Phosphorylation of cardiac sarcomeric proteins plays a major role in the regulation of the physiological performance of the heart. Phosphorylation of thin filament proteins, such as troponin I and T, dramatically affects calcium sensitivity of the myofiber and systolic and diastolic functions. Phosphorylation of the regulatory protein tropomyosin (Tpm) results in altered biochemical properties of contraction; however, little is known about the physiological effect of Tpm phosphorylation on cardiac function. To address the in vivo significance of Tpm phosphorylation, here we generated transgenic mouse lines having a phosphomimetic substitution in the phosphorylation site of α-Tpm (S283D). High expression of Tpm S283D variant in one transgenic mouse line resulted in an increased heart:body weight ratio, coupled with decreased expression of extracellular signal-regulated kinase 1/2 kinase signaling. Physiological analysis revealed that phosphorylated Tpm impairs diastolic function in the intact heart, without altering calcium sensitivity or cooperativity of myofibers. Our findings provide the first extensive in vivo assessment of Tpm phosphorylation in the heart and its functional role in cardiac performance.

Phosphorylation plays a major role in the regulation of cardiac function by affecting numerous membrane, cytoplasmic, and sarcomeric proteins. In the heart, phosphorylation of cardiac troponin I, cardiac troponin T, myosin-binding protein C, and titin regulate myofibrillar contraction, relaxation, and cross-bridge cycling (1–3). In contrast, relatively little is known regarding the importance of tropomyosin (Tpm) phosphorylation. Previous investigations established that Tpm is phosphorylated at serine 283, the penultimate amino acid of the protein (4–8). Tpm phosphorylation in the heart is developmentally regulated, with phosphorylated α-Tpm being the predominant isof orm during fetal and newborn stages (~60–70%); in the adult mouse heart, the level of α-Tpm phosphorylation is decreased to ~30% (3). To address this developmental decrease in cardiac Tpm phosphorylation, we previously found there is a continuous decrease after birth until 5 months, whereupon increases occur by 15 months (5). We, and others, determined there is differential Tpm phosphorylation within the four cardiac chambers, with atria having the highest levels of phosphorylated Tpm (9). In vitro investigations by Heeley, Mak, and co-workers (6–8) reveal that phosphorylated α-Tpm enhances head-to-tail polymerization in the overlap region of adjacent Tpm molecules that enhances the interaction of Tpm with cardiac troponin T. Although phosphorylation does not appear to affect F-actin binding properties of
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Tpm, the actin-activated myosin S1 ATPase activity is significantly higher with phosphorylated Tpm (10). In addition, Tpm phosphorylation appears to increase bond duration between actin–myosin interactions (11). Thus, phosphorylated Tpm may have an allosteric effect on actin to modify the actin–myosin interaction between the blocked, closed, and open states (12, 13), with a possibility of increasing the number of closed states and providing additional contact points for myosin (11). We have further demonstrated that Tpm pseudo-phosphorylation slows muscle relaxation at both reconstituted thin filament and myofibril levels without altering reconstituted thin filament calcium sensitivity (10). This process is facilitated by long-range cooperative activation along the thin filament (14). Together, these biochemical data suggest Tpm phosphorylation modulates muscle function by altering relaxation without altering contraction (11, 15).

Although various biochemical properties are attributed to phosphorylated Tpm based on in vitro studies, the in vivo significance of this post-translational modification is unknown. To address the functional significance of Tpm phosphorylation on sarcomeric and cardiac performance, we generated transgenic mice that express a phosphomimetic α-Tpm protein (α-Tpm S283D) specifically in their hearts. Exogenous protein expression from the transgene ranges from 40 to 90%, dependent upon the transgenic mouse line. Morphological analysis shows there are significant cardiomyopathic changes in the hearts, including dilation of the ventricular chambers, an increased heart:body weight ratio, with mild fibrosis. Physiological analysis shows there is diastolic dysfunction that is maintained with β-adrenergic stimulation. Surprisingly, there are no changes in measures of cardiac contraction, sarcomeric pCa–force relations, ATPase activity, or cooperativity. In addition, an examination of the kinases associated with Tpm phosphorylation found casein kinase 2 is integrally involved in this process. These studies provide the first extensive in vivo assessment of Tpm phosphorylation in the heart and its functional role in influencing cardiac performance.

