Deletion of the titin N2B region accelerates myofibrillar force development but does not alter relaxation kinetics

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ABSTRACT
Cardiac titin is the main determinant of sarcomere stiffness during diastolic relaxation. To explore whether titin stiffness affects the kinetics of cardiac myofibrillar contraction and relaxation, we used subcellular myofibrils from the left ventricles of homozygous and heterozygous N2B-knockout mice which express truncated cardiac titins lacking the unique elastic N2B region. Compared with myofibrils from wild-type mice, myofibrils from knockout and heterozygous mice exhibit increased passive myofibrillar stiffness. To determine the kinetics of Ca\(^{2+}\)-induced force development (rate constant \(k_{\text{ACT}}\)), myofibrils from knockout, heterozygous and wild-type mice were stretched to the same sarcomere length (2.3 \(\mu\)m) and rapidly activated with Ca\(^{2+}\). Additionally, mechanically induced force-redevelopment kinetics (rate constant \(k_{\text{TR}}\)) were determined by slackening and re-stretching myofibrils during Ca\(^{2+}\)-mediated activation. Myofibrils from knockout mice exhibited significantly higher \(k_{\text{ACT}}\), \(k_{\text{TR}}\) and maximum Ca\(^{2+}\)-activated tension than myofibrils from wild-type mice. By contrast, the kinetic parameters of biphasic force relaxation induced by rapidly reducing [Ca\(^{2+}\)] were not significantly different among the three genotypes. These results indicate that increased titin stiffness promotes myocardial contraction by accelerating the formation of force-generating cross-bridges without decelerating relaxation.

KEY WORDS: Crossbridge kinetics, Muscle relaxation, Passive tension, Titin genotype effects, Diastolic dysfunction

INTRODUCTION
Titin is a giant protein in striated muscle that spans the half sarcomere from the Z-disc to the M-band. The I-band region of titin contains a multifunctional spring that determines the elastic properties of the passive sarcomere. The passive tension of titin is important for centering the A-band in the middle of the sarcomere (Horowits and Podolsky, 1987). When the cardiac ventricle is filled in diastole, the sarcomeres get stretched. Because the compliant I-band region of titin takes up most of the strain, when the cross-bridges are detached, titin is the main determinant of passive stiffness of the sarcomere. The extensible I-band region of titin contains three types of viscoelastic elements: (1) the tandem Ig segments composed of serially linked immunoglobulin-like (Ig-like) domains, (2) the PEVK region, rich in proline (P), glutamate (E), valine (V), and lysine (K) and (3) the N2B region (Granzier and Labeit, 2002; Granzier and Labeit, 2004; Trombitas et al., 1999). Whereas the Ig-like domains and the PEVK region are found in both cardiac and skeletal muscle, the unique N2B region is exclusively expressed in cardiac muscle (Linke et al., 1999).

The high elastic and low viscous modulus of the N2B region enables the cardiac sarcomere to achieve a high efficiency during repeated contraction-relaxation work loop cycles (Nedrud et al., 2011). The elasticity of the N2B region can be modulated by PKA- or PKG-mediated phosphorylation to adapt to the mechanical needs (Krüger et al., 2009; Yamasaki et al., 2002). Recently, we have generated a mouse model deleting exon 49, which encodes the N2B region and leaves the remainder of the gene encoding titin intact. The homozygous N2B-knockout mice exhibit diastolic dysfunction with increased diastolic wall stress and passive myocyte stiffness. Passive skinned myocytes of N2B-knockout mice are threefold stiffer than those of wild-type mice, which can be explained by the increased strain of the remaining I-band region of titin (Radke et al., 2007). The knockout mice have been used to test the impact of titin stiffness and titin-based passive tension on length-dependent activation (LDA), i.e. the increase in the Ca\(^{2+}\) sensitivity of force development due to sarcomere stretch that provides the basis of the Frank-Starling mechanism of the heart (Lee et al., 2013; Lee et al., 2010). These studies revealed that LDA is enhanced in the knockout mice compared with LDA of wild-type mice and that LDA correlates under various conditions with titin-based passive tension.

It is not known whether titin affects cross-bridge kinetics during Ca\(^{2+}\)-induced myocardial contraction and relaxation. A recent study investigated the effect of altered titin stiffness on force kinetics using myofibrils of skeletal muscle from rats harboring a mutation of the multifunctional splicing factor RBM20 that resulted in longer more-compliant titins (Guo et al., 2012; Mateja et al., 2013). This revealed a slowed Ca\(^{2+}\)-induced force development and reduced active force (Mateja et al., 2013). Cardiac trabeculae from the same mutant rats exhibit reduced passive stiffness, slowed tension redevelopment, reduced active tension and impaired LDA (Patel et al., 2012). However, previous studies of myocardial preparations from several species, including mouse, rat and human, provided evidence that turnover kinetics of cross-bridges are unaffected by LDA and by changes of sarcomere length that alter passive tension (Edes et al., 2007; Wannenburg et al., 2000; Wannenburg et al., 1997). This suggests that, despite the increased strain of titin, the enhanced force at greater sarcomere length results from the recruitment of new cross-bridges rather than from rate-modulation of the transition of cross-bridges to force-generating states. Thus, it remains unclear whether the mechanical properties of titin affect cross-bridge
turnover kinetics and force kinetics of the myofibrillar contraction-relaxation cycle in the heart.

