ANNUAL REVIEWS

Annual Review of Physiology

Titin Gene and Protein Functions in Passive and Active Muscle

Wolfgang A. Linke[1,2,3]

[1]Institute of Physiology II, University of Münster, 48149 Münster, Germany; email: wlinke@uni-muenster.de

[2]Deutsches Zentrum für Herz-Kreislaufforschung, Partner Site Göttingen, 37073 Göttingen, Germany

[3]Cardiac Mechanotransduction Group, Clinic for Cardiology and Pneumology, University Medical Center, 37073 Göttingen, Germany

Annu. Rev. Physiol. 2018. 80:389–411

First published as a Review in Advance on November 13, 2017

The *Annual Review of Physiology* is online at physiol.annualreviews.org

https://doi.org/10.1146/annurev-physiol-021317-121234

Keywords

cardiac muscle, skeletal muscle, muscle mechanics, sarcomere, cardiomyopathy, heart failure

Abstract

The thin and thick filaments of muscle sarcomeres are interconnected by the giant protein titin, which is a scaffolding filament, signaling platform, and provider of passive tension and elasticity in myocytes. This review summarizes recent insight into the mechanisms behind how titin gene mutations cause hereditary cardiomyopathy and how titin protein is mechanically active in skeletal and cardiac myocytes. A main theme is the evolving role of titin as a modulator of contraction. Topics include strain-sensing via titin in the sarcomeric A-band as the basis for length-dependent activation, titin elastic recoil and refolding of titin domains as an energy source, and Ca^{2+}-dependent stiffening of titin stretched during eccentric muscle contractions. Findings suggest that titin stiffness is a principal regulator of the contractile behavior of striated muscle. Physiological or pathological changes to titin stiffness therefore affect contractility. Taken together, titin emerges as a linker element between passive and active myocyte properties.

1. INTRODUCTION

Sarcomeres represent the basic contractile units of cardiac and skeletal muscles. Although the biogenesis and turnover of sarcomeres are still poorly understood, much insight has been obtained into the molecular mechanisms of force generation by sarcomeric thin (actin-containing) and thick (myosin-containing) filaments. Elastic "third" or titin filaments run through one half of each sarcomere and provide striated muscle with unique structural and functional properties. Whereas the thick and thin filaments represent the active contractile element of muscle (1), the elastic filament is widely considered a passive component. However, there is ample evidence that the passive and active properties are tightly coupled to one another to determine the overall mechanical properties of skeletal and heart muscle. One example is the Frank-Starling law of the heart, which poses that increased filling of the cardiac ventricles immediately enhances the developed pressure. The molecular mechanisms behind this length-dependent activation (LDA) involve the interplay between passive and active sarcomere proteins upon stretching (2–5). Another example is the stretch activation of insect indirect flight muscle (6), which is also seen, to a lesser degree, in vertebrate striated muscles (7). When the muscle is rapidly stretched, active force develops with a short time delay—but importantly, at low Ca^{2+} concentrations (6). The stretch signal might be transmitted via titin-like elastic proteins (8, 9) to the overlap zone of the thick and thin filaments as a prerequisite for their activation (10, 11). These examples demonstrate that "passive" muscle proteins have the potential to affect active contraction in a more or less direct manner.

This review aims to discuss recent evidence underscoring the functional link between passive and active sarcomere components, with an emphasis on the role of titin in vertebrate heart and skeletal muscle. The first part introduces fundamental structure–function relationships of the titin gene and protein. Established passive functions are highlighted, and recent insight into titin's role in human disease is summarized. Thereafter, the review covers evolving mechanisms that indicate a more active role of titin in contractile force generation.

