Hyperphosphorylation of Mouse Cardiac Titin Contributes to Transverse Aortic Constriction-Induced Diastolic Dysfunction

Bryan Hudson, Carlos Hidalgo, Chandra Saripalli, Henk Granzier

Rationale: Mechanisms underlying diastolic dysfunction need to be better understood.
Objective: To study the role of titin in diastolic dysfunction using a mouse model of experimental heart failure induced by transverse aortic constriction.
Methods and Results: Eight weeks after transverse aortic constriction surgery, mice were divided into heart failure (HF) and congestive heart failure (CHF) groups. Mechanical studies on skinned left ventricle myocardium measured total and titin-based and extracellular matrix-based passive stiffness. Total passive stiffness was increased in both HF and CHF mice, and this was attributable to increases in both extracellular matrix-based and titin-based passive stiffness, with titin being dominant. Protein expression and titin exon microarray analysis revealed increased expression of the more compliant N2BA isoform at the expense of the stiff N2B isoform in HF and CHF mice. These changes are predicted to lower titin-based stiffness. Because the stiffness of titin is also sensitive to titin phosphorylation by protein kinase A and protein kinase C, back phosphorylation and Western blot assays with novel phospho-specific antibodies were performed. HF and CHF mice showed hyperphosphorylation of protein kinase A sites and the proline glutamate valine lysine (PEVK) S26 protein kinase C sites, but hypophosphorylation of the PEVK S170 protein kinase C site. Protein phosphatase I abolished differences in phosphorylation levels and normalized titin-based passive stiffness levels between control and HF myocardium.
Conclusion: Transverse aortic constriction-induced HF results in increased extracellular matrix-based and titin-based passive stiffness. Changes in titin splicing occur, which lower passive stiffness, but this effect is offset by hyperphosphorylation of residues in titin spring elements, particularly of PEVK S26. Thus, complex changes in titin occur that combined are a major factor in the increased passive myocardial stiffness in HF. (Circ Res. 2011;109:858-866.)

Key Words: diastolic dysfunction ■ passive stiffness ■ titin

A ltered hemodynamics, such as elevated blood pressure and aortic valve diseases, lead to pathological cardiac hypertrophy and ultimately heart failure (HF), characterized by left ventricular dilation, decreased contractility, and increased myocardial stiffness.1 Although the molecular mechanisms that underlie decreased contractility have been investigated extensively, the molecular basis of diastolic dysfunction is less well-understood.2–4 Understanding the mechanisms that govern diastolic dysfunction is important for understanding a wide range of cardiac diseases, including heart failure with preserved ejection fraction (HFpEF), a prevalent disease without effective therapy.4–7 The extracellular matrix (ECM) is often considered to dominate passive myocardial stiffness, but work focused on the intracellular protein titin has shown that titin is also important for generating passive muscle stiffness.5

Titin spans from Z-disk to M-line and is extensible in the I-band region of the sarcomere, where it functions as a molecular spring that develops passive force in sarcomeres stretched beyond their slack length (≈1.9 μm).8 Differential splicing results in two main isoforms: the N2B isoform that is stiff (≈3.0 MDa) and the larger N2BA isoform that is more compliant (≈3.4 MDa).9 These isoforms are coexpressed in the adult heart, with alterations in their expression ratio giving rise to changes in myocardial stiffness.10 In addition, titin phosphorylation also alters myocardial stiffness. Both protein kinase A (PKA)11 and protein kinase G (PKG)12 phosphorylate titin (most likely at the same site in the N2B spring element13) and reduce myocardial stiffness (increased compliance). Evidence suggests that hypophosphorylation of PKA/PKG sites on titin contribute to reduced compliance in HFpEF patients.13 Recently, it was found that titin is also a

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Here, we studied titin in mice with heart failure (HF) induced by transverse aortic constriction (TAC) and focused on extracellular matrix (ECM) remodeling for animal use. Institutes of Health Animal Care and Use Committee and followed the U.S. National Institutes of Health Using Animals in Intramural Research guidelines for animal use.

Methods

Echocardiography

Echocardiography was performed as described using a Vevo 770 System (Visual-Sonics). Details provided in Supplemental Materials and Methods.

Gel Electrophoresis

SDS–agarose electrophoresis was performed as previously described, with additional details in the Supplemental Materials and Methods.

Phosphorylation Assays

Detection of LV protein phosphorylation and recombinant protein was by protein labeling (32P), phosphoprotein stain (Pro-Q diamond), and Western blots probed with phospho-specific antibodies (PS26 BL Biochem and PS170 GenScript) against pS26 and pS170 of titin found in the PEVK element and against various calcium handling and myofibrillar proteins (Supplemental Materials and Methods).

Microarray Studies

The titin exon microarray experiments were performed as described previously.

Measurement of Passive Tension

Small muscle strips were attached to a motor and a force transducer via aluminum clips and passive tension was measured in relaxing solution. Sarcomere length (SL) was measured online by laser diffraction. Passive stiffness was determined from the slope of the linear regression line that was fit to the passive tension SL data in the 1.95- to 2.05-μm SL range, which encompasses the physiological SL range.

For the protein phosphatase-1 (PP1) experiments, muscles were incubated with PP1 (0.5 U/mL) for 2 hours, and passive stiffness was measured at 20-minutes intervals.

Statistics

Data are presented as mean±SEM. Group significance was defined using ANOVA followed by Tukey-Kramer multiple comparison test.

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**Figure 1.** Postmortem (A, B) and echocardiographic (C, D) analysis. Heart failure (HF) mice have increased left ventricle weight (LVW/body weight (BW) ratios and lung weight (LW/BW) ratios, with the largest changes in the congestive heart failure (CHF) group (A, B). Echocardiography revealed increased LV chamber volumes (C), with the largest changes in the CHF group. Doppler echocardiography indicates an increase in mitral valve (MV) early (E)/late (A) ratio (D), Ctrl, control.

***P<0.001 vs sham-operated controls. **P<0.01 vs sham-operated controls. *P<0.05 vs sham-operated controls. #P<0.001 vs HF transverse aortic constriction (TAC) mice.
SL significance was determined using two-way ANOVA. Student \( t \) test was used in Figure 6A (inset). \( P<0.05 \) was considered significant.

**Results**

**TAC-induced HF**

Using echocardiography and tissue weight analysis, we evaluated hypertrophy, LV chamber dimensions, and LV function in sham-operated (control, \( n=12 \)), HF (\( n=10 \)), and CHF (\( n=6 \)) mice. The LVW was significantly increased by 34% in HF and 92% in CHF and when normalized to BW, LVW/BW ratios were significantly increased by 36% and 226%, respectively (Supplemental Table I and Figure 1A). Lung weight/BW was also significantly increased in both HF and CHF, with a modest 20% increase in HF and a 346% increase in the CHF group (Supplemental Table I and Figure 1B). Echocardiography confirmed the hypertrophy and also revealed increased chamber dimensions in both experimental groups with changes that were most severe in the CHF mice (Supplemental Table II and Figure 1C). Fractional shortening (%) and ejection fraction (%) were both significantly reduced in the two experimental groups (Supplemental Table II). We measured expression of β-myosin heavy chain, an indicator of HF, and found a trend toward an increase in β-myosin heavy chain in HF mice and a significant increase in CHF mice (Supplemental Figure I). We also found trends toward a reduction in SERCA2a and PLB expression and decreased phosphorylation levels of S23/24 of cTnI and S16 of PLB in HF and significant changes in CHF (Supplemental Figure II A–D). Thus, the combined data from tissue weights, echocardiography, and protein data all reveal that the TAC mice had HF, with the most deleterious changes in the CHF group. Doppler echocardiography was used to measure the ratio of peak transmitral early (E) and late (A) diastolic velocities, and the deceleration time of the E wave (DT). The E/A ratio was significantly increased by 68% in the HF group and by 207% in the CHF group (Figure 1D), changes that are attributable to a modest increase in E and a more severe reduction in the A parameter (Supplemental Table II). These changes are likely to reflect increased ventricular chamber stiffness in both experimental groups, with the largest increase in CHF. Consistent with this hypothesis is the reduced DT in CHF (DT is considered to be inversely related to LV chamber stiffness\(^{23} \)).