In particular, it is unknown whether the kinetics of cardiac myofibrillar relaxation are affected by increased titin-based passive tension. Knowledge of the latter is important to better understand the sarcomeric mechanisms causing diastolic dysfunction. Both the passive tension of titin and the active tension of cross-bridges can elevate sarcomere tension during relaxation and thus impair ventricular filling. Incomplete inactivation, either due to an incomplete reduction in $[\text{Ca}^{2+}]$ to fully relaxing concentrations or due to an incomplete switching off of the regulatory troponin-tropomyosin system, causes residual active tension and slows relaxation (Iorga et al., 2008; Kruger et al., 2005; Stehle et al., 2009). Here, we use the N2B-knockout mice as a genetic model of diastolic dysfunction to investigate whether increased titin-based stiffness also slows down cardiac myofibrillar relaxation. At sarcomere lengths above the slack length, titin-based passive stiffness is expected to slow relaxation. We therefore explored the effect of titin stiffness on relaxation kinetics in cardiac myofibrils by setting the sarcomere length at relaxation to 2.3 μm, where titin exerts a substantial passive tension and restoring forces do not apply. Cardiac myofibrils rapidly equilibrate with the solution and this enables residual active tension and restoring forces do not apply. Cardiac myofibrils rapidly equilibrate with the solution and this enables incomplete inactivation, either due to an incomplete reduction in $[\text{Ca}^{2+}]$ to fully relaxing concentrations or due to an incomplete switching off of the regulatory troponin-tropomyosin system, causes residual active tension and slows relaxation (Iorga et al., 2008; Kruger et al., 2005; Stehle et al., 2009). Here, we use the N2B-knockout mice as a genetic model of diastolic dysfunction to investigate whether increased titin-based stiffness also slows down cardiac myofibrillar relaxation. At sarcomere lengths above the slack length, titin-based passive stiffness is expected to slow relaxation. We therefore explored the effect of titin stiffness on relaxation kinetics in cardiac myofibrils by setting the sarcomere length at relaxation to 2.3 μm, where titin exerts a substantial passive tension and restoring forces do not apply. Cardiac myofibrils rapidly equilibrate with the solution and this enables incomplete inactivation, either due to an incomplete reduction in $[\text{Ca}^{2+}]$ to fully relaxing concentrations or due to an incomplete switching off of the regulatory troponin-tropomyosin system, causes residual active tension and slows relaxation (Iorga et al., 2008; Kruger et al., 2005; Stehle et al., 2009).

RESULTS

Three different genotypes were explored: homozygous wild-type mice ($n=6$) expressing full-length titin, homozygous knockout mice ($n=4$) lacking exon 49, which encodes the compliant N2B region of titin, and heterozygous mice ($n=4$), i.e. mice lacking exon 49 only from one of the two alleles. Titin isoform expression in the three genotypes was quantified by densitometry of 1% vertical SDS-agarose gels loaded with left ventricular samples from 5–7-month-old sex-matched mice. Compared with the wild-type, in the knockout and heterozygous mice the N2B and N2BA titin isoforms exhibited a higher mobility, confirming the expression of truncated N2B (tN2B) and truncated N2BA (tN2BA) isoforms (Fig. 1A). Interestingly, samples from heterozygous mice contained significantly more ($P=0.01$) truncated (tN2B+tN2BA) than full-length (N2B+N2BA) titins. Instead of the 50% expected for the heterozygous genotype, the truncated titins amounted to 55±2% ($n=7$; ±s.e.m.) of total titin.

In addition, we tested whether titin remains stable during the preparation of the myofibrils. Subcellular myofibrils were prepared as described in Materials and Methods for the mechanical experiments, and titin isoform expression was determined by gel electrophoresis (supplementary material Fig. S1). The content of degraded titin in myofibrils was 11–12% for all three genotypes and was not significantly different to that found in myocardial samples, indicating the stability of titin during the preparation of myofibrils.

