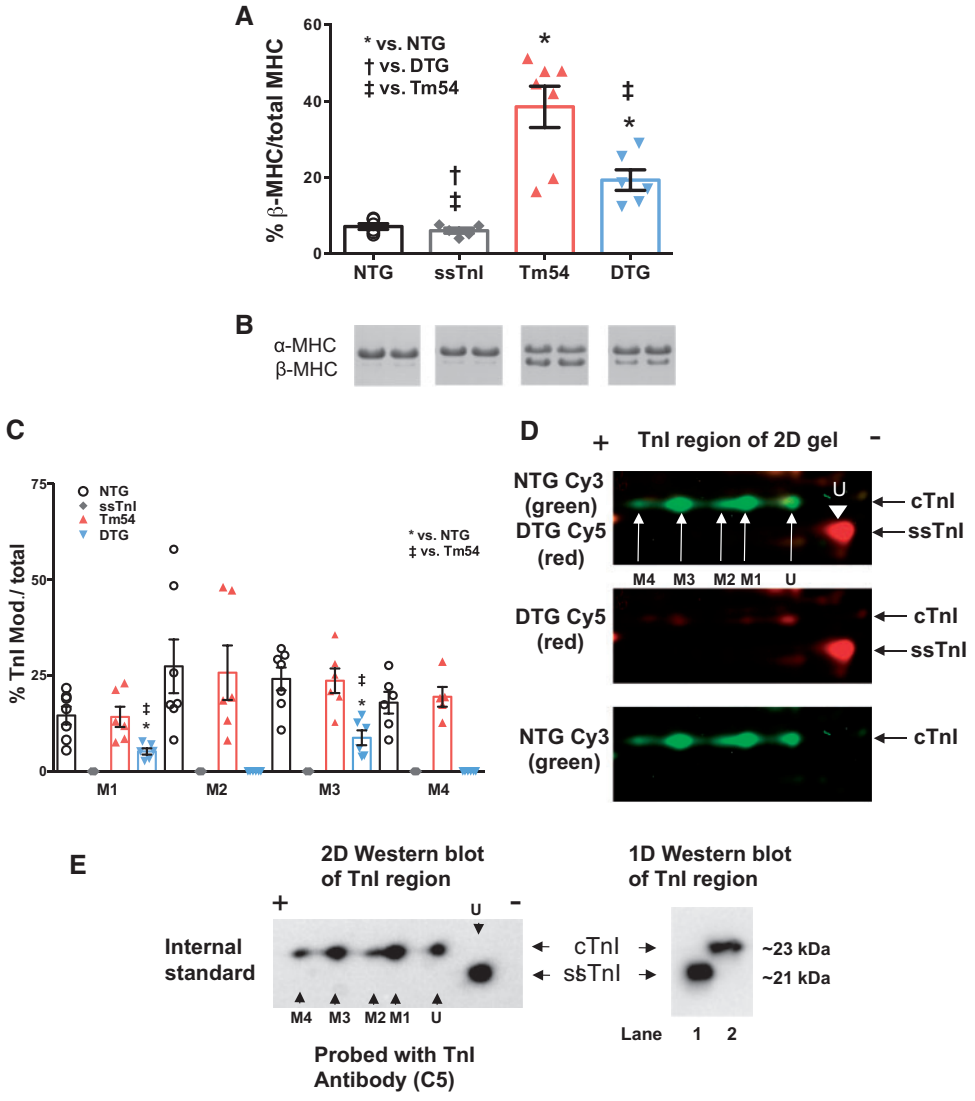

Figure 5 Expression of cardiac β-myosin heavy chain (MHC) was significantly reduced in DTG vs. Tm54 via SDS_PAGE analysis and troponin I (TnI) analysis via 2D-DIGE revealed significantly reduced overall phosphorylation of DTG vs. Tm54. Data shown as mean ± SEM; $P < 0.05$, *Significantly different from NTG, †significantly different from DTG, ‡significantly different from Tm54 based on an 1-way ANOVA with a Newman-Keuls post-hoc test. $n = 5–7$ per group. (*A*) % expression of β-MHC in each group of mice and (*B*) two representative lanes of MHC from each group of mice stained with Coomassie blue. (*C*) Histogram of the 2D-DIGE TnI quantitation (*D*) Representative 2D-DIGE region of interest image comparing the NTG vs. DTG (Tm54 + ssTnI) shown in green and red respectively. (*E*) Representative Western blot analysis of both 1D and 2D blots probed with troponin I C5 antibody recognizing both ssTnI and cTnI. cTnI = cardiac troponin I, ssTnI = slow skeletal troponin I, M = site of modification, U= unmodified spot. The 2D blot confirms spots in the 2D-DIGE are troponin I and ssTnI has only one unmodified spot. Lane 1 represents ssTnI sample and lane 2 represents cTnI sample. The 1D blot confirms ssTnI samples do not contain cTnI. Note the modifications 1-4 are likely phosphorylation based on previous phosphatase data,[37] but may also include other modifications.