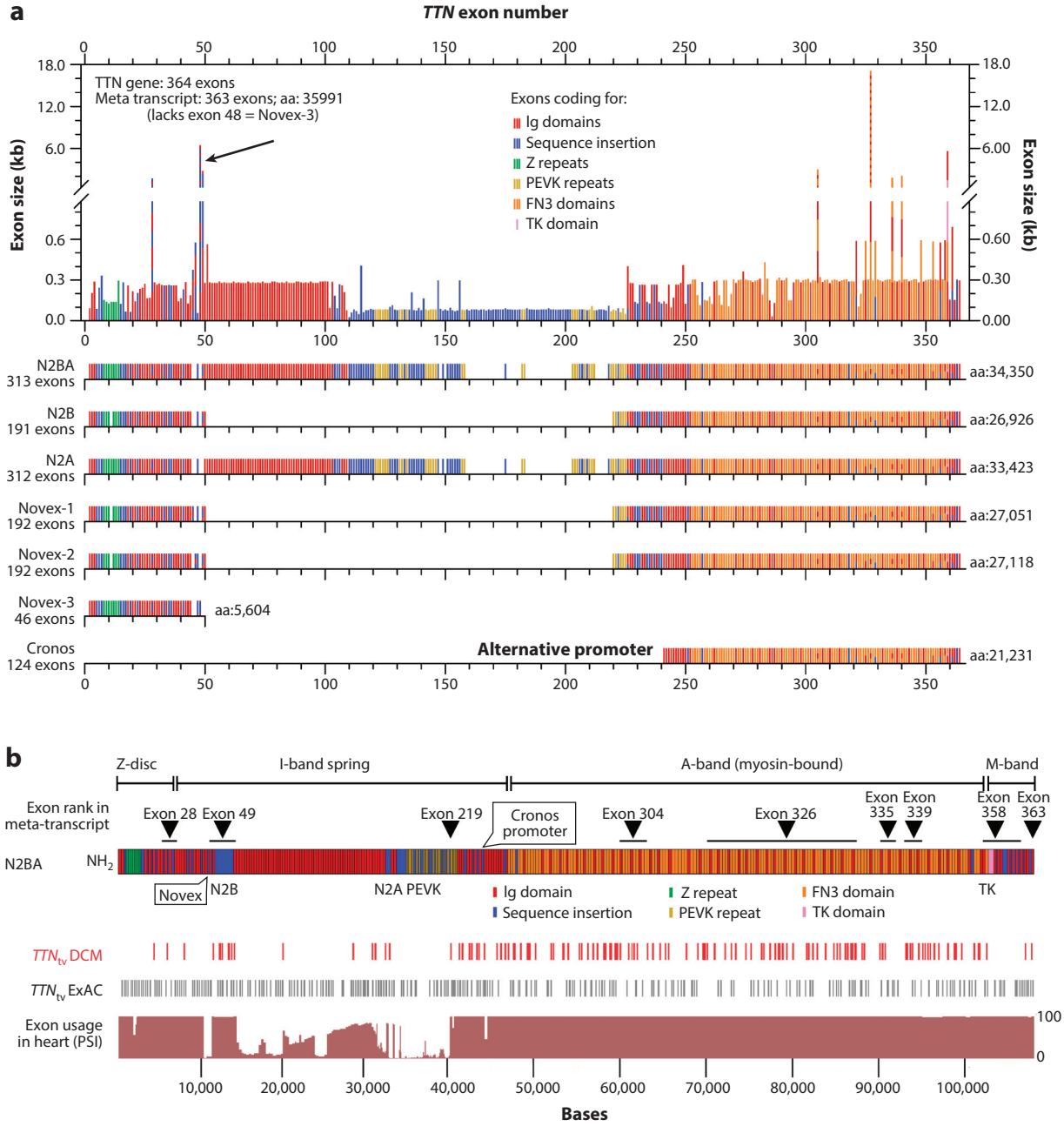
2. TITIN EXON AND PROTEIN DOMAIN COMPOSITION: IMPLICATIONS FOR TITIN FUNCTION

2.1. Titin Exon Structure and Alternative Splicing

Titin is a massive protein of striated muscle encoded by one of the largest genes, *TTN*. Human *TTN* encompasses 364 exons (**Figure 1*a***), of which exon 48 (Novex-3) contains a premature

Figure 1

Exon structure of the *TTN* gene and titin domain composition. (*a*) Exon sizes and numbers for the 364-exon human *TTN* gene (*top*) and exon usage in the different titin isoforms. The maximum possible number of exons spliced-in is indicated for each isoform; the maximum possible number of amino acids (aa) per isoform is also listed. N2BA represents the principle cardiac long isoform/canonical titin [NCBI: NM_001256850.1 (transcript), NP_001243779 (protein); UniProt: Q8WZ42-1]. Color codes refer to the type of protein domain or sequence expressed: immunoglobulin (Ig) domains; Ig-like domains; Z repeats; titin motif structures in the Z-disc; PEVK repeats (motifs rich in proline, glutamic acid, valine, and lysine); FN3 (fibronectin type 3) domains; and TK (titin kinase) domain. Blue-colored sequence insertions have been classified as unique sequence elements in the UniProtKB database. Note that at least some of the sequence insertions interspersed with the PEVK repeats may in fact be PEVK motifs but are not recognized as such in the database. (*b*) Domain structure of the canonical human titin protein (*top*). Exons larger than 1 kb are highlighted, along with exon 219 (beginning of constitutively expressed PEVK) and exon 363 (the last *TTN* exon in the meta-transcript). Shown is the distribution of *TTN* truncating variants (*TTN*tv; frameshift, nonsense, essential splice site) in human dilated cardiomyopathy (DCM) patients (54, 55) and in a population of healthy humans according to the Exome Aggregation Consortium (ExAC) (56, 57); only truncations located in exons with a percentage spliced-in (PSI) >15% are shown. Exon usage, visualized as PSI, describes the relative incorporation into transcripts expressed in human heart tissue (55, 56).