Effects of the N2B deletion on the passive-tension–sarcomere-length relationship of myofibrils

We tested whether the N2B deletion leads to increased passive stiffness in myofibril preparations, as shown in previous studies on cardiomyocytes (Radke et al., 2007). To do this, we stretched myofibrils at pCa ($-\log[\text{Ca}^{2+}]$) 8 to different lengths. After remaining at the stretched length for 30 s, an image of the myofibril was taken to evaluate the actual sarcomere length, and the passive force of the myofibril was measured. Force was normalized to the cross-sectional area (CSA) calculated from myofibrillar diameter and passive tension (passive tension=active force/CSA) and was plotted against the actual sarcomere length. The resulting passive-tension–sarcomere-length relationships of the three genotypes are shown in Fig. 1B. In line with previous findings on skinned cardiomyocytes (Radke et al., 2007), at a sarcomere length of >2.1 μm, myofibrils from knockout mice exhibited ~2.6-fold higher passive tension than myofibrils from the wild-type controls. The newly explored heterozygous genotype in this study yielded a myofibrillar passive tension that was ~2.1-fold higher than that of the wild-type myofibrils and was more similar.
Effects of N2B deletion on myofibrillar force kinetics

To explore the effect of titin stiffness on the kinetics of contraction and relaxation, myofibrils from the left ventricles of wild-type, heterozygous and knockout mice were mounted in a setup between a micro-needle and an atomic-force microscope cantilever. In this setup, a rapid solution change that is based on switching between two microflows is applied to the myofibril. By changing rapidly (within ~5 ms) from a flow of low [Ca\textsuperscript{2+}] (pCa 8) to high [Ca\textsuperscript{2+}] (pCa 4.6), or vice versa (Steihle et al., 2002b), the myofibril can be rapidly activated and relaxed. The protocol used for measuring the active force and force kinetic parameters is illustrated in Fig. 2 (for the non-normalized force transients see supplementary material Fig. S2). The observed force kinetics following a step increase of [Ca\textsuperscript{2+}] from pCa 8 to pCa 4.6 is consistent with previous reports for cardiac myofibrils of murine left ventricles (Steihle et al., 2002b), revealing a mono-exponential increase in force (starting at t=0.5 s in Fig. 2A) with a rate constant k\textsubscript{ACT}. ANOVA analysis revealed a highly significant effect (P<0.001) of the genotype on k\textsubscript{ACT}. Pairwise post-tests yielded highly significantly (P<0.001) elevated k\textsubscript{ACT} values for myofibrils from knockout compared with wild-type mice (Fig. 3A). Accordingly, maximum force (F\textsubscript{max}) per CSA of the myofibrils tested by ANOVA was genotype-dependent (P<0.01), and myofibrils of knockout but not of those from heterozygous mice exhibited a significantly higher F\textsubscript{max}/CSA (i.e. maximum tension) compared with myofibrils from wild-type mice in post-test analysis (Fig. 3B). The CSA of the myofibril bundles was not different between the three genotypes (supplementary material Fig. S3A), which confirms that the higher tension of myofibrils from knockout compared with wild-type mice was not related to differences in the geometry of the myofibrils that were selected for the force measurements. Furthermore, the difference in maximum tension between myofibrils from knockout and wild-type mice was not due to the higher passive tension of myofibrils from knockout mice. This finding might, in part, be explained by the higher amount of truncated titin compared with full-length titin in the myofibrils of heterozygous mice (see Discussion).

Contraction kinetics were further explored by inducing force development using a mechanical release-restretch maneuver applied to the myofibril while the [Ca\textsuperscript{2+}] was kept constant at pCa 4.6. Following the restretch (at t=1.5 s in Fig. 2), the force redevelops in a single exponential manner yielding the rate constant of tension redevelopment k\textsubscript{TR}. The value k\textsubscript{TR} reflects cross-bridge cycling kinetics, i.e. the sum of apparent rate constants limiting the forward turnover of cross-bridges from non-force-generating to force-generating states (f\textsubscript{app}) and from force-generating to non-force-generating states (g\textsubscript{app}), i.e. k\textsubscript{TR}=f\textsubscript{app}+g\textsubscript{app} (Brenner and Eisenberg, 1986). As in the former case of Ca\textsuperscript{2+}-induced force development, the kinetics of mechanically induced force redevelopment were genotype-dependent (P<0.01 tested by ANOVA). Post-tests revealed that the values of k\textsubscript{TR} were significantly higher (P<0.05) for myofibrils from both heterozygous and knockout mice compared with those of the wild-type mice (Fig. 3C). By contrast, there was no difference between heterozygous and knockout myofibrils. This apparent dominant effect of N2B deletion on contraction kinetics might be, at least in part, related to the higher amount of truncated versus full-length titin in the myofibrils of heterozygous mice. Overall, the effects of the genotype on k\textsubscript{ACT} and k\textsubscript{TR} indicate that the deletion of the elastic
N2B region of titin accelerates force development, revealing a novel role for titin stiffness in modulating the rate of contraction, regardless of whether contraction is induced by changing [Ca\textsuperscript{2+}] or is induced by returning from unloaded to isometric contraction.

The kinetics of force relaxation were induced by lowering the [Ca\textsuperscript{2+}] from pCa 4.6 to 8 (at \(t=2.5\) s in Fig. 2A or \(t=0\) s in Fig. 2B). Myofibrils from all genotypes exhibit the typical biphasic force decay, as reported previously for cardiac myofibrils of different species, including mice (Stehle et al., 2002b). Force starts to decay with a slow linear decay, which has a rate constant \(k_{\text{LIN}}\) and lasts for time \(t_{\text{LIN}}\), followed by a rapid exponential decay with a rate constant \(k_{\text{REL}}\). However, the myofibrils from neither heterozygous nor from knockout mice exhibited significant differences in any of the three relaxation parameters, \(k_{\text{LIN}}\), \(t_{\text{LIN}}\) or \(k_{\text{REL}}\), compared with the wild-type myofibrils (Fig. 4). This implies that the passive tension of titin does not influence relaxation kinetics.