Heart rate (HR) during these measurements was not different between the groups (Supplemental Table I and Figure 1B).

**Passive Tissue Mechanics and Passive Stiffness in HF and CHF TAC Mice**

Mechanical experiments were performed on skinned myocardial tissue. Maximal active tension was significantly reduced in both HF (\( n=5 \)) and CHF (\( n=4 \)) heart failure groups as compared to controls (\( n=4 \); 34.2±1.5 mN/mm\(^2\) and 30.3±2.4 mN/mm\(^2\), respectively, vs 41.8±2.6 mN/mm\(^2\) in controls; Table). This active tension reduction is consistent with other studies of rodents with experimental CHF (30% reduction in tension in CHF rats\(^{24} \) vs 28% in our work in CHF mice) and might be attributable to increased PKCα signaling that causes hyperphosphorylation of myofilament proteins.\(^{24} \)

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<th>Control</th>
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<th>CHF</th>
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<td>Maximal active tension, mN/mm(^2)</td>
<td>41.8±2.6</td>
<td>34.2±1.5*</td>
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<td>Total passive stiffness, mN/(mm(^2) μm/SL)</td>
<td>33.0±5.9</td>
<td>80.4±4.2‡</td>
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<td>ECM-based passive stiffness, mN/(mm(^2) μm/SL)</td>
<td>7.4±2.1</td>
<td>22.4±4.0‡</td>
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<tr>
<td>Titin stiffness, mN/(mm(^2) μm/SL)</td>
<td>25.6±6.4</td>
<td>64.8±5.3‡</td>
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CHF indicates congestive heart failure; ECM, extracellular matrix; HF, heart failure.

Maximal active tension: pCa 4.0; activated at sarcomere length 2.0.* \( P<0.05. \)
† \( P<0.001 \) vs sham-operated controls.
‡ \( P<0.01. \)
§ \( P<0.05 \) vs HF transverse aortic constriction mice.

Stiffness is defined as the slope of the linear fits of the tension–sarcomere length relation in the physiological sarcomere length range of 1.95–2.05 μm.

Figure 2. Effect of transverse aortic constriction (TAC)-induced heart failure (HF) on passive tension in skinned myocardium. Total passive tension (A) is significantly higher in HF and congestive heart failure (CHF) groups because of an increase in extracellular matrix (ECM)-based (B) and titin-based (C) passive tension. Right panels show the fold change in passive tensions at sarcomere lengths (SL) 1.95, 2.05, and 2.15 μm. Results indicate a significant increase in both HF and CHF at all three SL. Titin-based passive tensions in CHF were slightly reduced relative to HF.
interaction, we performed experiments in the presence of 20 mmol/L BDM, a known inhibitor of actomyosin interaction. BDM had no effect on passive tension (results not shown), suggesting that the increased passive tension in HF and CHF is attributable to only passive material properties of the myocardium. It is also important to highlight that although differences in calcium handling proteins exist, in both expression level and phosphorylation status (Supplemental Figure II), these changes are unable to explain our findings considering that the experiments were performed on muscle that had been demembranated with detergent (Triton X-100).

To evaluate whether elevated passive tension might be attributable to the oxidizing conditions that are known to exist in HF, we also evaluated the phosphorylation levels of PKA and PKG phosphorylation, with PKA and PKG phosphorylation declining in CHF to approximately 60% and ECM-based tension increases to approximately 40% of total.

**Differential Splicing and Phosphorylation of Titin**

Differential splicing results in the expression of two main cardiac titin isoforms, the stiff N2B and the larger and more compliant N2BA isoforms. Using high-resolution gel electrophoresis, we evaluated the titin isoform expression ratio in TAC mice with the expectation that upregulation of the stiff N2B isoform might explain the increased titin-based passive tension of the HF and CHF tissues. Consistent with earlier studies, the control animals have a N2BA:N2B expression ratio of approximately 0.2 (Figure 3A, B), confirming that the shorter stiffer N2B isoform is dominant in the mouse heart. The HF and CHF mice showed an increase in the more compliant N2BA isoform (Figure 3B, Supplemental Table III) that correlates with the LVW/BW ratio (P<0.001; Figure 3C). The increased expression of the compliant N2BA isoform is opposite of what was expected from the measured increase in titin-based passive stiffness. To test whether this discrepancy might be attributable to changes in expression levels of titin, we determined the relative amount of titin per milligram of tissue and the amount of titin relative to the expression level of myosin heavy chain. The HF and CHF groups were not significantly different in their level of titin expression (Supplemental Table III). Finally, we also evaluated the titin degradation product, T2, and found no significant change after aortic constriction (Supplemental Table III).

Thus, the only significant change that was found is the upregulation of the compliant N2BA isoform at the expense of the stiff N2B isoform. Because this finding was unexpected, we also evaluated titin expression at the transcript level by performing a titin exon expression analysis using a homemade microarray on which all 358 exons found in the mouse heart were represented. We found significant upregulation of a large group of PEVK and Ig exons in CHF tissues compared to controls (Supplemental Table IV), confirming that CHF mice express a larger titin than control heart tissue.

Because the stiffness of titin also can be varied through phosphorylation, with PKA and PKG phosphorylation decreasing stiffness and PKC phosphorylation increasing stiffness, we also evaluated the phosphorylation levels of...
increase in passive stiffness. Therefore, we developed phospho-specific antibodies to titin’s PKCα phosphorylation target proline (P) glutamate (E) valine (V) lysine (K) (PEVK) sites, (A) pS26 and (B) pS170, were evaluated in heart failure (HF) and congestive heart failure (CHF) samples as compared to control (Ctrl). Representative western blot images (top). Analysis shows (A) hyperphosphorylation of PEVK S26 and (B) a large reduction of S170 phosphorylation (bottom). *Significant vs wild-type (WT; P<0.05). ***Significant vs WT (P<0.001).

Figure 4. Determination of protein kinase A (PKA) phosphorylation status. A, Representative results on mouse skinned left ventricle (LV) incubated in [γ-32P]ATP without (−) or with (+) preincubation with PKA. Comassie blue (CB)-stained gel (top) and corresponding autoradiograph (AR; bottom). AR detects only available phosphorylation; therefore, less [γ-32P] incorporation is equivalent to less available phosphorylation sites. B, Both heart failure (HF) and congestive heart failure (CHF) samples have reduced [γ-32P] incorporation, indicating hyperphosphorylation of their PKA sites. *Significant vs control (Ctrl; P<0.05).

Discussion

There is heightened awareness that it is critically important to fully understand the underlying mechanisms of diastolic dysfunction because of its clinical importance and absence of effective therapies. In this work, we studied a TAC-induced HF mouse model, we segregated mice in HF and CHF groups, and we performed a myocardial passive stiffness analysis with a focus on titin. A large increase in passive myocardial stiffness was found in both the HF and CHF groups, and it was most severe in HF and somewhat less severe in CHF mice. The data further indicate that heart failure-induced fibrosis results in an increase in ECM-based passive stiffness and, importantly, that titin plays a major role in increased myocardial passive stiffness. The contribution of titin to passive myocardial stiffness is greatly increased in HF mice, relative to that of sham mice, and this might be attributable to hyperphosphorylation of a PKC site in the PEVK region of titin. In CHF mice, titin-based stiffness is increased as well, but not as much as in HF mice, which can be explained by the increased expression of the compliant N2BA titin that we found.