stop codon, resulting in the expression of a truncated titin protein, the Novex-3 isoform (12). The *TTN* meta-transcript consists of 363 exons (NCBI: NM_001267550.1), which code for a theoretical protein of 35,991 amino acids (NCBI: NP_001254479). From this transcript, many real titin isoforms are generated via alternative splicing (**Figure 1*a***). Heart muscle expresses two main titin variants: N2BA, which has up to 313 exons [NCBI: NM_001256850.1 (transcript), NP_001243779 (protein); UniProt: Q8WZ42–1] but exists in many different-length isoforms,

and N2B consisting of 191 exons. The isoforms Novex-1 (includes exon 45) and Novex-2 (includes exon 46) are present at low levels (12). Skeletal muscles express the N2A isoforms, which have up to 312 exons and always lack exon 49 coding for the N2B element (**Figure 1*a*,*b***). Alternative splicing generates many N2A isoforms of different length. The exact exon composition is known for only a few of the numerous potential splice pathways of the cardiac N2BA and skeletal muscle N2A isoforms. It is not currently clear why most exons in the range of 160–200 are not expressed in any of the sequenced isoforms. A novel C-terminal titin isoform is Cronos (13), which is expressed under an alternative promoter located before exon 241 of the human *TTN* gene. Most of the alternative splicing of titin exons occurs in a region that encodes the elastic spring segment located in the sarcomeric I-band (14). However, I-band titin also contains constitutively expressed segments at the proximal (near the Z-disc) and distal (near the A-band) ends. Almost all titin exons that encode the sarcomeric Z-disc, A-band, and M-band segments are constitutively expressed.

2.2. Role of Constitutive and Alternatively Spliced Titin Regions

The *TTN* exon expression pattern has important implications for the functional properties of the different titin segments in myocytes.

2.2.1. Constitutive titin regions. The Z-disc, A-band, and M-band titin segments have primarily structural roles by binding to other principal constituents of the sarcomere, including α-actinin and actin (Z-disc), myosin heavy chain (MHC) protein and myosin-binding protein C (MyBPC) (A-band), and myomesin (M-band) (15–17). In the myosin-bound portion of titin, individual domains of the immunoglobulin (Ig)-like and fibronectin type 3 (FN3) fold are arranged in super-repeat patterns, whose periodicity matches that of the myosin heads in the MyBPC-containing C-zone of the A-band (18). The A-band part of titin could be a ruler for thick-filament assembly (19, 20) and be involved in mechanosensing (21). Titin's M-band part also has scaffolding functions and probably conveys mechanosensitivity (22, 23).

2.2.2. I-band titin splicing and variability of sarcomere elasticity. The differentially spliced I-band region provides titin with highly variable elastic properties. The titin springs consist of Ig domain segments interspersed with unique regions (**Figure 1*b***), notably the cardiac-only N2B-unique sequence (N2Bus) and the PEVK element [named after the high content of proline, glutamic acid, valine, and lysine (24)], all of which are mechanically active (25, 26). By splicing in more Ig domains and PEVK repeats, the titin springs become longer and are therefore more compliant than those with fewer exons spliced in (25). For instance, fetal rodent hearts express long N2BA titin and have compliant sarcomeres that easily extend under low stretch forces, whereas adult rodent hearts express high proportions of N2B isoform and little N2BA; their sarcomeres thus have relatively high tensile stiffness (27–29). In contrast, the N2BA isoform predominates in adult myocardium of large mammals, such that titin-based passive tension is lower in cardiomyocytes (CMs) of pig, goat, or cow than in those of mouse, rat, or rabbit (30, 31). Normal adult human hearts have N2BA:N2B expression ratios of 30:70 to 40:60, and their cardiac titin stiffness ranges in between that of rodents and large mammals (31, 32). In skeletal muscle, the titin–isoform diversity is large among different adult muscle types of the same species (33), and titin–isoform size also depends on developmental stage (34). All of this gives rise to substantial variability in titin-based passive tension.

The titin–isoform pattern is under the control of splicing factors, notably RNA-binding motif protein-20 (RBM20) (35, 36). It is also modified by thyroid hormone and insulin (37, 38), perhaps

via effects on RBM20 (39). In-depth reviews of the relationship between titin–isoform expression and titin-based passive tension, the mechanism of titin–spring extension in sarcomeres, and the elastic properties of titin as an entropic spring can be found elsewhere (40, 41).

2.2.3. Titin as a signaling hub and target of posttranslational modification. Sarcomeric titin provides binding sites for at least 30 other muscle proteins and is an important signaling platform in CMs (16, 42). Specific I-band and M-band regions in titin are known to bind proteins involved in pathways of protein quality control (42). Moreover, titin is a major substrate for posttranslational modifications. Oxidation (43, 44) and phosphorylation (45–48) of the titin springs are recognized as modifiers of titin-based passive stiffness, particularly in CMs. The main focus of the phosphorylation studies has been on the cardiac N2B and PEVK elements, which can be phosphorylated by select protein kinases (PKs). There is consensus that phosphorylation of the N2Bus, for example, by PKA, PKG, or calcium/calmodulin–dependent PKIIδ, lowers titin stiffness (45, 46, 48), whereas phosphorylation of the (constitutively expressed) PEVK region, for example, by PKCα, increases it (47). However, in vivo quantitative phosphoproteomics has revealed many more phosphorylation sites in titin (and in the globular domains of I-band and A-band titin) (48), the functional relevance of which is unknown (49). Altered I-band titin phosphorylation is found in failing hearts and thought to cause pathological myocardial stiffening in patients with systolic or diastolic heart failure. These topics have been exhaustively reviewed recently (42, 49, 50) and will not be further discussed here.

2.3. Titin Splicing, Heart Failure, and Hereditary Cardiomyopathy

Alternative splicing of titin exons plays several roles in the pathophysiology of human heart disease.