**Effects of N2B deletion on the Ca\textsuperscript{2+} sensitivity of myofibrils**

We tested whether the accelerated force development of myofibrils from knockout compared with wild-type mice are related to altered Ca\textsuperscript{2+} sensitivity of contraction. The active forces of myofibrils from wild-type, heterozygous and knockout mice were determined by initiating contraction at different [Ca\textsuperscript{2+}] and the same sarcomere length (2.3 \(\mu\)m), as was performed to obtain \(k_{\text{ACT}}\) and \(k_{\text{TR}}\). The force–pCa relationships of individual myofibrils were evaluated for pCa\textsuperscript{50} (i.e. the –log[Ca\textsuperscript{2+}] required for half-maximal force production) and for the Hill coefficient \(n_H\), as an indicator for the cooperativity of the force–pCa relationship (Fig. 5C,D). The averaged force–pCa relationships, derived from data pooled from all myofibrils of a genotype, are shown in Fig. 5A. Myofibrils of heterozygous or knockout mice exhibited no significant difference in pCa\textsuperscript{50} values compared with the myofibrils of wild-type mice (Fig. 5C). This result suggests that the accelerated contraction of myofibrils from heterozygous and knockout mice does not result from an enhanced Ca\textsuperscript{2+} sensitivity of force development. However, there is a trend towards higher cooperativity with the expression of the N2B-deleted titin (Fig. 5B,D). ANOVA revealed a significant difference (\(P<0.05\)) in the \(n_H\) values among all genotypes. Post-test analysis revealed the \(n_H\) value of myofibrils from knockout mice to be significantly (\(P<0.05\)) higher than that of the wild-type myofibrils (Fig. 5D). This opens the interesting possibility that faster force development kinetics of myofibrils with stiffer titin might, to some extent, relate to enhanced Ca\textsuperscript{2+} sensitivity.
cooperative formation of force-generating cross-bridge interactions during Ca\(^{2+}\)-mediated activation.

**DISCUSSION**

Here, we present the first functional analysis of myofibrils from N2B-deficient mice. A major advantage of the myofibril preparation over multicellular preparations is that it is suitable for studying force kinetics during a contraction-relaxation cycle induced by rapid defined changes in [Ca\(^{2+}\)]. We find that increasing myofibrillar stiffness by deleting the elastic N2B region of titin does not affect relaxation kinetics but accelerates force-activation kinetics.

**Titin stiffness does not affect force-relaxation kinetics**

Diastolic function of the heart can be separated into an early and a late phase related to the isovolumic relaxation and filling. Cardiac myofibrils allow the study of sarcomere function of both early and late diastolic processes by analysing the force kinetics during relaxation and the passive tension after relaxation, respectively. As titin is the main contributor to the passive tension of the relaxed sarcomere, the increased passive tension of an N2B-deficient titin might affect relaxation. Here, we show that the increased passive force does not alter the relaxation kinetics of cardiac myofibrils. This is an important finding because it restricts the main effects of titin-based passive tension to the filling phase.

Our results demonstrate that myofibrils lacking the N2B region of titin exhibit an increased steepness of the sarcomere-length–passive-tension relationship, i.e. increased passive myofibrillar stiffness. This corroborates the results and interpretations of previous studies on cardiomyocytes and papillary muscle from these N2B-deficient mice, confirming the basic mechanical function of the unique cardiac N2B region as a highly extensible spring, strongly contributing to the elasticity of the relaxed sarcomere (Nedrud et al., 2011; Radke et al., 2007). Increased passive tension upon sarcomere stretch of cardiac myofibrils lacking this highly elastic region compared with those containing full-length titin can be explained by the increased strain of the remaining elastic I-band regions of titin. Myofibrils from heterozygous mice also exhibited strongly increased passive stiffness, further corroborating the importance of the extensible N2B region for cardiac sarcomere compliance. Thus, both myofibrils from heterozygous and knockout mice can be regarded as a model to investigate the effect of increased titin-based passive tension on the dynamics of the Ca\(^{2+}\)-controlled contraction-relaxation cycle.

Here, we find that the rate constant \(k_{\text{REL}}\) of the rapid relaxation phase leading to the relaxed state does not depend on the final passive tension. The rapid phase of relaxation occurs while sarcomeres re-lengthen from the contracted to the relaxed state (Stehle et al., 2002a). Hence, during ongoing relaxation, titin will become gradually more and more strained and titin-based passive tension increases. Nevertheless, this does not seem to significantly slow down the transition of the cardiac myofibril to the relaxed state. The rate constant \(k_{\text{LIN}}\), the time of the initial slow linear force decay \(t_{\text{LIN}}\) and the rate constant of the final exponential force decay \(k_{\text{REL}}\) did not differ among the three genotypes, which is consistent with the notion that a decrease in active force rather than an increase in passive force determines the kinetics of the force decay during relaxation (Stehle et al., 2006).