Total passive myocardial stiffness was increased approximately 2.5-fold in the HF mice and approximately two-fold in the CHF mice (Figure 7A). The total stiffness increase in the HF group is slightly higher than recently reported by Bradshaw et al in papillary muscle of TAC mice (~1.6-fold...
increase), which might be explained by the more severe hypertrophy in our study. ECM-based stiffness was found to be approximately three-fold higher in the HF group and approximately four-fold higher in the CHF group than in the sham mice (Table). These increases can be explained by the increased ECM collagen content and increased collagen cross-linking that are known to take place in the TAC mouse model. The increase in titin-based stiffness is consistent with the recently reported increased passive tension of skinned cardiac myocytes isolated from mice with severe TAC. The increase in titin-based stiffness is consistent with the recently discovered disulfide cross-linking that occurs in the N2B element under oxidative stress, a state that is common during heart failure. Because such cross-linking increases titin-based passive stiffness, we tested the possibility that the increase in stiffness in HF mice was attributable to increased disulfide cross-linking by performing experiments under reducing conditions (10 mmol/L DTT). Results indicate that oxidation of titin was not the cause of the increase in passive stiffness in heart failure. Titin-based passive stiffness can be regulated by differential splicing of titin; titin molecules with a longer extensible region increase sarcomere compliance. Two cardiac titin isoforms are coexpressed within the sarcomere, the N2B and N2BA isoforms with short (stiff) and long (compliant) extensible regions, respectively, and their expression ratio is an important determinant in titin-based passive stiffness. Furthermore, it has been demonstrated in a canine tachycardia-induced model of dilated cardiomyopathy and titin, and this increase is expected to contribute to elevated diastolic stiffness.

A possible explanation for the increase in titin-based stiffness is the recently discovered disulfide cross-linking that occurs in the N2B element under oxidative stress, a state that is common during heart failure. Because such cross-linking increases titin-based passive stiffness, we tested the possibility that the increase in stiffness in HF mice was attributable to increased disulfide cross-linking by performing experiments under reducing conditions (10 mmol/L DTT). Results indicate that oxidation of titin was not the cause of the increase in passive stiffness in heart failure. Titin-based passive stiffness can be regulated by differential splicing of titin; titin molecules with a longer extensible region increase sarcomere compliance. Two cardiac titin isoforms are coexpressed within the sarcomere, the N2B and N2BA isoforms with short (stiff) and long (compliant) extensible regions, respectively, and their expression ratio is an important determinant in titin-based passive stiffness. Furthermore, it has been demonstrated in a canine tachycardia-induced model of dilated cardiomyopathy and titin, and this increase is expected to contribute to elevated diastolic stiffness.

Figure 6. A, Titin-based passive tension and phosphorylation of S26 and S170 after dephosphorylation with protein phosphatase I (PP1). Before PP1 treatment, passive tension is significantly higher in heart failure samples (confirming results of Figure 2). Whereas tensions were unchanged in control (Ctrl) samples after PP1 treatment, heart failure (HF) samples had a significant reduction in their passive tensions. Inset shows percent change in titin-based passive stiffness (slope of passive tension, sarcomere length [SL] relation in 1.95 to 2.05 μm SL length range) after PP1 treatment. B, Western blot analysis of PS26 and PS170 titin sites in Ctrl and HF samples after PP1 treatment (same samples as in A), WB normalized to Ponceau S-based protein levels and results of control samples set to 100%. No differences in phosphorylation levels are detected after PP1 treatment. *Significant vs Ctrl (P<0.05). **Significant vs Ctrl (P<0.01). #HF significant vs HF + PP1 (P<0.05).

Figure 7. Passive stiffness in heart failure (HF) and congestive heart failure (CHF) myocardium. A, Comparison of total, extracellular matrix (ECM), and titin passive stiffness (from Table). B, Schematic showing effects on passive stiffness of increase in S26 phosphorylation (top) and upregulation of N2BA titin (bottom).
in spontaneously hypertensive rats\textsuperscript{16} that a decrease in the expression of N2BA titin is accompanied by an increase in myocardial passive stiffness. Therefore, we hypothesized that the increase in titin-based myocardial stiffness might be attributable to increases in the stiffer N2B isoform, resulting in more passive tension when stretched to a specific sarcomere length. In contrast to the expected increase in expression level of the stiffer N2B isoform, both protein (Figure 3) and transcript (Supplemental Table IV) data showed that the compliant N2BA isoform of titin actually increases its expression level (Figure 3B). Although these data are opposite from what we expected, the result does correspond to isoform changes previously seen in human patients with ischemic human heart disease,\textsuperscript{37} dilated cardiomyopathy,\textsuperscript{38,39} HFpEF,\textsuperscript{13} and aortic stenosis.\textsuperscript{13} Thus, although differential splicing is a prominent mechanism for altering passive stiffness, the change in splicing that occurs in HF cannot explain the increased titin-based stiffness that we found and, in fact, it is expected to lower passive stiffness instead.

The stiffness of titin can also be altered through phosphorylation, with disparate effects via the PKA/PKG and PKC pathways. Both PKA and PKG have been found to phosphorylate the cardiac-specific N2B element and reduce stiffness of titin.\textsuperscript{11,12} However, PKC\textalpha phosphorylates the PEVK region of titin and this increases passive stiffness.\textsuperscript{15–16} Recent studies have shown that the PKA/PKG pathway is important in explaining the increased passive stiffness of myocytes in HFpEF patients in whom a reduction has been found in the phosphorylation level of PKA/PKG sites in titin and in whom treating the skinned cardiac myocytes from HFpEF patients with PKA lowers passive stiffness toward that of the control cells.\textsuperscript{13} Therefore, we thought that the observed shift from the stiffer N2B isoform to a more compliant N2BA isoform in our study could be thought of as a compensatory mechanism for increased myocardial stiffness, and that a reduction in the basal phosphorylation level of PKA accounts for the increased stiffness. Yet again, in contrast to our hypothesized results, PKA phosphorylation analysis showed hyperphosphorylation of PKA sites (Figure 4). Similar to the increased compliant isoforms that we found, these changes are predicted to decrease passive tension. One hypothesis for why these results are different from those in the human study discussed is as follows. Recently, Hamdani et al\textsuperscript{40} reported differences in phosphorylation levels of regulatory proteins in a variety of animals ranging from mice to human. Data revealed that the basal phosphorylation level is species-dependent and that reduced PKA-mediated phosphorylation was found only in end-stage failing human myocardium. We hypothesize that mice with HF are less sensitive to defects in \(\beta\)-adrenergic signaling than humans and speculate that this explains why the PKA sites of titin are not hypophosphorylated in our study. Hyperphosphorylation of PKA sites might potentially be explained by the recent finding that the PKA sites of titin in the N2B region of titin can also be phosphorylated by PKG\textsuperscript{12} and the elevated PKG activity in TAC mice that has been reported.\textsuperscript{41} Clearly, myofilament protein phosphorylation is a complex process with alternative pathways that can have similar or disparate results.