Results

Generation of α-Tpm S283D TG mice

To assess the in vivo significance of Tpm phosphorylation in the heart, we generated and analyzed four transgenic (TG) mouse lines that express a phosphomimetic α-Tpm protein (α-Tpm S283D) specifically in their hearts. Exogenous protein expression from the transgene ranges from 40 to 90%, dependent upon the transgenic mouse line. Morphological analysis shows there are significant cardiomyopathic changes in the hearts, including dilation of the ventricular chambers, an increased heart:body weight ratio, with mild fibrosis. Physiological analysis shows there is diastolic dysfunction that is maintained with β-adrenergic stimulation. Surprisingly, there are no changes in measures of cardiac contraction, sarcomeric pCa–force relations, ATPase activity, or cooperativity. In addition, an examination of the kinases associated with Tpm phosphorylation found casein kinase 2 is integrally involved in this process. These studies provide the first extensive in vivo assessment of Tpm phosphorylation in the heart and its functional role in influencing cardiac performance.

Cardiac α-Tpm S283D transcript and protein expression in TG mice

Message levels from the transgene and control transcripts were assayed using Northern blotting hybridization. Transcript levels of the transgene were highest in lines 143 and 149, which correlates with their corresponding transgene copy numbers, whereas the expression of the endogenous α-Tpm mRNA was similar in the nontransgenic (NTG) and transgenic mouse hearts (data not shown).

To determine α-Tpm S283D protein expression, myofibrillar proteins from NTG and TG hearts were run on one-dimensional IEF gels (PI: 4.2–4.9) and subjected to Western blotting analysis using a striated muscle Tpm specific antibody. This process is facilitated by long-range cooperative activation along the thin filament (14). Together, these biochemical data suggest Tpm phosphorylation modulates muscle function by altering relaxation without altering contraction (11, 15).

Figure 1. A, DNA construct used for generating Tpm S283D mice. B, genomic Southern blotting showing TG DNA band. C, Western blotting (from an IEF slab gel) of cardiac myofibrillar proteins immune-reacted with the CH1 Tpm specific antibody. D, Western blotting (repeat of C) with the high exposure, showing the levels of endogenous α-Tpm. E, Western blotting (from an IEF slab gel) of cardiac myofibrillar proteins treated with phosphatase and immune-reacted with a Tpm antibody. HGH, human growth hormone 3’-UTR.

copies. To negate the possibility that a deletion or mutation occurred in the α-Tpm S283D DNA sequences during transgenesis, we conducted nucleotide sequencing of Tpm mouse genomic DNA using construct-specific primers. The results verified the presence of the designed mutation and indicated no additional changes in α-Tpm sequence.

Figure 1. A, DNA construct used for generating Tpm S283D mice. B, genomic Southern blotting showing TG DNA band. C, Western blotting (from an IEF slab gel) of cardiac myofibrillar proteins immune-reacted with the CH1 Tpm specific antibody. D, Western blotting (repeat of C) with the high exposure, showing the levels of endogenous α-Tpm. E, Western blotting (from an IEF slab gel) of cardiac myofibrillar proteins treated with phosphatase and immune-reacted with a Tpm antibody. HGH, human growth hormone 3’-UTR.
the endogenous α-Tpm isoforms are visible in all samples, along with the predominant exogenous α-Tpm S283D protein in the TG samples (Fig. 1D). Surprisingly, expression of all three protein species is very high in line 954, whereas there is a significant decrease in endogenous Tpm expression in the other TG lines. The reason for the increased total Tpm expression in line 954 is unknown. When the myofibrillar protein samples were subject to phosphatase treatment prior to electrophoresis, the NTG sample shows a single band (Fig. 1E). Under these dephosphorylating conditions, TG line 149 shows only the α-Tpm S283D band, whereas the remaining TG hearts express both α-Tpm S283D and endogenous α-Tpm in varying amounts. Quantification of these bands demonstrates that the percentage of transgenic/endogenous Tpm protein expression is highest in line 149 (100%), and more moderate in lines 143, 951, and 954 (90, 67, and 43%, respectively). These protein expression levels are consistent with the data from the genomic Southern blotting analysis.

**Dilated cardiomyopathy in response to α-Tpm S283D expression**

Morphological examination of the α-Tpm S283D TG mouse hearts was conducted at various time intervals from 0.5 to 5 months after birth. Most high expression α-Tpm S283D protein mice (line 149) develop a severe dilated cardiac phenotype by 15 days postpartum (Fig. 2, A–C). In addition, there is an increased heart:body weight ratio with these mice: 2.00 ± 0.12 versus 0.62 ± 0.04 for TG versus control littermates (p < 0.001) (Fig. 2D). Many of these mice die by 1 month postpartum. In the moderate expression TG lines (lines 143, 951, and 954), a less severe dilated cardiomyopathy phenotype develops by 6 months with the hearts exhibiting mild myocyte hypertrophy and fibrosis. This moderate phenotype does not appear to progress to a more severe condition, even after 1 year. Surprisingly, there are no significant differences in the heart:body weight ratio between NTG and moderate expression TG mice at either 3 or 6 months of age.