In terms of cross-bridge turnover kinetics, and similarly to the ratio of ATPase per unit force (called tension cost), \(k_{\text{LIN}}\) can be interpreted as a measure of \(g_{\text{app}}\), i.e. the apparent rate by which cross-bridges leave force-generating states under isometric conditions (Stehle et al., 2002a; Tesi et al., 2002). The finding that deletion of the elastic N2B region does not affect \(k_{\text{LIN}}\) is in agreement with a recent study by de Tombe’s group exploring the contraction-relaxation kinetics of myofibrils from tibialis anterior skeletal muscle of RBM20-deficient rats with altered titin isoform.
expression (Mateja et al., 2013). They showed that $k_{\text{LIN}}$ is not altered by reduced titin stiffness. However, there have been some divergent results concerning whether the value of $g$ reported by the tension cost is affected by sarcomere-length-dependent changes in passive tension (Mateja et al., 2013; Wannenburg et al., 1997). The only significant difference in the tension cost between RBM20-deficient rats and wild-type rats was observed at low sarcomere length, where it was higher for the wild-type myofibrils. As sarcomere length is reduced below the slack length, titin exerts a restoring force (Helmes et al., 1996) that might accelerate cross-bridge detachment. At sarcomere lengths above the slack length, where our study was conducted, restoring forces do not apply. Both our study of mice expressing a shorter titin and previous work in RBM20-deficient rats expressing longer titin isoforms (Mateja et al., 2013), which evaluate the isolated passive properties of titin at sarcomere lengths above the slack length, suggest that titin-based passive tension does not affect relaxation kinetics.

Furthermore, our results could be affected by the loss of post-translational modifications on the N2B region of titin. Phosphorylation within the N2B region of titin by PKA or PKG reduces passive tension and improves diastolic function (Krüger et al., 2009; Yamasaki et al., 2002). Accordingly, loss of PKA and PKG sites in the N2B region would be expected to have similar effects to those that we expected from increased titin-based passive tension in slowing down the final phase of relaxation phase. However, because $k_{\text{SEL}}$ of myofibrils from all three genotypes was similar and not reduced by N2B deficiency in myofibrils from knockout and heterozygous mice compared with the wild-type myofibrils, it is unlikely that we failed to see an effect of titin stiffness on relaxation kinetics.

**Modulation of force development kinetics by titin**

We show that deletion of the elastic N2B region from titin in the myofibrils of knockout mice enhances passive stiffness, increases maximum Ca$^{2+}$-activated force and increases the rate constant of force development, regardless of whether force is induced by increased [Ca$^{2+}$] (rate constant $k_{\text{ACT}}$) or by mechanical manipulation (rate constant $k_{\text{TR}}$). By contrast, the group of de Tombe and Moss have recently shown that myofibrils from skeletal muscle and skinned cardiac fibres of adult RBM20-deficient rats expressing a long compliant titin isoform in their muscles exhibit reduced passive stiffness, reduced active force and lower rate constants of force development (Mateja et al., 2013; Patel et al., 2012). However, the RBM20-deficient rat model is based on a spontaneous mutation of the splicing factor RBM20. This deficiency does not only influence the splicing of titin, where it is important for the proper splicing of the middle I-band Ig domains and the PEVK region, it also influences additional splicing of other targets, such as calmodulin-dependent protein kinase-IIα (CaMKIIα) (Guo et al., 2012). CaMKIIα also phosphorylates titin (Hidalgo et al., 2013). Although only the N2B element is deleted in the knockout mice studied here, secondary effects cannot be completely excluded. However, in combination, the recent findings in RBM20-deficient rats and our present findings in the N2B-knockout mice provide clear evidence for a positive correlation between the rate constant of force development and the passive stiffness of titin.

Force-redevelopment kinetics reflect cross-bridge turnover kinetics; $k_{\text{TR}}$ reports the sum of the apparent rate constants $f$ and $g$ that rate-limit the transition of cross-bridges from non-force-generating to force-generating states and from force-generating to non-force-generating states, respectively (Brenner and Eisenberg, 1986). Because the value of $g$ reported by $k_{\text{LIN}}$ is not significantly different between the three genotypes studied here, increased titin stiffness predominantly affects cross-bridge turnover by increasing the probability of the transition of cross-bridges from weakly bound, non-force-generating states to force-generating states. Because force is proportional to the apparent duty ratio [$f/(f+g)$], an increase of $f$ at constant $g$ results in a higher force, which could explain, to some extent, the 22% higher active tension of myofibrils from knockout compared with wild-type mice. 