Recently, our laboratory has found that titin is also a target of PKC\textalpha and that PKC\textalpha phosphorylation of titin increases cardiac stiffness.\textsuperscript{15,16} Two sites were found in the PEVK region of titin (S26 and S170 in the PEVK region of the N2B isoform) that are present in all known cardiac and skeletal muscle titin isoforms. We produced phospho-specific antibodies against the two sites (Supplemental Figure V) and used them to assay site-specific phosphorylation events within titin. The results showed hypophosphorylation of the S170 PKC\textalpha site and hyperphosphorylation of S26 (Figure 5). Because we have shown that PKC phosphorylation increases passive stiffness both at the single molecule\textsuperscript{14} and single cell levels,\textsuperscript{15,16} it is possible that the hyperphosphorylation of S26 plays an important role in the increased titin-based stiffness. The hyperphosphorylation of PKC sites is consistent with the increased expression levels of PKC\textalpha that have been found in mice subjected to TAC.\textsuperscript{41} That hyperphosphorylation of titin is a mechanism for increased passive stiffness is supported by the dephosphorylation studies in which passive stiffness was measured before and after PP1 treatment (Figure 6) and that showed that PP1 significantly lowered the high passive stiffness of HF myocardium.

It is interesting that two phosphorylation sites 144 residues apart (PEVK S26 and S170) and phosphorylated by the same kinase have such different phosphorylation levels in HF myocardium. Previous studies on PKC phosphorylation sites showed that PKC\textalpha prefers basic residues N-terminal and C-terminal to the phosphorylation site.\textsuperscript{42} These studies also indicate that PKC preferentially phosphorylates serines with basic residues within three amino acids of both the C-terminal and N-terminal sides of it. Based on these studies, PKC would have a stronger affinity toward S26 than S170 because of the closer proximity of the neighboring basic amino acids (lysine [K] and arginine [R]; Supplemental Figure VII), which is consistent with the experimental PKC phosphorylation data on recombinant PEVK proteins.\textsuperscript{15} Other possibilities exist as well, such as protein binding at or near S170.

The PP1 experiments lowered passive stiffness (Figure 6), and because PKA/PKG and PKC sites have been shown to be dephosphorylated by PP1,\textsuperscript{12,15} either one or both might explain the passive stiffness reduction. However, dephosphorylation of PKA sites increases passive stiffness,\textsuperscript{12} and the S170 PKC site already has a low phosphorylation level before PP1 treatment (Figure 5B) and, thus, they are unlikely candidates. However, S26 is more promising because single molecule studies have shown that it is more important than S170 in determining passive stiffness,\textsuperscript{14} and S26 is hyperphosphorylated in HF tissue, where stiffness is high and its dephosphorylation (PP1 treatment) is expected to cause a reduction of stiffness, as we found. Thus, phosphorylation of S26 is likely to be crucial in determining the contribution of titin to myocardial stiffness. The significant increase in phosphorylation of S26 in HF is consistent with the increased PKC\textalpha activity that has been reported to occur in CHF rats.\textsuperscript{24} Considering that the PKC\textalpha-based passive stiffness modulation pathway was only discovered recently, it is unknown whether our findings in the HF mouse model extrapolate to different disease models. It is interesting to note, however, that a recent HFpEF study reported that cellular passive
stiffness after PKA treatment reduced passive tension but remained higher than in control cells (in fact, N2BA titin was upregulated, similar to what we found, and this upregulation predicts lower tension than in the control cells). This discrepancy can be explained by an elevated phosphorylation level of the PKCα-site S26. Thus, the role of S26 in the increased passive stiffness that was revealed in our study of the TAC-induced HF mouse model might be relevant in other diseases as well.

In summary, we found a significant increase in passive myocardial stiffness in HF and that titin plays an important role in this increase. Phosphorylation analysis and PP1 studies identified hyperphosphorylation of S26 of titin as a possible candidate for explaining part of the increased passive stiffness in HF. Lower titin-based stiffness was found in CHF than HF, which might be attributable to the upregulated N2BA titin in CHF compared to HF mice. Thus, altered hemodynamics trigger complex changes in the spring of titin, with hyperphosphorylation of S26 initially causing an increase in passive stiffness, in addition to titin-splicing changes during progression to CHF that lower passive stiffness (Figure 7B). Considering that hyperphosphorylation of S26 appears to be a dominant effect in passive stiffness changes during the progression to HF, the PKC pathway warrants further investigation because it is a potential target for therapeutics aimed at lowering diastolic stiffness.

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Disclosures
None.

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**Novelty and Significance**

**What Is Known?**

- Diastolic dysfunction is a prominent aspect of heart failure (HF).
- Mechanisms that underlie diastolic dysfunction are not well-understood.

**What New Information Does This Article Contribute?**

- We focused on passive stiffness of the left ventricle (LV) using a mouse model of HF and studied the extracellular matrix (ECM) and titin.
- HF results in increased ECM-based and titin-based passive stiffness. Changes in titin include isoform switching and hyperphosphorylation of the molecular spring region of titin.
- Complex changes in titin take place that in combination are a major factor in the increased passive myocardial stiffness in HF.

Elucidation of the mechanisms that govern diastolic dysfunction is important for understanding a wide range of cardiac diseases, including HF with preserved ejection fraction. The ECM is often considered to dominate passive myocardial stiffness, but work focused on the intracellular protein titin has shown that titin is also important. We studied diastolic dysfunction and the role of titin in a mouse model of experimental HF. Mechanical studies on skinned LV myocardium showed that total passive stiffness was increased in HF, and this was attributable to increases in both ECM-based and titin-based passive stiffness, with titin being dominant. Although the expression level of titin isoforms were affected, these changes cannot account for the increase in titin stiffness in HF. Investigations into the role of several kinases (protein kinase A and protein kinase C) and phosphatases suggest that the increase in titin-based stiffness could be explained in large part by hyperphosphorylation of titin spring elements. Considering that hyperphosphorylation of ser-26 in the pro-glu-val-lys spring element appears to be an important determinant of changes in passive stiffness during progression to HF, the protein kinase C pathway warrants further investigation because it is a potential target for therapeutics aimed at lowering diastolic stiffness.
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Supplemental Material

-Tables and Captions
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-Figures (Figs. I-VII)

-Material and Methods
### Online Table I. Postmortem Analysis

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>HF</th>
<th>CHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>12</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>BW (g)</td>
<td>32.2 ± 1.1</td>
<td>31.7 ± 1.0</td>
<td>26.0 ± 0.7 ** ##</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>155 ± 2.3</td>
<td>203 ± 9.7 ***</td>
<td>305.4 ± 6.9 *** ####</td>
</tr>
<tr>
<td>LVW(mg)</td>
<td>120.9 ± 2.0</td>
<td>162.0 ± 9.7 ***</td>
<td>232.6 ± 6.2 *** ####</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>4.8 ± 0.1</td>
<td>6.5 ± 0.4 ***</td>
<td>11.3 ±0.2 *** ####</td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>3.80 ± 0.12</td>
<td>5.17 ± 0.36 ***</td>
<td>8.59 ±0.30 *** ####</td>
</tr>
<tr>
<td>LW/BW (mg/g)</td>
<td>4.25 ±0.15</td>
<td>5.11 ± 0.27 **</td>
<td>18.96 ± 0.43 *** ####</td>
</tr>
<tr>
<td>Tibia Length (mm)</td>
<td>18.0 ± 0.1</td>
<td>17.9 ± 0.1</td>
<td>17.7 ± 0.2</td>
</tr>
</tbody>
</table>

Body Weight (BW), Heart Weight (HW), Left Ventricle weight (LVW), Lung weight (LW). *P<0.05, **P<0.01, ***P<0.001 vs sham-operated controls. #P<0.05, ##P<0.01, ###P<0.001 vs Heart Failure TAC mice.
# Online Table II. Echocardiography

<table>
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<th>CHF</th>
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<tbody>
<tr>
<td>No. of animals</td>
<td>13</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Heart Rate (BPM)</td>
<td>503 ± 20</td>
<td>486 ± 15</td>
<td>502 ± 21</td>
</tr>
</tbody>
</table>