**Work-performing heart model**

The work-performing heart model was used to obtain an ex vivo assessment of cardiac performance (19). These measurements were conducted on TG mouse hearts at 6 months of age. As seen in Fig. 3 and Table 1, the rate of relaxation is significantly reduced in the TG lines, concomitant with an increase in half-time to relaxation. There were no differences in the heart rates, nor in the rates of contraction or time to peak pressure. Previous work demonstrates that TG mice that overexpress WT α-Tpm show no significant alterations in cardiac function when compared with NTG control mice (17, 18).

We also determined responses to isoproterenol to ascertain whether the observed diastolic dysfunction was associated with impaired β-adrenergic responses. The reduced rate of relaxation by hearts was assessed during stimulation with isoproterenol, a β-adrenergic agonist that augments muscle contraction and relaxation by cAMP/protein kinase A-dependent kinase. Interestingly, a blunted response was observed in relaxation performance in the TG lines at all concentrations of isoproterenol (10⁻¹¹ to 10⁻⁷ mol/liter) (Fig. 4).

The Starling law reflects the increase in cardiac performance in response to increased intraventricular pressure or volume load. As cardiac muscle is stretched, cardiac myofibers respond with increased force generation. The NTG and TG hearts...
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![Graphs showing physiological analyses of control and S283D left ventricular function.]

Table 1

| Parameters | Wild type (n=6) | TG line 143 (n=5) | TG line 149 (n=3) | TG line 954 (n=5) |
|------------|----------------|-------------------|-------------------|-------------------|
| SP, mmHg   | 149±6          | 139±7             | 144±12            | 141±5             |
| DP, mmHg   | 4.3±1.3        | 11.5±2.2          | 10.5±3.5          | 5.0±1.7           |
| +dP/dt, mmHg/s | 4419±303 | 3970±285          | 4167±335          | 3917±412          |
| −dP/dt, mmHg/s | 3571±166 | 2349±140          | 2596±360          | 2822±151          |
| TPP, ms/mmHg | 0.35±0.01   | 0.39±0.02         | 0.38±0.02         | 0.38±0.03         |
| RT, ms/mmHg | 0.56±0.04    | 0.74±0.03         | 0.7±0.03          | 0.72±0.05         |
| HR, beats/min | 298±9      | 295±10            | 295±10            | 285±18            |

* P<0.05 Line 143, 955, 149 versus NTG
** P<0.01 Line 143, 954, 149 versus NTG

Figure 3. Physiological analyses of control and S283D left ventricular function. A comparison of contractile parameters in NTG and S283D mouse hearts at 6 months is shown. B and D, rates of relaxation (−dP/dt) (B) are significantly reduced, and the time to half-relaxation (RT) (D) increased in the TG mouse hearts. A and C, rates of contraction (+dP/dt) (A) and the TPP (C) are unchanged.

Table 1 Contractile parameters in WT and S283D TG mice

SP, left ventricular systolic pressure; DP, left ventricular diastolic pressure; EDP, left ventricular diastolic pressure; +dP/dt, maximal rate pressure development; −dP/dt, maximal rate pressure decline; TPP, time to peak pressure, normalized to half relaxation pressure; HR, heart rate; n, number of mice.

To examine the correlation between physiological results from whole hearts versus sarcomeres, experiments were conducted to compare the relation between Ca²⁺ and tension developed by myofilaments obtained from control versus α-Tpm S283D left ventricular fiber bundles of 4.5–6-month-old TG mice. In the first set of experiments, we compared the pCa-tension relations for fiber bundles obtained from NTG (n=8), and moderate expression TG line 954 (n=10), and line 143 (n=8) mice. As shown in Fig. 6A, there are no significant changes in the maximum tension, pCa₅₀ (log of free [Ca²⁺]), or cooperativity as measured by the Hill coefficient between the NTG and TG fiber bundles. Measurements of pCa-ATPase activity show there are no significant differences regarding ATPase activity in response to increasing Ca²⁺ concentrations, the Hill coefficient, or the pCa₅₀ values between the TG and NTG myofibers (Fig. 6C). In addition, there is no difference between the TG and NTG myofibers with respect to tension cost (ATPase production and increased tension) (Fig. 6D).