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<th>$k_{\text{TR}}$</th>
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<td>(5.01 s$^{-1}$)</td>
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This alone does not seem to be sufficient to explain the 22% higher active tension of myofibrils from knockout compared with wild-type animals. An additional explanation might be that increased titin stiffness better maintains the structural integrity of the sarcomere during repeated activations (for example, by better keeping the A-bands centered in the sarcomere) and that this enhances active tension. Consistent with this idea, we show that in the knockout myofibril, less active tension is lost after a series of activations than in the wild-type myofibril (supplementary material Fig. S4). In summary, our results indicate that enhanced strain by titin increases active force in part by promoting the turnover of cross-bridges to force-generating states and possibly also by maintaining the structural integrity of the sarcomeres.

The rate constant $f$ (i.e. the probability of the cross-bridge transition to force-producing states) can be regarded as a product of the fraction of cross-bridges attached to actin in a pre-force-generating state multiplied by the rate constant of the forward transition from this pre-force state to force states. One possibility would be that the higher passive tension transmitted from titin to the myosin filament reduces the lattice spacing in the A-band, so that non-force-generating cross-bridges get closer to the thin filament, enhancing their attachment in the pre-force-generating configuration to the activated thin filament. The idea of improved cross-bridge attachment at reduced radial spacing is related to the inter-filament spacing hypothesis that has been a widely held hypothesis for explaining LDA (McDonald and Moss, 1995), but which has become controversial (Konhilas et al., 2003). In our study, the activation of myofibrils was initiated at a sarcomere length of 2.3 μm, at which myocardium from knockout relative to wild-type animals exhibits significantly increased LDA (+0.07 pCa) and modestly but significantly reduced myofilament lattice spacing (~1.9 nm) (Lee et al., 2013; Lee et al., 2010). However, whereas there is consensus among studies that lattice compression using up to 4% dextran usually slightly increases the maximum force, which might explain the increased $F_{\text{max}}$ in our study (Kawai and Schulman, 1985; Konhilas et al., 2002; Lee et al., 2013; McDonald and Moss, 1995; Millman, 1998), there is divergence on whether lattice compression slightly decelerates (Kawai and Schulman, 1985) or slightly accelerates cross-bridge turnover (McDonald and Moss, 1995). We consider it unlikely, therefore, that the higher $k_{\text{TR}}$ obtained with myofibrils from heterozygous and knockout mice can be fully explained by decreased lattice spacing.

In our experimental system, we did not detect significant differences in the Ca$^{2+}$ sensitivity of force generation between
myofibrils from wild-type and knockout mice – unlike the previously published higher Ca\(^{2+}\) sensitivity (+0.07 pCa) of skinned knockout papillary muscles compared with wild-type papillary muscles (Lee et al., 2013; Lee et al., 2010). Thus, it is unlikely that the faster cross-bridge kinetics of myofibrils from knockout compared with wild-type mice results from an increased Ca\(^{2+}\) sensitivity or LDA.

The present study reveals an increased maximum force (\(F_{\text{max}}\)) of myofibrils from knockout compared with wild-type mice, which had not been detected in studies using skinned myocytes (Radke et al., 2007) or skinned papillary muscle (Lee et al., 2010). The reason for this divergent finding in myofibrils compared with the other preparations of higher structural complexity is not clear. However, each preparation has advantages and disadvantages. As the force exerted by myofibrils results almost solely from their unidirectional aligned sarcomeres it provides a more pure preparation for probing sarcomere function but it does not include additional effects resulting from the higher structural complexity and extracellular properties of myocardium. Additionally, owing to the short diffusion distances in myofibrils, the build-up of metabolites that can depress force (e.g. ADP, P\(_i\)) will be much less than in myocytes and papillary muscle, and the myofibril might provide more accurate force levels.

The mechanism of how strain in the I-band region of titin modulates cross-bridge turnover kinetics is unknown. However, because titin attaches to the myosin filament, passive tension is communicated to the thick filament, and this might affect cross-bridge behavior. Consistent with this hypothesis, X-ray diffraction studies reveal that the proximity of myosin heads to the thin filaments, as indicated by the intensity ratio \(I_{12}/I_{10}\) of equatorial reflections, is favored at high passive tension (Lee et al., 2013). Furthermore, a recent X-ray diffraction study performed by Farman and co-workers showed that LDA might be based on the ordering of weakly bound cross-bridge orientation prior to activation (Farman et al., 2011). Titin winds along the myosin filament (Al Khayat et al., 2013), and it is tempting to speculate that increased passive tension affects myosin such that it enhances the probability of cross-bridge transitions to force-generating states, as reflected by the higher \(k_{\text{ACCT}}\) and \(k_{\text{TR}}\) in myofibrils from knockout compared with wild-type mice in our study. Regarding the physiological situation when the sarcomeres are stretched during diastolic filling of the heart, the acceleration of cross-bridge cycling by increased titin-based passive tension might compensate for the potential slowing down of cross-bridge cycling resulting from the reduction of filament overlap. Further studies are required to dissect the individual contributions of the I-band regions of titin and the A-band lattice in priming the cross-bridges prior to activation for a faster transition to force-generating states upon Ca\(^{2+}\)-induced contraction.