## M-Mode Protocol

<table>
<thead>
<tr>
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<th>HF</th>
<th>CHF</th>
</tr>
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<tbody>
<tr>
<td>LV Vol;d (ml)</td>
<td>58 ± 2.7</td>
<td>83 ± 7.4 **</td>
<td>97 ± 3.9 ***</td>
</tr>
<tr>
<td>LV Vol;s (ml)</td>
<td>26 ± 2.4</td>
<td>48 ± 6.7 **</td>
<td>56 ± 4.5 ***</td>
</tr>
<tr>
<td>LVID;d (mm)</td>
<td>3.7 ± 0.1</td>
<td>4.3 ± 0.2 **</td>
<td>4.6 ± 0.1 ***</td>
</tr>
<tr>
<td>LVID;s (mm)</td>
<td>2.6 ± 0.1</td>
<td>3.4 ± 0.2 **</td>
<td>3.6 ± 0.1 ***</td>
</tr>
<tr>
<td>IVS;d (mm)</td>
<td>1.0 ± 0.04</td>
<td>1.1 ± 0.03</td>
<td>1.2 ± 0.05 **#</td>
</tr>
<tr>
<td>IVS;s (mm)</td>
<td>1.2 ± 0.04</td>
<td>1.3 ± 0.04</td>
<td>1.5 ± 0.05 **##</td>
</tr>
<tr>
<td>LVPW;d (mm)</td>
<td>1.0 ± 0.04</td>
<td>1.1 ± 0.04</td>
<td>1.2 ± 0.04 **#</td>
</tr>
<tr>
<td>LVPW;s (mm)</td>
<td>1.7 ± 0.04</td>
<td>1.3 ± 0.04</td>
<td>1.5 ± 0.05 **##</td>
</tr>
<tr>
<td>%EF (%)</td>
<td>57 ± 3</td>
<td>44 ± 3 **</td>
<td>43 ± 3 **</td>
</tr>
<tr>
<td>% FS (%)</td>
<td>29 ± 2</td>
<td>23 ± 2 *</td>
<td>21 ± 2 **</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>117 ± 6.6</td>
<td>169 ± 14.1 **</td>
<td>217 ± 9.6 ***#</td>
</tr>
</tbody>
</table>

## Pulse-Wave Doppler analysis

<table>
<thead>
<tr>
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<th>HF</th>
<th>CHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure gradient (PG, mm Hg)⁹</td>
<td>------</td>
<td>56.3 ± 4.6 ***</td>
<td>85.6 ± 4.6 ***##</td>
</tr>
<tr>
<td>MV E (m/s)</td>
<td>0.69 ± 0.03</td>
<td>0.76 ± 0.05</td>
<td>0.87 ± 0.05 **</td>
</tr>
<tr>
<td>MV A (m/s)</td>
<td>0.42 ± 0.02</td>
<td>0.33 ± 0.03 *</td>
<td>0.21 ± 0.03 ***#</td>
</tr>
<tr>
<td>MV E / A</td>
<td>1.58 ± 0.05</td>
<td>2.66 ± 0.51 *</td>
<td>4.86 ± 0.90 ***#</td>
</tr>
<tr>
<td>DT (msec)</td>
<td>21.0 +/- 0.8</td>
<td>21.8 +/- 0.6</td>
<td>19.6 +/- 0.9</td>
</tr>
</tbody>
</table>

LV diastolic volume (LV Vol;d), LV systolic volume (LV Vol;s), LV internal diastolic diameter (LVID; d), LV internal systolic diameter (LVID;s), Inter ventricular septum, diastolic (IVS;d), Inter ventricular septum, systolic (IVS;s), LV posterior wall, diastolic (LVPW;d), LV posterior wall, systolic (LVPW;s), LV Ejection fraction (EF), LV Fractional shortening (FS), Mitral valve E velocity (MV E), Mitral valve A velocity (MV A), and the deceleration time of the E wave (DT) *P<0.05, **P<0.01, ***P<0.001 vs sham-operated controls. #P<0.05, ##P<0.01 vs Heart Failure TAC mice. ⁹Calculated from the peak blood velocity (Vmax) (m/s) (PG=4 Vmax²) measured by Doppler across the aortic constriction.
### Online Table III Protein Analysis

<table>
<thead>
<tr>
<th></th>
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<th>HF</th>
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</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>MHC (OD/mg)</td>
<td>33.9 ± 4.2</td>
<td>35.7 ± 4.1</td>
<td>33.0 ± 4.0</td>
</tr>
<tr>
<td>Total Titin (OD/mg)</td>
<td>8.93 ± 1.00</td>
<td>7.14 ± 1.54</td>
<td>8.13 ± 1.75</td>
</tr>
<tr>
<td>Total Titin/MHC</td>
<td>0.24 ± 0.01</td>
<td>0.26 ± 0.04</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>N2BA/N2B</td>
<td>0.22 ± 0.02</td>
<td>0.29 ± 0.03 *</td>
<td>0.36 ± 0.02 ***</td>
</tr>
<tr>
<td>T2/Total Titin</td>
<td>0.13 ± 0.02</td>
<td>0.09 ± 0.01</td>
<td>0.10 ± 0.02</td>
</tr>
</tbody>
</table>

*P<0.05, ***P<0.001 vs sham-operated controls.
### Online Table IV. Transcript analysis.

Titin exon microarray analysis comparing CHF with control LV tissues. All differentially expressed exons are shown. 35 exons are upregulated in CHF and 3 down regulated. All differential exons are in the Z-disk or titin’s extensible I-band.

<table>
<thead>
<tr>
<th>Upregulated exons</th>
<th>Upregulated exons</th>
<th>Downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTE1 2.69 +/- 0.10 **</td>
<td>MTE2 2.52 +/- 0.10 **</td>
<td>MTE115 1.68 +/- 0.05 ***</td>
</tr>
<tr>
<td>MTE3 2.53 +/- 0.04 ***</td>
<td>MTE6 2.35 +/- 0.06 ***</td>
<td>MTE117 1.68 +/- 0.04 ***</td>
</tr>
<tr>
<td>MTE4 1.57 +/- 0.09 *</td>
<td>MTE5 2.22 +/- 0.07 **</td>
<td>MTE242 1.53 +/- 0.06 **</td>
</tr>
<tr>
<td>MTE6 2.35 +/- 0.06 ***</td>
<td>MTE15 1.68 +/- 0.05 **</td>
<td>MTE162 1.58 +/- 0.11 *</td>
</tr>
<tr>
<td>MTE16 1.78 +/- 0.12 *</td>
<td>MTE163 1.61 +/- 0.06 **</td>
<td>MTE163 1.58 +/- 0.11 *</td>
</tr>
<tr>
<td>MTE54 1.81 +/- 0.09 **</td>
<td>MTE173 2.02 +/- 0.11 **</td>
<td>MTE164 1.58 +/- 0.11 *</td>
</tr>
<tr>
<td>MTE66 1.56 +/- 0.09 *</td>
<td>MTE190 1.98 +/- 0.05 ***</td>
<td>MTE165 1.58 +/- 0.11 *</td>
</tr>
<tr>
<td>MTE72 2.24 +/- 0.08 **</td>
<td>MTE191 1.77 +/- 0.08 **</td>
<td>MTE173 1.58 +/- 0.11 *</td>
</tr>
<tr>
<td>MTE81 1.69 +/- 0.10 *</td>
<td>MTE192 1.83 +/- 0.08 **</td>
<td>MTE189 1.58 +/- 0.11 *</td>
</tr>
<tr>
<td>MTE82 1.76 +/- 0.04 **</td>
<td>MTE194 1.89 +/- 0.12 **</td>
<td>MTE200 1.94 +/- 0.07 **</td>
</tr>
<tr>
<td>MTE85 1.54 +/- 0.08 *</td>
<td>MTE202 1.69 +/- 0.06 **</td>
<td>MTE208 1.82 +/- 0.11 *</td>
</tr>
<tr>
<td>MTE86 1.80 +/- 0.07 **</td>
<td>MTE208 1.51 +/- 0.04 **</td>
<td>MTE214 1.51 +/- 0.04 **</td>
</tr>
<tr>
<td>MTE87 1.85 +/- 0.05 **</td>
<td>MTE215 1.52 +/- 0.08 *</td>
<td>MTE215 1.52 +/- 0.08 *</td>
</tr>
<tr>
<td>MTE130 2.18 +/- 0.06 **</td>
<td>MTE216 1.54 +/- 0.07 **</td>
<td>MTE192 1.83 +/- 0.08 **</td>
</tr>
</tbody>
</table>

Blue: Z-disk exons; Extensible I-band region: --Red: Ig exons; --Yellow: PEVK exons.