2.3.1. Titin isoform transitions in failing hearts. End-stage human hearts that are failing due to ischemic or dilated cardiomyopathy (DCM) show a titin expression pattern that is shifted toward higher proportions of N2BA isoforms, compared to the N2BA:N2B isoform composition in healthy adult human hearts (32, 51, 52). The same is true for the hearts of patients with diastolic heart failure (42, 53). Several rodent models of heart disease also show this isoform shift toward N2BA (42). The higher percentage of more compliant N2BA–titin springs (coexpressed with stiffer N2B–titin springs) would lower sarcomeric stiffness, which could be a compensatory mechanism counteracting the increased titin-based stiffness due to altered titin phosphorylation and/or the increased extracellular matrix stiffness frequently present in failing hearts. Titin–isoform transitions toward N2B have occasionally been observed in animal models of heart disease but almost never in human heart failure (42).

2.3.2. Position-dependence of dilated cardiomyopathy-associated *TTN* truncating mutations. *TTN* is a major human disease gene. Interestingly, titin splicing has important consequence on the disease phenotype in hereditary cardiomyopathy caused by *TTN* mutations. Truncating mutations in *TTN* have been identified in 20–25% of human patients with adult-onset DCM, which frequently has a genetic etiology (54, 55). These heterozygous *TTN* truncating variants (*TTN*tv) include frameshift, nonsense, and essential splice site mutations; they represent the single most common genetic cause of DCM, with equivalent prevalence to variants in all other known disease genes combined. However, the location of *TTN*tv mutation within titin is important. *TTN*tv in DCM cases are most abundant in A-band titin (54) and, with slightly lower prevalence, in constitutive I-band exons, including the proximal and distal segments of the titin spring (56) (**Figure 1*b***). Notably, *TTN*tv are also present in approximately 1–3% of healthy

individuals, but they are randomly distributed across the entire titin molecule in the general population (54–57) (**Figure 1*b***). The role of *TTN*tv mutations in apparently healthy humans is poorly understood, but some *TTN*tv may predispose to eccentric remodeling of the heart (56).

Alternative splicing of I-band *TTN* exons appears to be the main reason why *TTN*tv in DCM patients are rarely found in the nonconstitutive parts of titin. These location-dependent effects can be appreciated from a graphical representation of *TTN* exon usage in human cardiac tissue (**Figure 1*b***), which describes the relative incorporation into transcripts as percent spliced-in (PSI; 100% means constitutive expression) (55, 56). The map suggests that the low usage of many I-band exons acts as a natural "exon skipping" mechanism in *TTN*tv-related DCM. An alternative explanation for the lower prevalence of I-band versus A-band *TTN*tv has been proposed, stating that upregulation of Cronos could rescue the effects of *TTN*tv that occur proximal, but not distal, to its transcriptional start site (13). However, conclusive evidence for this scenario is lacking (56, 58).

2.3.3. Pathomechanism of *TTN*tv in dilated cardiomyopathy. The pathomechanism of *TTN*tv is subject to ongoing research. Most *TTN*tv are not detectable at the protein level (55, 56, 59), although truncated mRNA is still translated (56), suggesting rapid *TTN*tv protein degradation. Thus, a "poisoned" polypeptide is probably not the predominant pathomechanism. In those rare cases in which a truncated protein appeared, such as in a DCM patient-derived CM line differentiated from induced pluripotent stem cells (iPSC-CMs), it was unlikely to be incorporated into the sarcomeres (59). Independently of whether or not truncated protein was detected in human iPSC-CMs heterozygous for *TTN*tv, myofibers engineered from these cells showed a deficit in active tension generation, and importantly, their contractile reserve was reduced (59). Collectively, work on iPSC-CMs, rodent models of *TTN*tv, and humans suggests that the pathomechanism in DCM-causing heterozygous *TTN*tv involves haploinsufficiency, as indicated by nonsense-mediated degradation of mutant alleles, a modest perturbation of cardiac metabolism and signaling, and heart physiology/mechanics that become impaired during cardiac stress but are almost normal in the absence of this stress (56, 59). However, more research is needed to establish whether *TTN*tv per se are pathogenic or whether the DCM phenotype appears only when a second or third stressor "hits."

2.4. Other Titinopathies

DCM is not the only disease phenotype of titinopathies. *TTN*tv are also found in women with peripartum cardiomyopathy, with a prevalence similar to that observed in DCM (60). Additionally, *TTN* mutations have been reported in hypertrophic cardiomyopathy (HCM) (61), restrictive cardiomyopathy (62), and arrhythmogenic right ventricular cardiomyopathy (63). However, *TTN*tv have been shown by high-throughput DNA sequencing technology to be no more frequent in patients with HCM than in the general population, suggesting a limited role or no role for them in this cardiomyopathy type (54). Furthermore, hereditary skeletal muscle disorders with or without cardiac involvement can be caused by *TTN* mutations. These include tibial muscular dystrophy and limb girdle muscular dystrophy type 2J, which are due to mutations at the extreme COOH-terminus of titin (64), as well as hereditary myopathy with early respiratory failure (65, 66) and a subtype of centronuclear myopathy (67). Collectively, research on *TTN* mutations has provided important insight into titin-related structure–function relationships in heart and skeletal muscle. Such studies are also a prerequisite for possible future targeted treatment strategies of these hereditary diseases.