Dominant effects of N2B-deleted titin in myofibrils from heterozygous mice

Interestingly, the passive stiffness of myofibrils from heterozygous mice was higher than expected from the 55%:45% ratio of N2B-deleted titin to wild-type titin and was almost as high as that of myofibrils from the knockout mice. Titin heterozygotes are unique in their mixed expression of titin molecules, as compared with homozygous animals, which express either only wild-type or only N2B-deficient isoforms. The net effect of how coexisting wild-type and N2B-deficient titin isoforms will affect passive stiffness is difficult to predict, especially as data on the spatial organization of titin within the intact sarcomere structure is scarce. Based on three-dimensional single-particle analysis of electron-microscopy micrographs, A-band titin might exist in pairs (Al Khayat et al., 2013; Zoghbi et al., 2008). The distal tandem Ig segment has been suggested to form a higher-order structure of a side-to-side hexamer (Houmeida, et al., 2008). Nevertheless, the orientation and localization of titin along the sarcomere is not sufficiently resolved. If titin mainly traverses the sarcomere I-band as dimers, then heterodimers consisting of an N2B-deleted and a wild-type titin strand would have a higher stiffness than the two monomeric strands, because the extension of the elastic N2B element of wild-type titin will be restricted owing to lateral interactions that keep it in register with the shorter N2B-deleted titin strand. This would result in a dominant effect of the shorter titin. If titin mainly exists as monomers, thereby acting as an independent passive spring, then passive stiffness should increase linearly with the amount of the stiff N2B-deleted titin isoform. In this case, passive stiffness of myofibrils from heterozygous mice would scale at 55% relative to that of wild-type (0%) and knockout (100%) myofibrils. By contrast, if titin mainly forms dimers, the passive stiffness of myofibrils from heterozygous mice would scale at 80% relative to that of wild-type (0%) and knockout (100%) myofibrils, calculated based on a random probability of the formation of wild-type and knockout homodimers (WT–WT and KO–KO) and heterodimers (WT–KO), consistent with our findings.

The dominant effect of the N2B-deletion is also observed on \(k_{\text{TR}}\), suggesting that cross-bridge turnover kinetics are accelerated by the increased titin stiffness in myofibrils of heterozygous mice. However, this was not associated with an elevated active force. Although an inhomogeneous distribution of wild-type and N2B-deficient titin could also result in a lower active force, it is unlikely that titins expressed from different alleles distribute non-uniformly among sarcomeres because, in a recent study, we always observed a homogeneous distribution of GFP-tagged titin versus non-tagged titin in heterozygous animals from different alleles (da Silva Lopes et al., 2011). However, even a homogeneous distribution of wild-type and N2B-deficient titin would not stabilize the filament lattice as expected from the increase in passive stiffness, because the unequal tensile forces that WT–WT titin double strands exert compared with those of WT–KO and KO–KO strands on a thick filament would shift the position of the thick filament from the center of the surrounding hexagonal array of the thin filaments. As a result, some of the cross-bridges cannot form force-generating interactions with the thin filament, which might explain why active force is not increased in myofibrils from heterozygous mice.

Conclusions

Incorporating titin-based stiffness does not affect the relaxation kinetics of cardiac myofibrils. This contrasts with our previous findings showing that incomplete inhibition of active tension by impaired regulatory function of cardiac troponin I slows down relaxation by decreasing the rate constant \(k_{\text{REL}}\). In combination, these findings will be useful for better differential diagnosis of diastolic dysfunction that is generally indicated by impaired filling and elevated end-diastolic pressure (EDP). However, elevated EDP can be either based on elevated passive tension or residual active tension and, thus, can underlie completely different pathomechanisms, requiring different therapies. We
therefore propose that impaired relaxation kinetics could be a specific indicator of the dysregulation of active tension.

By contrast, our results indicate that titin-based passive tension affects systolic function by promoting the turnover of cross-bridges to force-generating states upon Ca\(^{2+}\)-induced contraction. Further work is required to determine whether this underlies similar mechanisms to the ones proposed for LDA. Independent of the detailed mechanism, the results suggest that titin-based tension tunes cross-bridge turnover kinetics during the cardiac cycle in an advantageous manner, by accelerating cardiac contraction without decelerating relaxation.

**MATERIALS AND METHODS**

**Preparation of myofibrils**
The generation of N2B-deficient mice expressing cardiac titin that lacks the elastic N2B region has been described previously (Radke et al., 2007). Age-matched (5–7-months-old) homozygous knockout, heterozygous and wild-type mice of either gender were sacrificed by cervical dislocation as approved by the institutional Animal Care and Use Committee. Immediately after heart excision, the papillary muscles were dissected from the left ventricle and pinned with needles at their ends on the Sylgard surface of a chamber. To dissolve all membranous structures, the papillary muscles were incubated for 2 h in 1% Triton X-100 skinning solution containing 5 mM potassium phosphate, 5 mM potassium azide, 2 mM magnesium acetate, 5 mM K\(_2\)EGTA, 3 mM Na\(_2\)ATP and 47 mM potassium creatine phosphate (pH 7.0). The solution was then replaced by an identical one without Triton X-100, and samples were stored for up to 24 h at 0°C. Immediately before the experiment, the skinned papillary muscles were homogenized at 4°C for 5–10 s at maximum speed with a blender (Ultra-Turrax T25, Janke & Kunkel, Staufen, Germany). Some of the myofibril suspension was used for protein analysis, and the rest was used for mechanical measurement.