different DCM mouse models. We and others have previously shown that in HCM and RCM mouse models that display increased myofilament sensitivity to Ca^{2+} bringing sensitivity closer to normal levels has beneficial effects.[33,34,39,40] Here, we provided support for the concept that early manipulation in myofilament Ca^{2+} sensitivity in a DCM model is also beneficial and can significantly prevent both morphological (*Figure 1*) and functional (*Figure 2 and 3*) changes. At 5 months of age DTG mice do not show any dilation and most systolic and diastolic parameters are not different from NTG mice. These beneficial changes are associated with increased myofilament sensitivity to Ca^{2+} slightly above the NTG level (*Figure 4*) at the same level of Tm54 expression. Importantly, this slight over sensitization did not result in any occurrence of arrhythmias during the echocardiographic assessments or episodes of SCD in DTG mice that we observed up to 5 months which was the time of final non-survival experiment. It has been previously reported that increased myofilament Ca^{2+} sensitivity causes susceptibility to cardiac arrhythmia in mice.[41,42] Although over sensitization of myofilament to Ca^{2+} can be dangerous and should be taken into account with regard to the

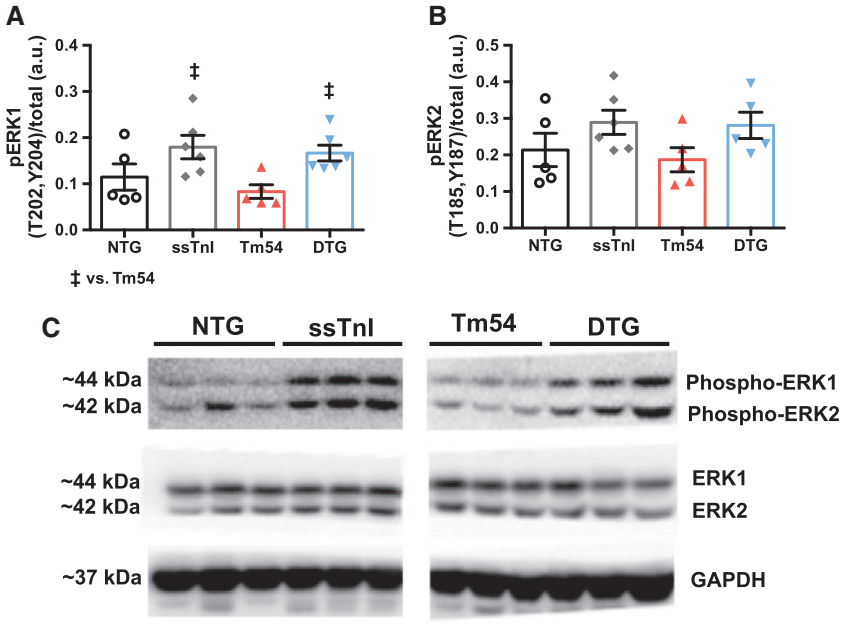

Figure 6 Phosphorylation of ERK1 was significantly increased in the DTG vs. Tm54 and pERK2 was increased in DTG vs. Tm54 but not significantly. (*A*) phosphorylation of ERK1 and (*B*) phosphorylation of ERK2. Data are presented as mean ± SE. Phosphorylated protein bands were normalized to total protein. No significant differences in total protein expression were observed. ‡significantly different from Tm54 based on post-hoc multiple comparison analysis (Newman-Keuls test). *n* = 5-6 per group. (*C*) Representative western blots for ERK1/2.

development of new therapies and establishing the proper therapeutic window of new drugs, in normal physiological conditions myofilaments sensitivity changes and they operate in a specific zone of Ca^{2+} sensitivity.