ERK signaling in S283D mice

The ERK1/2 signaling pathway regulates a balance between concentric (hypertrophic cardiomyopathy) and eccentric (dilated cardiomyopathy) growth (20). In response to cardiomyopathic stimulation, several MAPK signaling pathways activate downstream kinases, including JNKs and ERKs (21, 22). Previous work established a correlation between activation of the ERK1/2 pathway and induction of concentric hypertrophy, whereas inhibition of the ERK pathway results in eccentric hypertrophy (20). To address whether there are alterations in the ERK pathway in the dilated cardiomyopathic TG S283D mice (line 143), we assayed expression of ERK1/2 (total levels), phosphorylated ERK1/2, phosphorylated 90-kDa ribosomal S6

reveal a positive correlation between workload and functional parameters of cardiac performance (data not shown). The TG myocardium worked at a similar rate of contraction; however, the TG hearts worked at a lower rate of relaxation. The slopes of the regression curves for the NTG and TG hearts were not different, indicating that the maximal −dP/dt and +dP/dt were sensitive to length-dependent regulation to an extent similar to that in the NTG hearts (Fig. 5).

Ca²⁺-force measurements in skinned fiber bundles

To examine the correlation between physiological results from whole hearts versus sarcomeres, experiments were conducted to compare the relation between Ca²⁺ and tension developed by myofilaments obtained from control versus α-Tpm S283D left ventricular fiber bundles of 4.5–6-month-old TG mice. In the first set of experiments, we compared the pCa-tension relations for fiber bundles obtained from NTG (n=8), and moderate expression TG line 954 (n=10), and line 143 (n=8) mice. As shown in Fig. 6A, there are no significant changes in the maximum tension, pCa₅₀ (log of free [Ca²⁺]), or cooperativity as measured by the Hill coefficient between the NTG and TG fiber bundles. Measurements of pCa-ATPase activity show there are no significant differences regarding ATPase activity in response to increasing Ca²⁺ concentrations, the Hill coefficient, or the pCa₅₀ values between the TG and NTG myofibers (Fig. 6C). In addition, there is no difference between the TG and NTG myofibers with respect to tension cost (ATPase production and increased tension) (Fig. 6D).

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kinase 3 (RSK3), and JNK1. The results show there are decreased expression levels in all of these kinases (Fig. 7), which correlates with decreased levels of expression in other mouse models of dilated cardiomyopathy, including the Tpm 54 model (23).

Casein kinase and Tpm phosphorylation

To investigate the biochemical process associated with Tpm phosphorylation, we conducted a computer-based phosphorylation motif search for kinases involved in Tpm phosphorylation.
tion that revealed several potential candidates. The initial search suggested that protein kinase C (PKC) and casein kinase 2 (CK2) may be involved in Tpm phosphorylation (Table S1). To test these candidates, Dr. J. Molkentin generously provided heart samples from knockout mice lacking PKCα(−/−), PKCβ(−/−), and PKC αγ (triple null). Western blots reveal

|          | F_{\text{max}} (mN/mm²) | Tension | nHill | pCa_{50} | n |
|----------|-------------------------|---------|-------|----------|---|
| NTG      | 25.67±1.46              | 2.77±0.25 | 5.79±0.03 | 8  |
| TG Line 954 | 24.62±1.07              | 2.58±0.14 | 5.82±0.01 | 10 |
| TG Line 143 | 27.13±1.69              | 2.93±0.14 | 5.81±0.02 | 8  |
| NTG      | 24.10±1.45              | 5.27±2.45 | 5.78±0.06 | 4  |
| TG Line 149 | 27.27±3.02              | 3.15±0.20 | 5.81±0.03 | 7  |

Figure 6. pCa–tension relations and ATPase activity of skinned fiber preparations obtained from control and S283D hearts. A and B, pCa force measurements. The maximum developed force, Hill coefficient, and pCa_{50} (half-maximum activation) were similar between NTG and TG skinned fiber bundles. C and D, pCa-ATPase measurements. ATPase production was measured with a −log of free [Ca²⁺] in the NTG and TG fiber bundles. There is no significant difference in maximum ATPase activities. E and F, stress-ATPase measurements in TG and NTG myofilaments. ATPase production was plotted as a function of stress. There is no significant difference between NTG and TG values. The moderate expression TG lines (lines 954 and 143) are in A, C, and E; the high expression TG line (line 149) is in B, D, and F. n = number of fibers. The results are means ± S.D.
that Tpm phosphorylation levels are not altered in these hearts, demonstrating that these PKC isoforms are not involved in this process (Fig. S1).