3. ESTABLISHED ROLES OF TITIN IN ACTIVE CONTRACTION

The evidence for titin as a modulator of active contraction is still evolving. However, some mechanisms are already more established than others and are reviewed first. More controversial topics are covered thereafter.

3.1. The A-Band Centering Function of Titin to Support High Active Contractile Force

The earliest evidence for a role of titin in active contraction came from work on skeletal myofibers exposed to low-dose ionizing radiation, which degraded titin but also affected additional proteins (68). If such myofibers were prestretched and activated by Ca^{2+} under isometric conditions, the thick filaments in the sarcomeres moved off-center toward the Z-discs, whereas in normal intact sarcomeres they were kept at a more central position due to the spring force developed by titin (68, 69) (**Figure 2*a***). The axial misalignment of the thick filaments following titin degradation caused a large reduction in active tension. These results suggested that titin-based elastic forces help maintain high Ca^{2+}-dependent tension development and that this effect increases with the stiffness of titin. It was concluded that a primary physiological function of titin may be to keep the thick filaments centered during passive stretch and to recenter them every time the muscle is relaxed after an active contraction.

3.2. Length-Dependent Activation and A-Band Mechanosensing via Titin

Another role for titin in contraction has been established in LDA (5). Although this phenomenon is best known in conjunction with the Frank-Starling mechanism of the heart, it also occurs in skeletal muscle (70). LDA is characterized by an immediate increase in the Ca^{2+} sensitivity of the myofilaments during a stretch of the sarcomere (**Figure 2*b***). This change can be quantified experimentally by determining the Ca^{2+} concentration at which a (permeabilized) muscle fiber reaches 50% of its maximum contractile force (pCa_{50}). Ca^{2+} sensitization occurs when the pCa_{50} on the force–pCa curve is shifted leftward. Conversely, a rightward shift indicates Ca^{2+} desensitization (**Figure 2*b***). Moreover, at a higher sarcomere length (SL), the maximum force is greater than at a lower SL, at least when a near-optimal overlap of the thin and thick filaments is maintained. This active force increase could have a different molecular origin than the increase in Ca^{2+} sensitivity (71).

3.2.1. Sarcomeric proteins participating in length-dependent activation. The molecular mechanism of LDA has been studied over many decades. Currently, there is good evidence for the involvement of several different sarcomeric components, such as troponin subunits on the thin filament (5, 71, 72) and the thick-filament proteins MHC (73–75), MyBPC (76, 77), and regulatory myosin light chain (MLC) (71, 74, 78). Titin-based passive force plays a central role in LDA by transmitting the stretch signal to the contractile apparatus (36, 79–81).

3.2.2. The myofilament lattice spacing hypothesis. The layout of titin springs in the sarcomere is not fully in parallel with the long axis of the sarcomere. This is because titin binds to the actin filament at the periphery of the Z-disc (82) and must run somewhat obliquely through the I-band to attach to the thick filament (79) (**Figure 3*a***). Therefore, tensile stretching of the sarcomere increases titin's elastic force in the longitudinal direction (F_L), but it also generates a radial force component (F_R), which pulls the actin and myosin filaments closer to one another when the SL increases (**Figure 3*a***). This titin-based radial force may underlie, at least in part, the well-known