This increased myofilament Ca^{2+} sensitivity observed in DTG mice can be explained mainly by expression of ssTnI instead of cTnI (*Figure 5*) and was not associated with changes in phosphorylation of TnT, Tm, myosin binding protein C, or myosin light chain 2 (see Supplementary material online, *Figures S1–4*). Moreover, we found no significant modifications in cTnI between NTG and Tm54 groups. As predicted, overall phosphorylation of TnI was reduced in DTG mice (*Figure 5C* and *E*), inasmuch as they mainly express ssTnI, which cannot be phosphorylated by PKA.[24,25] In addition we found that Tm54 mice express β-MHC and its level was significantly reduced in DTG mice (*Figure 5A* and *B*). Expression of β-MHC was also reported in other mouse models of DCM ΔK210 TnT.[7] Although, the DTG mice still express higher relative amounts of β-myosin in the myosin isoform population compared to NTG group, they show improved relaxation. There are several potential mechanisms that may contribute to this observation. Overall, DTG hearts show reduced remodeling compared to Tm54 hearts that may in part explain our finding that expression of ssTnI enhanced myocardial relaxation in the DTG model despite increased sensitivity to Ca^{2+}. Moreover, the DTG mice most likely maintain better responsiveness to β-adrenergic stimulation. The better responsiveness to β-adrenergic stimulation would allow increasing the rate of relaxation through phosphorylation of other cellular proteins such as phosholamban. It is also possible that the Ca^{2+} fluxes are altered in Tm54 mice and maintained close to normal in DTG mice.

It has been previously suggested that mutations that cause DCM abolish the relationship between TnI phosphorylation and myofilament Ca^{2+} sensitivity and that re-coupling by pharmacological drugs may be of potential therapeutic significance for treating of cardiomyopathies.[16,43] Our data as well as others[6] suggest that this uncoupling between TnI phosphorylation and myofilament Ca^{2+} sensitivity may be present in some, but not all DCM cases. It should be noted that in the work reported by Memo *et al.*[16] a reconstituted system with human heart muscle TPM1 E54K demonstrated a Ca^{2+} response similar to controls. Although this finding may indicate a difference between the human and mouse mutant Tm, this conclusion is made difficult by the use of an unloaded system lacking a full complement of sarcomeric proteins, especially titin and myosin binding protein C. In other words, the different findings reported here may reflect the significance of investigating full native preparations rather than reconstituted preparations. Clearly more work is necessary with the human samples to sort this out.

The increased Ca^{2+} sensitivity in Tm54 myofilaments controlled by ssTnI resulted not only in improved heart morphology and function, but also increased pERK1 and a trend toward increased pERK2 compared to Tm54 mice. The role of ERK1/2 in regulating the balance between concentric vs. eccentric hypertrophy has been reported using mice either lacking ERK1/2 or expressing activated Mek1.[44] The authors showed that activation of the ERK1/2 pathway induced concentric hypertrophy, whereas inhibition of ERK1/2 pathway resulted in eccentric hypertrophy as it is seen in pressure vs. volume overload hypertrophy. We and others have shown that HCM is associated with increased ERK1/2 activity and can be reversed in rescued models.[33,34,45,46]

Although, there are some studies in treating the ΔK210 TnT mouse model with the Ca^{2+} sensitizers, pimobendan, they are limited. Du *et al.*[6] reported that treatment of 4-week and 20-week old DCM ΔK210 TnT mice for 4 weeks with pimobendan, improved cardiac function and prolonged survival, but the authors presented only a limited set of data and these data were mainly for treatment of 4-week old mice. Moreover,