Previous work showed that CK2 can phosphorylate cytoplasmic Tpm (24). To test the hypothesis that CK2 is also involved in striated muscle α-Tpm phosphorylation, we conducted in vitro biochemical experiments using recombinant α-Tpm in combination with CK2 enzyme. Results show CK2 can phosphorylate recombinant Tpm and be inhibited by TBB and heparin (Fig. 8, A and B) (25). These reactions were performed with GTP as the sole energy source, another selective feature of CK2. This work demonstrates the ability of CK2 kinase to phosphorylate striated muscle Tpm. Also, results show that recombinant Tpm S283A protein (where the penultimate serine residue is changed to an alanine) is not phosphorylated by CK2 (Fig. 8C) and that CK2 holoenzyme phosphorylates Tpm more efficiently than CK2α alone (Fig. 8D).

Because our data demonstrate CK2 can phosphorylate Tpm in vitro, we sought to determine whether this occurs in cardiac muscle tissue. Toward this end, we determined whether Tpm forms a protein complex with CK2 (CKII) in mouse cardiac whole homogenates. Immunoprecipitation with the CH1 Tpm specific antibody removed all striated muscle Tpm from the heart homogenates. As shown in Fig. 9 (A and B), both Tpm and CK2 (CK2β) are detected when immunoprecipitated with Tpm and then probed with their respective antibodies. Fig. 9 (C and D) shows Tpm and CK2 were detected when heart homogenates are immunoprecipitated with CK2β antibody and probed with their respective antibodies. Immunoprecipitating and subsequently detecting with the same antibody (Fig. 9, A and D) was for quality control because immunoprecipitating protein should be also detected along with any binding partners. Thus, both Tpm and CK2 associate and form a protein complex in the heart.

To confirm the interaction between Tpm and CK2, we conducted a yeast two-hybrid assay where CK2 was the “bait” and α-Tpm was the “prey,” and they were cloned on separate plasmids. Both plasmids were transformed together into yeast, and the interaction of CK2 and Tpm was tested on nutritional selec-
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Figure 9. Tpm associates with CK2 (CKII) in the heart. Western blotting of immunoprecipitates generated with antibodies to Tpm and CK2 in whole heart homogenates. A and B, precipitated with Tpm; C and D, precipitated with CK2. The immunoprecipitates were probed with Tpm (A–C) or CK2 (B and D). STD, molecular weight standards; WH, mouse whole heart homogenates (positive control); IP, immunoprecipitated with Tpm (A and B) or immunoprecipitated with CK2 (C and D). Note Tpm was not stripped off membrane in A prior to probing for CK2 in B; thus, both bands are present in B. E, yeast two-hybrid assay of Tpm with CK2. Growth is seen in the yeast co-transformed with α-Tpm and CK2 after three cycles of selective medium. The “bait” plasmid is pGBK77 (negative control), and the “prey” plasmid is pGAD77 (negative control).

Discussion

In this investigation we addressed the functional consequences of Tpm phosphorylation using an in vivo model. Results show Tpm pseudo-phosphorylation leads to a dilated cardiomyopathic phenotype and activation of the associated ERK1/2 signaling pathway. The primary physiological effect on the heart is a decreased rate of relaxation, without altering contractile indices, myofilament calcium sensitivity, or ATPase activity. Additional results demonstrate that casein kinase 2 plays an integral role in the Tpm phosphorylation process.

The results of this investigation are in contrast to in vitro studies (6–8) but supported by isolated muscle studies (10), and most likely indicate differences between biochemical and in situ properties of myofibers. In the few TG line 149 mice that survived and could be evaluated in the work-performing heart and skinned fiber bundle analyses, the contractile and relaxation functions were similar to that from other moderate expression S283D lines; we are surprised at this finding in that the physiological performance was not affected more dramatically. We believe the severe cardiac pathology in most of the line 149 hearts is due to the near 100% expression of the S283D protein, whereas the hearts from the other TG lines express both endogenous and exogenous Tpm.

The results from several investigations indicate that multiple kinases may play a role in Tpm phosphorylation. Phosphoinositide 3-kinase and death-associated protein kinase appear to phosphorylate cytoskeletal Tpm isoforms (26, 27). Cardiac Tpm has also been identified as a target of PKCζ, as has a kinase isolated from chicken embryos (28, 29). This current study demonstrates that casein kinase 2 also plays a role in this post-translational process. Future studies will identify whether unique conditions (i.e., fetal versus adult stages, skeletal versus cardiac muscle, normal physiological conditions versus hyper-trophic stress versus physiological stress) cause specific kinases to phosphorylate Tpm and which Tpm isoforms are the major targets of these kinases.