*P<0.05, **P<0.01, ***P<0.001 vs sham-operated controls.
On line Figure I. Myosin Heavy Chain (MHC) expression in LV of control (Ctrl), heart failure (HF), and congestive heart failure (CHF) mice. A) Representative examples of αMHC and βMHC isoform expression in representative samples. A 1-day old neonatal sample that expresses both αMHC and βMHC is shown for reference. Following TAC-induced heart failure, there is a clear shift from the predominant αMHC isoform towards the βMHC isoform, with the most pronounced shift occurring in the CHF hearts. Bar graphs in B show expression ratio of 6 Ctrl, 6 HF, and 6 CHF samples (mean ± SEM).
Online Figure II. Protein expression and phosphorylation levels in LV of Ctrl, HF, and CHF mice. A-E: WB-based expression of A) SERCA2a, B) phospholamban (PLB), C) pS16 of PLB, D) pThr17 of PLB, and E) pS23/23 of cTnI. GAPDH is used as a loading control. Expression of SERCA2a, PLB, PLBpS16 and TnIpS23/24 is significantly reduced in the CHF mice whereas PLBpThr17 is unaltered. Bar graphs are mean ± SEM expression levels normalized to GAPDH (n=6 per group). Levels are relative to those in the control mice. D-H) Pro-Q Diamond staining to reveal total protein phosphorylation of cMyBP-C, TnT, TnI and MLC2V. F: Representative samples. G-J): Phosphorylation levels of cMyBP-C (G), TnT (H), TnI (i) and MLC2V (J). ProQ Diamond staining normalized to Coomassie Blue staining. Because Pro-Q Diamond reports total phosphorylation, disparate results between Pro-Q diamond staining and specific phosphorylation sites probed with phospho-antibodies (compare B and G) can be expected. (4 mice per group.)
Online Figure III. Effect of increased DTT concentration on myocardial passive stiffness. To evaluate the effect of potential oxidizing conditions on myocardial stiffness we incubated the LV samples in high concentrations of the reducing agent, DTT (10mM) and stretched to SL 2.15. Passive stiffness was not altered following incubation with 10mM DTT.
**On line Figure IV. Sources of passive stiffness.** Comparison of the relative contribution of titin and collagen to the overall passive tension in Ctrl, HF, and CHF groups. Results indicate that titin is the major contributor to passive tension in all three groups.
Online Figure V. Validation of pS26 and pS170 antibodies against PKCα-induced phosphorylation of titin and effect of protein phosphatase 1 (PP1). (A) Validation using recombinant PEVK protein without (-) or with (+) PKCα phosphorylation; both pS26 and pS170 recognize phosphorylation following PKCα incubation in wt PEVK. Each antibody is specific, as mutation of the target serine to alanine abolishes antibody binding (S170A and S26A). In the double mutation (2X), where both S26 and S170 have been mutated to alanine, neither antibody has any specific binding. (B) We also tested the antibodies on mouse LV without (-) or with (+) PKCα. Both antibodies strongly label phosphorylated titin. (C) Effect of PP1 on PKCα phosphorylated WT PEVK (top pS26, bottom pS170). PKCα phosphorylates both S26 and S170 and PP1 dephosphorylates these sites.
On line Figure VI. ProQ-diamond stain does not detect PEVK phosphorylation. Phosphorylation of recombinant N2B protein with PKA can be easily detected with both autoradiography and ProQ-diamond whereas PKCα phosphorylation of PEVK protein is only detected with autoradiography and not ProQ-diamond. (* PKCα which autophosphorylates)
On line Figure VII. PEVK sequence alignment around S26 (left) and S170 (right). More basic residues are found within a 3 residue span from the S26 site than the S170 site. See text for details.
Supplemental Materials and Methods

Transverse Aortic Constriction. For Transverse Aortic Constriction (TAC) surgeries, a mixture of Avertin (2,2,2 tribromoethanol, Aldrich T4,840.2) (0.5mg/kg), Acepromazin (1mg/kg), and Ketamine (1mg/kg) was injected intraperitoneally in 60 day old male C57BL/6J mice. This mixture provides an adequate depth of anesthesia for 50–60 min which is long enough to finish the surgery. The aortic banding procedure was performed similar to that previously described 1-2 with minor adaptations. Briefly, the chest cavity was opened and the ascending portion of the aorta was bluntly dissected from the pulmonary trunk. A curved forceps was then placed under the transverse aorta, 7-0 silk was grasped by the forceps and moved underneath the aorta, and a loose double knot was made. A 27-gauge needle with OD 0.42 mm was delivered through the loose double knot and placed directly above and parallel to the aorta. The loop was then tied around the aorta and needle and secured with the second knot (this was done very quickly, to minimize ischemia). The needle was immediately removed to provide a lumen with a stenotic aorta. Following the surgery, all layers of muscle and skin were closed with 6-0 continuous absorbable and nylon sutures, respectively, and the wound was treated with Betadine. Immediately after the operation, 0.5 ml of 37°C saline was given intraperitoneally, and a dose of analgesic (buprenorphine, 0.1 mg/kg) was also given subcutaneously; further analgesic was administered every 8 h for the next 48 h. For the sham operation (control) the mice underwent the identical procedure, except placing of the ligature. The surgical survival rate following TAC was ~90% and mortality rate within the first month following TAC was low (<5%). Successful surgical ligation of the transverse aorta, determined by a Doppler flow velocity measurement (see below), indicated a large pressure gradient following constriction (see online Table II). TAC and sham animal heart function were evaluated 8 weeks after surgery, using echocardiography. Following this, animals were sacrificed, weighed (BW) and total heart weights (HW), LV weights (LVW), lung weights (LW), and tibia length were determined. Hearts were immediately dissected (see Animals and Tissue collection below) (removal of papillaries for muscle mechanics and splitting the LV into bilateral halves). Bilateral halves were either frozen or stored in RNAlater. Animals were divided into 2 groups based on both LVW/BW (HF 4.25mg/g-6.75mg/g; CHF>6.75mg/g) and LW/BW (HF
4.5mg/g-12mg/g; CHF>12mg/g) (see Figure 1), since LV mass and pleural edema both correlate with degree of heart failure development.