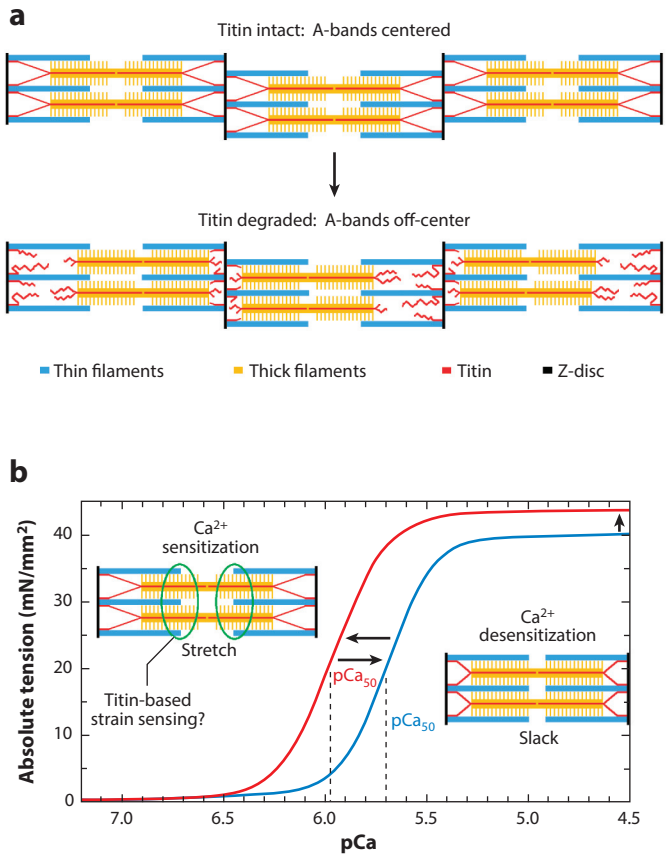


Figure 2

Established contributions of titin to the active contractile properties of sarcomeres. (*a*) Intact titin maintains high contractile force in stretched sarcomeres as it centers the A-bands. Degraded titin cannot perform this function, and the A-bands move uncontrolledly toward the Z-discs during and after contraction, which reduces active force. (*b*) Titin is a main determinant of length-dependent activation, presumably via its strain-sensing function. Sarcomere stretch increases activation, as quantified by the increased Ca^{2+} sensitivity of the contractile apparatus (shown here in cardiomyocytes): The Ca^{2+} concentration at half-maximum activation (pCa_{50}) on the active force–pCa curve is shifted leftward (pCa is the negative decadal logarithm of the Ca^{2+} concentration). The absolute force level at high Ca^{2+} concentration also increases. These effects are reversible.

decrease in interfilament spacing (and A-band diameter) that occurs upon stretching (83). It was thought that the reduction in interfilament spacing directly causes the increased Ca^{2+} sensitivity, as the myosin heads would more easily reach over to actin and thus generate higher forces at the same Ca^{2+} concentration (84–86). However, when the interfilament spacing was adjusted by osmotic compression to match that induced by sarcomere stretching, it was found that myofilament Ca^{2+} sensitivity was affected by SL per se, rather than by decreased lattice spacing (87). Furthermore, the relative reduction in myosin lattice spacing with SL was largely independent of the stiffness of titin expressed in the sarcomeres, although the absolute values differed (70, 75, 88) (**Figure 3*b***). Hence, there is no consistent (inverse) relationship between the stiffness of the titin springs and the degree of lattice spacing reduction upon stretching. Collectively, these findings have called into question the lattice spacing hypothesis as a main explanation for LDA.

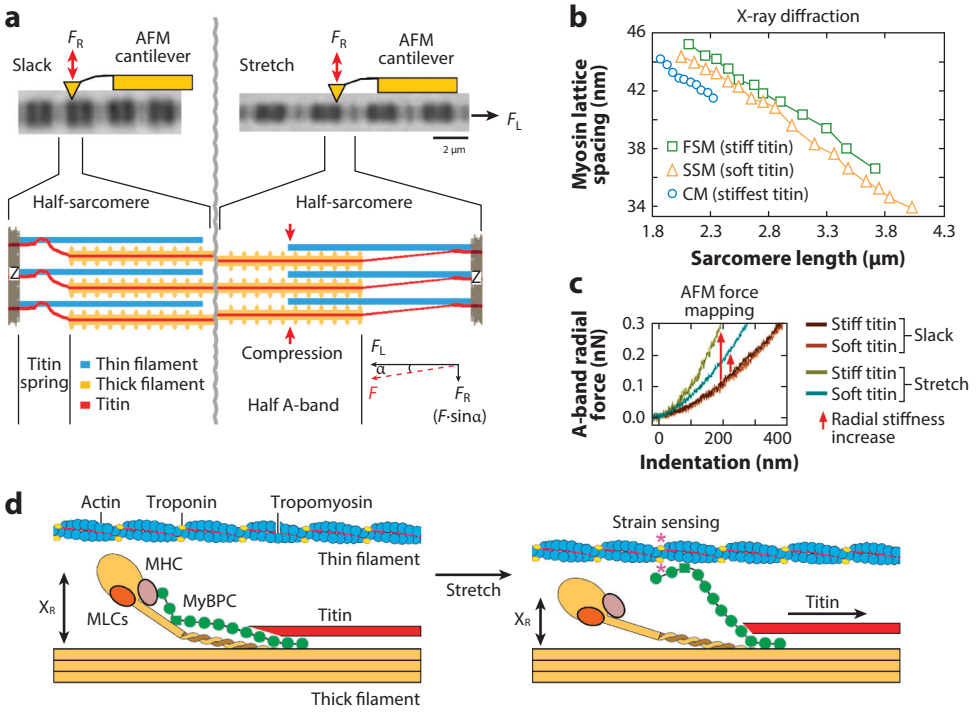


Figure 3

The role of titin stiffness in LDA and A-band strain sensing. (*a*) Sarcomeres at slack (*left*) and after a stretch (*right*). Phase-contrast images show part of a rabbit skeletal myofibril, on which the passive force in the longitudinal direction (F_L) can be measured with a force transducer, and the radial force component (F_R) is measured via force mapping using AFM. The half-sarcomere schematics below highlight the slightly oblique layout of the titin spring, which gives rise to both F_L and F_R when titin is stretched in the sarcomere by a force F. (*b*) Averaged interfilament lattice spacing as a function of sarcomere length for rat muscle fibers expressing titin of different stiffness. Panel adapted with permission from Reference 70. Copyright 2002, John Wiley and Sons. The relative decrease in lattice spacing with sarcomere stretch is similar in all muscle types. (*c*) AFM force mapping reveals differences in radial stiffness of stretched sarcomeres expressing compliant and stiff titin, respectively (88). (*d*) Proposed molecular model of titin strain sensing in the sarcomeric A-band, which triggers structural rearrangements of thick and thin filament proteins as the underlying mechanism of LDA. Abbreviations: AFM, atomic force microscopy; CM, cardiac muscle; FSM, fast skeletal muscle; LDA, length-dependent activation; MHC, myosin heavy chain; MLC, myosin light chain; MyBPC, myosin-binding protein C (the square indicates the M-domain); SSM, slow skeletal muscle; X_R, radial distance of cross-bridge (myosin head) from the thick-filament backbone.