Previous work reported that Tpm is highly phosphorylated in murine hearts of the developing fetus, with levels decreasing in the adolescent mouse myocardium (4). This process reverses itself after 5 months of age. The reason for this decrease in Tpm phosphorylation, followed by an increase, is unknown but may relate to changes in contractile protein isoform expression. A select number of the contractile proteins, such as β-myosin heavy chain, decrease their cardiac muscle expression postnatally, only to increase expression in aged mice; a similar effect may be occurring with Tpm phosphorylation. Interestingly, there is a significant difference in Tpm phosphorylation levels in the atria versus ventricles of the heart; higher Tpm phosphorylation occurs in the atria (9). A possible explanation is that unlike other contractile proteins that have isoforms that are preferentially expressed in the atria or ventricles, such as the atrial and ventricular light chains, and the α- and β-MHC isoforms, α-Tpm is the principle isoform in both cardiac chambers. The β-Tpm isoform is expressed at higher levels in prenatal myocardium, but these levels are still relatively low (i.e. 20% β-Tpm in the murine heart) (30). Thus, phosphorylation of Tpm is a biochemical process that cardiomyocytes may employ to regulate some of the physiological properties of Tpm in the sarcomere. Nevertheless, considering drastic physiological alterations occur with phosphorylation of other cardiac contractile proteins, it is somewhat surprising that phosphorylation of Tpm appears to exhibit a more modest effect on cardiac function. Because previous studies determined that Tpm phosphorylation is altered during both hypertrophic and dilated cardiomyopathic conditions (16, 31), studies are currently in progress to determine whether this post-translational process plays a role in ameliorating or augmenting the response to cardiomyopathic diseases.

Previously, we addressed the significance of Tpm dephosphorylation by generating transgenic mice, which substituted an alanine residue for serine 283 (5). The results show these mice exhibit a physiological hypertrophic phenotype without alterations in cardiac function, myofilament calcium sensitivity, cooperativity, or response to β-adrenergic stimulus. Additional studies examined whether decreasing Tpm phosphorylation may improve cardiac function in the context of a chronic, intrinsic stressor. To examine this hypothesis, we generated transgenic mice that incorporated both the S283A mutation and a cardiac hypertrophic E180G mutation (18, 31); the result-
ing animals are “phenotypically rescued,” exhibit no signs of cardiac hypertrophy, and display improved cardiac function. These studies, coupled with the current investigation, illustrate the significance of Tpm phosphorylation on cardiac morphology and physiology in the context of the whole animal. Importantly, studies in human patients demonstrate increases in skeletal muscle Tpm phosphorylation in various skeletal muscle myopathies, including Tpm3 myopathy (32). This influence of Tpm phosphorylation not only affects sarcomeric function but also activates various signaling pathways, such as p38-MAPK and ERK, which are associated with physiological and cardiomyopathic processes (27, 33, 35). The development of drug targets associated with Tpm phosphorylation and/or dephosphorylation may prove useful as therapeutic agents in the treatment of cardiac disease.

Experimental procedures

The Institutional Animal Care and Use Committee of the University of Cincinnati College of Medicine approved the handling and maintenance of animals.

Generation of S283D α-Tpm TG mice

Mouse striated muscle α-Tpm cDNA was subjected to QuikChange II site-directed mutagenesis (Agilent Technologies) utilizing the primer 5′-CAC GCT CTC AAC GAT ATG ACT GAC ATA TAA GTT TCT TTG CTT CAC-3′ mutating the penultimate serine to an aspartic acid. The mutation was verified through sequencing of the construct by Genewiz. The 5′-UTR and poly(A) tail sequence (36). Transgenic mice were generated using the FVB/N strain as previously described (37), and founder mice were identified using PCR. Previously published methods were used to determine copy numbers of the transgene in the TG mice (38). In brief, 10 μg of genomic DNA was isolated from founder mice, digested overnight with EcoR1, and Southern blotted using the 3′-UTR α-Tpm as a probe. The endogenous 3′-UTR band present in both NTG and TG samples is 1.7 kb, and the exogenous transgenic band is 3.4 kb. Quantification of the transgene copy numbers was conducted using ImageQuant 5.1 software comparing band signal intensities from the endogenous and TG bands. Nucleotide sequencing of TG mouse DNA verified the sequence of the α-Tpm S283D transgene.