**Echocardiography.** Echocardiography was performed similar to as described previously. Briefly, anesthesia induction was performed with 2% ISOFL0 (Abott, Abott Park, IL) in a Univentor 400 anesthesia chamber. Following anesthesia, the mouse was placed in dorsal recumbence on a heated, tilt platform for echocardiography. Body temperature was maintained at 37°C, and anesthesia was continued with 0.5-1.5% ISOFL0. Transthoracic ECHO images were obtained with a Vevo 700 High Resolution Imaging System (Visual-Sonics, Toronto, Canada), using the model 707B scan head designed for murine cardiac imaging. A standoff was created for the ultrasound transducer using conductivity gel. Care was taken to avoid animal contact and excessive pressure which can induce bradycardia. Imaging was performed at a depth setting of 1 cm. Images were collected and stored as a digital cine loop for off-line calculations. Standard imaging planes, M-mode, Doppler, and functional calculations were obtained according to American Society of Echocardiography guidelines. The parasternal long-axis four-chamber view of the left ventricle (LV) was used to guide calculations of percentage fractional shortening, percentage ejection fraction, and ventricular dimensions and volumes. A right supra-clavicular view was used to measure cardiac output, calculated with aortic diameter and aortic flow velocity time index. In addition, the left atrial dimension was measured in the long-axis view directly below the aortic valve leaflets. Passive LV filling peak velocity, E (cm/s), and atrial contraction flow peak velocity, A (cm/s), were acquired from the images of mitral valve Doppler flow from a four chamber view. The E-A velocity time index (E-A VT1) was the computed velocity time integral of both E and A wave forms. The E/E-A VT1 ratio is an index of diastolic function independent of preload. The Ea and Aa were the tissue peak velocity of the LV adjacent to the mitral valve annulus. For pulsed-wave Doppler recording, a sample size of 0.3 mm was used. A sweep speed of 100 mm/s was used for M-mode and Doppler studies.

**Animals and tissue collection.** Mouse left ventricular (LV) and papillary muscles were collected from male BL6 mice 8 weeks following TAC surgery (see below). Mice were anesthetized with isoflurane (Abbott Laboratories, Chicago, IL) and sacrificed by cervical dislocation. The hearts were rapidly excised and the muscles dissected (collection of papillaries and LV) in oxygenated HEPES pH 7.4 (in mM: NaCl, 133.5; KCl, 5; NaH2PO4,
1.2; MgSO₄, 1.2; HEPES, 10) and weighed (Online Table 1). Dissected LV muscles were then divided into two pieces and: 1) snap frozen in liquid nitrogen, or 2) stored in RNAlater. Papillary muscles were skinned in relaxing solution pH 7.0 (RX) (in mM: BES 40, EGTA 10, MgCl₂ 6.56, Na₂ATP 5.88, DTT 1, K-propionate 46.35, creatine phosphate 15) (chemicals from Sigma-Aldrich, MO, USA) with 1% Triton-X-100 (Pierce, IL, USA) overnight at ~3°C, then washed thoroughly with relaxing solution and stored for one month or less at -20°C in relaxing solution containing 50% (v/v) glycerol. To prevent protein degradation, all solutions contained protease inhibitors (phenylmethylsulfonyl fluoride (PMSF), 0.5 mM; Leupeptin, 0.04 mM; E64, 0.01mM). All animal experiments were approved by the University of Arizona Institutional Animal Care and Use Committee and followed the U.S. National Institutes of Health “Using Animals in Intramural Research” guidelines for animal use.

**Gel Electrophoresis.** For titin protein analysis, SDS–agarose electrophoresis was performed as previously described⁵. Briefly, muscle samples were solubilized in a urea and glycerol buffer and analyzed by vertical SDS–agarose electrophoresis. The 1% agarose gels were run at 15 mA per gel for 3 h and 20 min. The gels were stained with Coomassie brilliant blue (CBB), and subsequently scanned and analyzed using One-D scan EX (Scanalytics Inc., Rockville, MD, USA) software. The integrated optical density of titin and MHC was determined as a function of the volume of solubilized protein sample that was loaded (a range of volumes was loaded on each gel). The slope of the linear range of the relationship between integrated optical density and loaded volume was obtained for each protein.

**Western Blots (WBs).** For titin Western blotting (WB) LV samples were run on 1% agarose gels, and transferred to PVDF membrane (Millipore, Immobilon®-FL Cat. No. IPFL00010) using a semi-dry transfer unit (Bio-Rad, Hercules, CA). The blots were stained with Ponceau S solution (SIGMA P7170) to visualize total transferred protein. The blots were then probed with rabbit polyclonal antibodies against titin’s pS26 (GenScript, 1:1000) and pS170 (Genscript, 1:250) against recombinant PEVK. On skinned fibers the antibodies were used against titin’s pS26 (GL Biochem, Shanghai, 1:1000) and pS170 (Genscript, 1:250). We also studied phosphorylation levels of S26 and S170 of skinned fibers that has been treated by PP1 (0.5 U/µl; for details, see Passive tension Measurement section, below) for 2 hours followed by WB analysis with
antibodies against pS26 and pS170. To normalize for loading differences, phosphorylated titin labeling was normalized to total protein, determined from the Ponceau S-stained membrane and analyzed with One-D scan EX. Secondary antibodies conjugated with fluorescent dyes with infrared excitation spectra were used for detection. One-color IR western blots were scanned (Odyssey Infrared Imaging System, Li-Cor Biosciences, NE, USA) and the images analyzed. We also performed a WB analysis of SERCA2A, PLB, pS23/24 cTnI, PLBpS16, and PLBpTrh17 with antibodies purchased from Badrilla LTD (Leeds, UK; catalog #A010-20), Badrilla LTD (Leeds, UK; catalog #A010-14), Cell Signaling Technology (Ma, USA, catalog # 4004S), Badrilla LTD (Leeds, UK; catalog #A010-12), Badrilla LTD (Leeds, UK; catalog #A010-13). Blots were dual labeled with an anti-GAPDH antibody (used as a loading control) that was from Abcam (Cambridge USA, ab8245).

Phosphorylation Assays

Detection of protein phosphorylation by protein labeling ($^{32}$P) and phosphoprotein stain (Pro-Q diamond). BL/6 mouse LV fibers were skinned in skinning solution pH 7.0 (in mM: 20 BES, 10 EGTA, 6.56 MgCl$_2$, 5.88 Na$_2$ATP, 1 DTT, 46.35 K-propionate, 15 creatine phosphate), and 1% (w/v) Triton X-100 (at 20°C). Subsequently, the skinned fibers were washed in relaxing solution (skinning solution without Triton X-100), and stored in relaxing solution containing 50% (v/v) glycerol at -20°C. Skinned fibers 2 mm in length, 0.5 mm in diameter were dissected. Two sets of skinned fibers were incubated with 1 U/µl of protein kinase A (PKA) catalytic subunit from bovine heart (Sigma) in relaxing solution or 0.066 U/µl of protein kinase C alpha (PKC$_\alpha$) (Enzo) in activating solution pH 7.0 (in mM: BES 16, CaCO$_3$-EGTA 4, MgCl$_2$ 2.5, Na$_2$ATP 2.4, DTT 0.85, K-propionate 18.1, creatine phosphate 6, NaCl 30, glycerol 5%), lipid activator (PS 0.2 mg/ml, DAG 0.02 mg/ml, triton X-100 0.6%), and 10 mM NaF, 2 mM Na$_3$VO$_4$). To one set 20 µCi of [$\gamma$-$^{32}$P]ATP stock solution, specific activity 3,000 Ci/mmol (PerkinElmer, USA) were added and the samples incubated 4h at RT. The reaction was stopped by adding solubilization buffer (6M urea, 2 M thiourea, 2.3% SDS, 58 mM DTT, 0.02% bromophenol blue, and 38.5 Tris HCl pH 6.8). The solubilized samples were electrophoresed on a 2 to 7% gradient SDS-PAGE and the gels were Coomassie blue stained, dried, and exposed to X-ray film. The dried gels and the autoradiography were scanned using Epson Expression 800 scanner. The images were analyzed with One-Dscan software (Scanalytics Inc) to obtain the integrated optical density. The titin
integrated OD of the autoradiograph was normalized to that of the Coomassie blue-stained gel, to normalize for protein loading. The other set of fibers was incubated as above but without radiochemical. The proteins were separated as above and the gel was stained with Pro-Q diamond to visualize proteins phosphorylated. The gel was fixed in 50% methanol 10% acetic acid, stained with Pro-Q diamond (Invitrogen), destained with 20% ACN 50 mM sodium acetate pH 4, and scanned with Typhoon 9400 (Amersham Biosciences) (excitation source 532 nm laser, emission filter 560 nm longpass). Then the gel was Coomassie blue stained to visualize total proteins and scanned as above. The Pro-Q diamond stain proteins were normalized to that of the Coomassie blue-stained gel to normalize for protein loading.