3.2.3. Toward a molecular mechanism of length-dependent activation. Titin stiffness appears to be important for LDA-defining mechanosensory processes in the sarcomeric A-band. A small-angle X-ray diffraction study on intact cardiac muscle fibers reported length-dependent changes in the myosin head orientation as a potential factor in LDA (89). Subsequently, time-resolved X-ray diffraction measurements revealed structural alterations in both myosin and troponin in response to stretch of normal wild-type rat cardiac muscle fibers, which mainly express the stiff N2B–titin isoform (75). Notably, the increase in SL prompted the myosin heads to move toward the myosin backbone. However, if a rat strain was used that expressed a giant, extremely compliant, cardiac N2BA–titin isoform, LDA was greatly reduced, and the SL-dependent changes in myosin and troponin structure were absent (75). These results suggested that the molecular

basis of LDA involves stretch-induced simultaneous structural rearrangements within the thin and thick filaments that correlate with titin strain, whereas altered interfilament spacing or movement of myosin toward actin did not appear to be relevant factors.

In a methodologically different approach, rabbit skeletal myofibrils were isolated for measurements of radial stiffness in the A-band region of nonactivated, osmotically compressed, individual sarcomeres, both before (slack) and after a controlled stretch (88) (**Figure 3*a***). For these recordings, atomic force microscopy (AFM) was used in "force mapping" mode, in which cantilever deflection is monitored in response to sample indentation. Importantly, these experiments were performed on myofibrils expressing either long, compliant (soft) or short, stiff titin springs. At slack length, A-band radial stiffness was found to be the same in the two myofibril types, as indicated by the identical slopes of the force-indentation curves (**Figure 3*c***). However, following the stretch, A-band radial stiffness was significantly higher in the sarcomeres expressing stiff titin than in those expressing soft titin, and this effect could not be explained by differences in the strain-induced reduction in myofilament lattice spacing (88). Findings were interpreted in terms of structural rearrangements in A-band proteins affecting radial stiffness, which depend on titin strain and possibly indicate titin stiffness sensing by these proteins. A shorter, stiffer titin isoform would induce a larger effect than a longer, compliant titin isoform. These data added another aspect to the overwhelming published evidence suggesting that decreased titin stiffness causes reduced LDA (36, 75, 79, 80, 90–92), and conversely, that increased titin stiffness enhances LDA (81).

A proposed working model of A-band strain sensing via titin attempts to incorporate crucial findings about the involvement of sarcomeric proteins in LDA (**Figure 3*d***). The model, which specifically relates to cardiac LDA, considers that the C-terminus of MyBPC associates with the myosin shaft and A-band titin (93). MyBPC can also interact via its N-terminus with the myosin head and probably with the regulatory MLC, as well as via the so-called M-domain with the myosin neck region (94). The N-terminus of MyBPC (including the M-domain) may also interact with the thin filament, either directly with actin or with tropomyosin, thereby activating the thin filament (95–97). Diastolic stretch induces titin strain that is transmitted to MyBPC in the A-band. Evidence does suggest that upon sarcomere stretch, there is a small movement of A-band titin relative to the thick-filament backbone (21). In the most speculative part of the model, the transmission of titin strain to MyBPC causes a redistribution of the N-terminus of MyBPC toward the thin filament, away from the myosin head/neck region and MLC. This rearrangement could, in theory, stiffen the A-band region in the radial direction (88), considering that MyBPC is a semiflexible molecule made of a string of Ig/FN3 domains connected by short linkers. Furthermore, the rearrangement of MyBPC would lead to a reduction in the radial distance of cross-bridge (X_R), positioning the myosin head closer to the thick-filament backbone, as observed (75). Titin strain could also trigger additional structural changes in myosin (21) and regulatory MLC, the latter of which might increase the maximum active force (71). In the model, MyBPC is expected to affect the structural state of the thin filament through alterations in the structure of troponin, which enhances thin-filament activation. This modification promotes cross-bridge formation upon activation by Ca^{2+}, manifests as Ca^{2+} sensitization of troponin C, and leads to increased systolic force (pressure) development. In short, this model suggests that titin mechanical strain is sensed by thick-filament proteins and transmitted via MyBPC to the thin filament, thus resulting in cardiac myofilament LDA. According to this scenario, the Frank-Starling mechanism may rest on the interplay between various sarcomere proteins, with titin as a mechanical trigger.

4. NOVEL ASPECTS OF CONTRACTILITY MODULATION VIA TITIN

The mechanisms discussed below suggest additional effects of titin on the mechanical properties of active muscle; however, their in vivo functional relevance is less certain.