Genotyping

DNA samples were obtained from 14-day-old mice, and PCR was utilized to determine which animals carried the transgene. Primers specific for the transgene are α-MHC forward 5′-GCC CAC ACC AGA AAT GAC AGA-3′ and α-Tpm reverse 5′-TCC AGT TCA TCT TCA GTG CCC-3′. GAPDH is used as an internal control, and primers are as follows: GAPDH forward, 5′-AGC GAG CTC AGG ACA TTC TGG-3′; and GAPDH reverse, 5′-CTC TTA ACC ACG CTC CTA GCA-3′.

Transgenic protein and Western blotting analyses

Myofibrillar proteins were extracted from NTG and α-Tpm S283D mouse ventricles as described previously (37). Protein concentrations were calculated, and samples were subjected to SDS-PAGE, followed by staining with Coomassie Blue to normalize and ensure equal loading of samples. For separation of Tpm isoforms, 3 μg of myofibrillar preparations were resolved on a 4.2–4.9 immobilized pH gradient isoelectric focusing strip. After isoelectric focusing, the samples were transferred to nitrocellulose membrane for Western blotting. Western blotting analyses were conducted using the Tpm muscle-specific antibody CH1 (Sigma–Aldrich) and a Tpm Ser-283 phosphorylation-specific antibody generated for our laboratory (Yen-Zyme) to visualize both endogenous, recombinant, and transgenic Tpm species. The intensity of the bands was quantified with ImageQuant 5.1 software.

Whole ventricular homogenates from NTG or α-Tpm S283D 3–4-month-old mice were utilized to visualize ERK/MAPK cell signaling proteins using Western blotting analyses. Samples were run on 12% SDS-PAGE, blotted to either nitrocellulose or polyvinylidene difluoride membrane, and probed with antibodies for ERK, RSK, and JNK (Cell Signaling).

Histopathological analysis

Mouse hearts from NTG and high expression S283D (line 149) mice at 15 days were isolated, weighed, and examined for histopathological changes. Heart weight–to–body weight ratios were calculated to determine evidence of cardiac hypertrophy. Images were taken on a Nikon SM2–2T dissecting microscope and an Olympus BX4C compound microscope.

Isolated anterograde-perfused heart preparation

These studies were performed on five TG mice from lines 143, 954, and a limited number of survivors from line 149 (n = 3). NTG and TG mice were anesthetized intraperitoneally with 100 mg/kg Nembutal sodium and 1.5 units of heparin to prevent intracoronary microthrombi (19). The heart was rapidly excised, and the aorta was cannulated with a 20-gauge needle, followed by retrograde perfusion with a modified Krebs–Henseleit solution. The buffer was equilibrated with 95% O2 and 5% CO2, with a pH of 7.4, and maintained at 37.4°C. Anterograde work-performing perfusion was initiated at a workload of 250 mm Hg ml/min as described previously (19). Heart rate, left ventricular pressure, and mean coronary perfusion pressure were continuously monitored. The pressure curve was used to calculate the rate of pressure development (+dP/dt) and decline (−dP/dt), time to peak pressure (TPP), and time to half-relaxation (RT1/2). Starling curves were generated by linear regression and Origin software (version 4.0, Microcal Software). For the regression lines, the average slopes were calculated using only the initial part of the Frank–Starling curve (at cardiac work from 0 to 350 mm Hg/ml/min). Multiple measurements were taken for each experiment. The data are presented as means ± S.E.

Skinned fiber bundle preparation and force measurements

The relation between stress (tension) and ATPase activity was measured simultaneously, over a range of pCa (−log of the molar free Ca2+) values, in detergent-extracted fiber bundles similar to that described previously (10, 17, 19). The fiber bundles (150–175 μm in diameter and 1.2 mm in length) were
dissected from the left ventricular papillary muscle of the mice hearts and skewed overnight in relaxing solution containing 33.99 mM potassium propionate, 6.92 mM MgCl₂, 6.22 mM Na₂ATP, 1 mM DTT, 5 mM NaN₃, 20 mM EGTA, 100 mM N,N-bis[2 hydroxyethyl]-2-aminoethanesulfonic acid, pH 7.0, and protease inhibitors (1 µM pepstatin A, 10 µM leupeptin, 100 µM phenylmethylsulfonl fluoride, and 1% Triton X-100. Fiber bundles were mounted at one end to a motor and to a force transducer at the other end. Resting sarcomere length was set to 2.2 µm using a laser diffraction pattern. Bathing solutions consisted of relaxing solution containing 10 mM phosphono-pyruvate, 4 mg/ml pyruvate kinase, 0.24 mg/ml lactate dehydrogenase, 10 µM oligomycin B, and 0.2 mM P⁴,P⁵-di(adenosine-5’)pentaphosphate. Tension and ATPase activity were determined over a range of pCa values generated by adding CaCl₂ to the bathing solution. Cross-sectional area of the fiber bundles was measured using an elliptical model, and the measurements of fiber width and height were obtained. Tension was expressed as force/cross-sectional area. The ATPase activity of the fiber bundles was measured by a linked enzyme assay coupled to the breakdown of NADH as described previously (17). The data were linearized using the Hill transformation, and the pCa tension or pCa ATPase activity relation was fitted to the Hill equation using nonlinear regression analysis to derive the pCa₅₀ value and Hill coefficient (17).