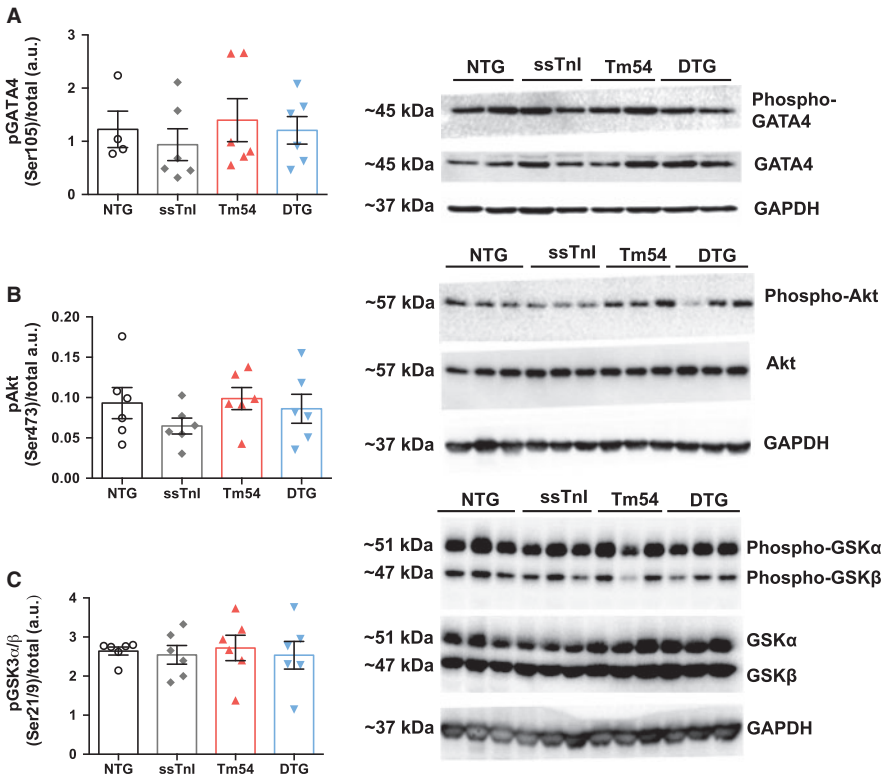

Figure 7 Phosphorylation of GATA4, Akt and GSK3α/β was not significantly altered. (*A*) phosphorylation of GATA4 at Ser105, (*B*) phosphorylation of Akt at Ser473, and (*C*) phosphorylation of GSK3α/β at Ser21/9. Data are presented as mean ± SE $n = 4–6$ per group. Phosphorylated protein bands were normalized to total protein. No significant differences in total protein expression or phosphorylation levels were observed.

pimobendan is also an inhibitor of phoshodieterase III (PDE3), which raises the concern of increased risk of arrhythmia.[47] Recently, Du *et al.*[8] reported that propyl gallate, a phenolic antioxidant in the same DCM TnT mouse model was able to increase survival, reduce pathological remodeling and improve some hemodynamic parameters, but most of the parameters were still different from the WT mice demonstrating persistent maladaptation.

Although our data provide proof of principle regarding the effectiveness of early intervention in preventing the progression of the disorders in DCM, there are limitations to our interpretations of the mechanistic basis of our findings. The effects of ssTnI expression may be better considered to be pleiotropic rather than specific with regard to enhanced Ca^{2+} sensitivity. Expression of ssTnI in the adult heart has been demonstrated to have effects other than increasing myofilament response to Ca^{2+}. These include effects to reduce length dependence of activation[24] as well as induction of metabolic remodeling.[27] Moreover, ssTnI lacks both PKA and PKC phosphorylation sites that may modulate contraction in DCM. The extent to which these other effects act in synergy with Ca^{2+} sensitization is not clear from our study. It is apparent that treatment with a specific sarcomere activator such Omecamtiv Mecarbil, without PDE III inhibition, may provide more definitive evidence that specific effects on myofilament response to Ca^{2+} are able to prevent DCM progression. Preliminary studies with skinned fibres[48] and human inducible pluripotent stem cells[49] indicate that Omecamtiv may in fact be effective. Even so it remains to be determined whether long term Ca^{2+} sensitization induces remodeling similar to that seen with long term ssTnI expression.

In summary, we have demonstrated that early sensitization of myofilaments to Ca^{2+} should be considered as a new and promising therapeutic intervention for DCM caused by mutations in thin filament proteins that show decreased myofilament Ca^{2+} sensitivity. Modification of TnI signaling in the myofilaments is an obvious and important target. It has been reported that a small-molecule activator of fast skeletal troponin is able to sensitize myofilaments and offers hope of a therapy for disorders of skeletal muscle.[50] It will be important to test thin filament activators in other DCM models, especially those linked to mutations in the thick filaments and titin. Moreover, there are no specific early interventions in children from families with familial cardiomyopathies,[51] although early identification of HCM and DCM mutations are possible and more common.[52] Our data point to the importance of taking advantage of early childhood diagnosis and to start prevention therapy before the disease develops. A first pilot randomized trial to modify the HCM diseases with diltiazem by treating carriers without LVH has just been published suggesting that pre-clinical administration of diltiazem is safe and may improve LV remodeling[53] supporting our conclusion for early treatment of DCM patients before the development of the phenotype.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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