**SDS-gel electrophoresis**
The titin protein analysis of fresh myocardial samples or myofibrillar preparations prepared as described above was performed as described previously (Warren et al., 2003). Myocardial samples were frozen in liquid N\(_2\) and homogenized with a mortar. The homogenized myocardium or the pellet of centrifuged myofibrillar suspensions was solubilized in lysis buffer containing 8 M urea, 50% (v/v) glyc erol, 80 mM dithiothreitol (DTT) and protease inhibitors (Roche). Titin isoforms were separated by using an SDS-agarose gel electrophoresis system followed by Coomassie Blue staining. Quantification of the expression of titin isoforms was performed using Phoretix software (Biostep, Jahnisdorf, Germany).

**Mechanical measurements**
The force measurement was performed at 10°C using an experimental setup described previously (Stehle et al., 2002a; Stehle et al., 2002b). Briefly, small myofibril bundles (diameters of 1.5–4 μm) were mounted in relaxing solution (pCa 8) between the tip of an atomic-force cantilever and the tip of a length-driving stiff tungsten needle. After mounting, the slack sarcomere length (SL\(_{d}\)) of each preparation was determined. Myofibril bundles from knockout mice had a slightly but not significantly shorter SL\(_{d}\) (1.959±0.009 μm, n=31; ±s.e.m) than bundles from heterozygous (1.985±0.007 μm, n=31) and wild-type mice (1.976±0.006 μm, n=44). The passive-force–sarcomere-length relationship was determined by stretching the myofibrils in relaxing buffer by 4%, 8%, 12%, 16% and 20% of their slack length. At 50 s after each stretch, an image of the myofibril was captured to allow the evaluation of the actual sarcomere length. Immediately after image capture, the myofibril was rapidly slackened to determine passive force from the drop of force to zero force. Prior to activation, all myofibrils were stretched to a sarcomere length of 2.3 μm. The activation and relaxation cycle was induced by a rapid solution change (within 10 ms) that was applied to the mounted myofibril bundles (Colombo et al., 1998; Stehle et al., 2002b). To determine the isometric steady force during activation (pCa 4.6) and force-recovery kinetics, a release–stretch protocol was applied to the myofibrils. After an initial activation at maximum [Ca\(^{2+}\)] (pCa 4.6), the myofibril was subjected to a series of submaximal activations at increasing [Ca\(^{2+}\)] and a final activation at pCa 4.6. Force–pCa relationships were produced by normalizing force data to the mean of the first and the last activation performed at pCa 4.6.

**Data analysis and statistics**
To determine the rate constant \(k_{\text{ACT}}\) of the Ca\(^{2+}\)-induced force development and the rate constant \(k_{\text{REL}}\) of the mechanically induced force development, the transients were fitted with a single exponential function. The kinetic parameters of relaxation, \(k_{\text{REL,1L},\text{pCa}}\) and \(k_{\text{REL,1}}\) were obtained by fitting the force decay following the rapid reduction of [Ca\(^{2+}\)] from pCa 4.6 to 8 by a function consisting of a linear and an exponential equation (Stehle et al., 2002b). To determine the \(-\log(\text{Ca}\^{2+})\) required for half-maximum activation (pC\(_{50}\)) and the Hill coefficient, force–pCa relationships of individual myofibrils were fitted by the Hill equation \(F=F_{\text{max}}(1-F_{\text{max}})/[1+10^{n_{H}(-\log(\text{Ca}\^{2+})-p\text{C}_{50})}]\), where \(F\) is the steady-state force measured at each pCa normalized to the steady-state force at pCa 4.6, \(F_{\text{max}}\) is the minimum force at low [Ca\(^{2+}\)], pC\(_{50}\) is the \(-\log(\text{Ca}\^{2+})\) at which the force is half maximal and \(n_{H}\) (Hill coefficient) is the slope of the force–pCa relationship.

One-way analysis of variance (ANOVA) was used to compare the means of functional parameters between groups. The significance of differences between two genotypes was determined by using Tukey’s multiple comparison test and is indicated in the results as *P<0.05, **P<0.01 and ***P<0.001. All values are given as the mean±s.e.m. obtained from n individual myofibril bundles from each genotype.

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Competing interests
The authors declare no competing interests.

Author contributions
F.E. performed and analyzed the mechanical experiments. M.H.R. performed and analyzed the biochemical experiments. G.P. revised the manuscript and wrote the manuscript. R.S. designed the experiments and wrote the manuscript.

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