Detection of recombinant protein phosphorylation by protein labeling ($^{32}$P) phosphoprotein stain (Pro-Q diamond), and WB. Two sets of purified recombinant titin fragments, N2B and PEVK, were incubated with 1 U/µl of PKA in relaxing solution or with 0.066 U/µl of PKCa in activating solution (described above). To one set 20 µCi of [$\gamma$-$^{32}$P]ATP was added and incubated for 4h at RT. Then the proteins were denatured with SDS-PAGE reducing sample buffer (0.5 M tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.1 mM 2-mercaptoethanol, 0.01% bromophenol blue), heated for 10 min at 95°C and electrophoresed on a 4-20% gradient SDS-PAGE. The gels were stained with Coomassie blue, dried, and then scanned with an Epson Expression 800 scanner using software v 1.01e. The dried gels were exposed to Kodak BioMax MS film with Kodak BioMax TranScreen HE at -80ºC and the film developed with Kodak X-omat 200A processor. The autoradiography was scanned as above. With each gel a 21 step neutral density filter (Stouffer, IN, USA) was included to standardize optical density of proteins bands. The images were analyzed using ONE-DScan 2.05. The integrated OD of the autoradiograph was normalized by the integrated OD of the Coomassie blue-stained gel, to normalize the data for differences in protein loading. The other set of recombinant proteins was incubated as above but without radiochemical. The proteins were separated as above and the gel was stained with Pro-Q diamond to visualize proteins phosphorylated. The gel was fixed in 50% methanol 10% acetic acid, stained with Pro-Q diamond, destained with 20% ACN 50 mM sodium acetate pH 4, and scanned with Typhoon 9400 (excitation source 532 nm laser, emission filter 560 nm longpass). Then the gel was Coomassie blue stained to visualize total proteins and scanned as above. The Pro-Q diamond stain proteins were normalized to that of the Coomassie blue-


stained gel to normalize for protein loading. Recombinant PEVK protein expressed in E.
coli was phosphorylated with 0.06 U/μl PKCα (Biomol) 2h at 30°C. The protein sample
was then split with one half solubilized and the other had its PKCα inhibited with 37 nM
bisindolylmaleimide I hydrochloride (Cell Signaling) and 0.7 μM chelerythrine chloride
(Sigma). The protein was then dephosphorylated by adding 1 U/μl PP1 (Calbiochem) 2h
at 30°C. The solubilized proteins were separated by SDS-PAGE, 2-4% gradient gel, and
transferred to PDVF membrane. The membranes were stained with Ponceau S for total
protein and immunoblotted with phospho-specific polyclonal rabbit antibodies PS26 and
PS170 (GenScript). Secondary antibody goat anti-rabbit conjugated with fluorescent
dye with infrared excitation spectra was used for detection. The membranes were
scanned (Odyssey Infrared Imaging System) and the images analyzed (See Online Fig.
5 for examples).

**Microarray studies.** We dissected the mice LV following TAC and collected them in
RNAlater. The microarray experiments were performed as described previously⁵. Briefly,
RNA was isolated using the Qiagen RNeasy Fibrous Tissue Mini Kit. RNA was amplified
using the SenseAmp kit (Genisphere) and Superscript III reverse transcriptase enzyme
(Invitrogen). Reverse transcription and dye coupling (Alexa Fluor 555 and Alexa Fluor
647 were used) was done using Invitrogen’s superscript plus indirect cDNA labeling
module. Half of each sample was incorporated with Alexa Fluor 555 and the other with
Alexa Fluor 647. All the mouse titin (50 mer oligonucleotides) were spotted in triplicate
on Corning Ultra GAPS glass slides. For each sample 750 ng of cDNA (Nanodrop,
Thermo scientific) from each sample was hybridized (Ambion: Slide-Hyb buffer #1) for
16 h at 42 °C after which slides were scanned at 595 nm and 685 nm with an Array
WoRx scanner. Spot finding was done with SoftWoRx Tracker and spot analysis with
CARMA⁷. The analysis detects relative changes in the fluorescence of a probe (adult as
compared to neonate). These changes are represented as a fold-difference and reflect
the ratio of adult exon expression to neonate exon expression. A positive fold change
indicates an increase in expression in adult relative to the neonate. A negative fold
change indicates an increase in expression in neonate relative to the adult sample.

**Measurement of passive tension.** Small muscle strips (100-200 µm in diameter, ~2mm
in length) were dissected from the stored skinned papillary preparations. Passive tension
was measured with a strain gauge force transducer and fiber length was controlled by a high-speed motor. Preparations were attached to the motor arm and the force transducer via aluminum clips and lowered into a chamber containing relaxing solution. The width and the height of the fiber were measured and the cross-sectional area (CSA) was calculated assuming the cross section is elliptical in shape. The measured forces were then converted to tension (force/CSA). Typically three preparations were studied per heart and the average was obtained for each mouse. Sarcomere length (SL) was measured on line at 1 kHz by laser diffraction. At the beginning of each experiment, muscles were activated at SL 2.0 µm (pCa 4.0) to measure residual active tension. Typically maximal active tension was between ~30-40 mN/mm². For Online Table III, tensions are converted to stiffness; stiffness is defined as the slope of the linear fits between the SL ranges of 1.95-2.05 µm as related to passive tension.

**Stretch-Hold Protocol:** Relaxed fibers were stretched (10%/sec) from their slack length (mouse ~1.85 µm) to a SL of 2.15 µm, followed by a 90 second hold. At the end of the “hold” the fiber was released back to slack length and allowed to rest 12 minutes before the next stretch. Muscles were stretched 4 times in order to show reproducibility of the force curve. Muscles were thoroughly rinsed in 20 mM BDM, a know inhibitor of actomyosin interaction that abolishes active isometric force development and stretches were repeated. For the PP1-treatment Protocol muscles were treated similar to the above protocol where a baseline force curve was determined prior to PP1-treatment. Following baseline determination muscles were treated in-chamber with PP1, α-isoform, rabbit muscle, recombinant, expressed in E. coli (Calbiochem) (0.5 U/µl) for 2 hours, stretching at every 20 minute interval. To determine titin and collagen contribution to passive force, thick and thin filaments were extracted from the sarcomere, removing titin’s anchors in the sarcomere⁸. The remaining force, assumed to be collagen based, was subtracted from the pre-extraction forces to determine titin-specific forces (for details, see⁸).

**Statistics.** Data are presented as mean ± SEM. Group significance was defined using ANOVA followed by Tukey-Kramer multiple comparison test; SL significance was determined using 2-way ANOVA; Student’s t-test was used in Fig. 6A, inset; probability values <0.05 were taken as significant and are indicated on figures as: HF and CHF significant from control, *P < 0.05; CHF significant from HF, †P < 0.05 (two symbols:
ANOVA with a linear model that incorporates terms to account for experimental variability was used in analysis of microarray data (for details see Greer et al., 2006). Linear regression analysis was used as necessary for correlations, \(*P < 0.05\).