Computer search of identification of Tpm kinase

To characterize the phosphorylation site and identify the putative kinase involved in Tpm phosphorylation, a web server-based program (KinasePhos 2.0) was employed that adapts the sequence-based amino acid coupling-pattern analysis and solvent accessibility as new features for SVM (support vector machine) (39). Because our previous experiments and the mutant S283A Tpm mouse confirmed that Ser-283 is the only Tpm amino acid that gets phosphorylated in a head to tail polymerization region (5), the following sequence was used as an input: EELDHALNDTSMIDAIKKKMQM. This amino acid sequence fetched the following sequence motif: NDMTSMDA to pull up the putative kinases (Table S1), in which CK2 had a very high score. The results were verified on NetPhos 3.1 Server (40) and the prediction results confirmed CK2 as one of the predominant kinases involved in phosphorylating the Tpm Ser-283 amino acid.

Casein kinase 2 assays

The assay was performed using the purified recombinant Tpm proteins that was generated in our laboratory (34, 38); the CK2 A1 and CK2 holoenzymes were purchased from New England Biolabs. The 1× assay buffer constituted of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 10 mM KCl, and 7.5 mM β-glycerophosphate was supplemented with 250 µM GTP, and the assay was performed at 30°C. After the assay, the samples were boiled with SDS loading buffer and Western blotted with phosphoTM antibody and CH1 antibody. The CK2 inhibitors used in the assay were heparin (2 µg/ml) and TBB (50 µM). Rabbit polyclonal CK2α (Santa Cruz, sc-9030) and rabbit polyclonal CK2b (Abcam, ab59399) were used for the immunoprecipitation and Western blotting studies.

Yeast two-hybrid analysis

Full-length CK2 cDNA construct was subcloned into the GAL4 DNA-binding domain of pGBKKT7 (Clontech) so as to serve as “bait.” The “prey” was full-length α-Tpm cDNA cloned next to the GAL4 activation domain of pGADT7 (Clontech). The bait and prey were transformed into Y2H Gold cells together. Functional interactions were tested by growth of the transformed yeast cells initially plated on medium stringency medium containing SD/–Leu/–Trp and then plated onto SD/–Ade/–His/–Leu/–Trp/X-α-Gal/AbA plates, which activate the expression of repression proteins. The empty pGBK7T7 vector served as a negative control.

Statistical analyses

The data from multiple experiments were quantified and expressed as means ± S.E. or means ± S.D., and differences between groups were analyzed by using two-tailed Student’s t test or, when not normally distributed, a nonparametric Mann–Whitney U test. p values less than 0.05 were considered significant in all analyses. The data were computed with GraphPad Prism version 6.0.

Author contributions—S. R., G. J., N. P., B. J. B., C. M. W., S. L., B. M. W., R. J. S., and D. F. W. conceptualization; S. R., G. J., N. P., B. J. B., C. M. W., R. J. S., and D. F. W. data curation; S. R., G. J., N. P., B. J. B., C. M. W., R. J. S., and D. F. W. formal analysis; S. R., B. M. W., R. J. S., and D. F. W. funding acquisition; S. R., N. P., B. J. B., R. J. S., and D. F. W. validation; S. R., B. J. B., C. M. W., S. L., B. M. W., and D. F. W. investigation; S. R., G. J., N. P., and D. F. W. methodology; S. R., G. J., N. P., B. J. B., C. M. W., R. J. S., and D. F. W. writing and editing; S. L., R. J. S., and D. F. W. supervision; D. F. W. resources; D. F. W. visualization; D. F. W. writing–original draft; D. F. W. project administration.

Acknowledgments—We acknowledge the generosity of Dr. J. Molkenfor providing various marine heart samples and the excellent technical assistance from Maureen Bender for excellent animal husbandry skills. We also thank Dr. Palanikumar Manoharan for assistance in the manuscript submission.

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