4.1. Storage and Release of Elastic Energy in the Titin Spring

Similar to any other spring that shortens against a weight, the titin spring has the potential to perform when it contracts from stretched length at the cost of energy stored in the spring.

4.1.1. Role of elastic recoil. The mechanical energy released by the recoiling titin springs was quantified in single rabbit skeletal and human cardiac myofibrils that were stretched in the nonactivated state and then allowed to shorten freely, only against the internal load (98, 99). In these passive "slack tests," titin elastic recoil was fast initially but was dampened by viscous drag, such that the passive shortening speed of the myofibrils slowed down greatly within tens of milliseconds. Because single myofibrils were used, contributions from other elastic elements present in myocytes, such as intermediate filaments (desmin), could be excluded. It was concluded that titin elastic recoil may speed up the early phase of active shortening if it begins from a stretched length, at which the elastic energy stored in titin is substantial. These findings were consistent with previous work demonstrating that stretched passive elements can accelerate the active unloaded shortening of cardiac and skeletal muscle (100–102), as well as the initial shortening of submaximally activated CMs under load (103).

4.1.2. Are in vivo sarcomere lengths high enough to allow for elastic energy storage in titin? A prerequisite for these effects to be relevant in vivo would be a working SL range, within which titin-based passive forces are relatively high. However, information regarding the in vivo SL range of living muscle is scarce. Conventional studies typically relied on SL measurements of chemically fixed samples, which are prone to shrinkage. To avoid this problem, minimally invasive optical microendoscopy was used to observe the second-harmonic frequencies of light generated in the muscle fibers. This allowed the visualization of the SLs in moving skeletal muscles of live mice and humans (104, 105). The in vivo SLs thus measured were highly variable and ranged from ~2.5 μm to well above 3.0 μm in mouse muscles and between 3.0 μm and 3.4 μm in human muscles. A later study, which used the same technique on mouse muscles, found somewhat shorter but still highly variable in vivo SLs (106). Within the SL range detected, the titin molecules are predicted to generate elastic forces of several piconewtons up to a few tens of piconewtons (107, 108). These findings suggest that the in vivo SLs of working muscle are high enough to allow for ample elastic energy storage in titin. In whole muscle, elastic elements apart from titin, such as other cytoskeletal and springy extracellular matrix proteins, may provide additional elastic energy storage. This energy could be used to support muscle contraction, especially in early phases of active shortening (98, 99).

4.2. Reversible Titin Ig Domain Unfolding and Consequences for Muscle Function

The elastic titin segment consists of up to ~100 Ig domains connected by short linker sequences and interspersed with the long unique sequence elements N2Bus and PEVK (**Figure 1**). Pioneering single-molecule mechanical studies demonstrated that these Ig domains can unfold under a stretch force applied under AFM (109, 110) or by optical tweezers (111, 112). However, for many years, the idea that titin Ig domain unfolding also occurs in normally working muscle (113, 114) had been rejected. A main reason was that the unfolding forces measured in vitro in constant-velocity AFM force-extension recordings (109, 110) and in silico (115) were 100 pN or higher, which is a force level considered far above that reached physiologically (116).

4.2.1. Titin domains unfold and refold at physiological forces and sarcomere lengths. Recent work demonstrated unequivocally that unfolding and refolding of titin Ig domains do occur at low force in vitro and also in skeletal myofibrils stretched by physiological amplitudes (117). When recombinant Ig domain fragments from the proximal I-band titin region were held by magnetic tweezers at constant forces between 4 and 10 pN over minutes or even hours, many cycles of Ig domain unfolding–refolding were observed. Because force levels of 6–10 pN/titin molecule may be reached in muscle fibers at higher physiological SLs (107, 108), nonactivated single rabbit myofibrils were stretched to an SL of ~3 μm, at which the force/titin is estimated to be ~6 pN (117). Then, a pair of proximal titin Ig domains from the same sarcomeric I-band, but separated by the Z-disc, which had been fluorescently labeled, was followed over time to measure the dynamics of its separation distance. This analysis demonstrated regular steps in the separation distance, which were ~10–15 nm in size and matched the unfolding–refolding step sizes measured in the recombinant Ig domain constructs held at a force of 6 pN. The steps were reproducibly detected in stretched myofibrils and absent in nonstretched sarcomeres. Because entropic elasticity theory predicts lengthening steps on the order of 10–12 nm when titin Ig domains unfold at a force level of 6 pN, the steps in the stretched myofibrils were interpreted as dynamic unfolding and refolding events of Ig domains (117). In the magnetic tweezers experiments, proximal Ig domains unfolded when the stretch force was at least 4 pN, and they refolded against a force of 10 pN or less. At ~6 pN, 50% of the previously unfolded Ig domains were folded, and at 4 pN, nearly all were folded. These unfolding–refolding transitions of Ig domains under force were also seen when isolated native titin molecules were held by optical tweezers at forces below 10 pN (118, 119). Thus, reversible Ig domain unfolding is part of titin's normal elastic response to stretch under physiological conditions (**Figure 4*a***). By limiting the stress sustained by stretched titin, this mechanism permits the sarcomere to cope with deformations of hundreds of nanometers. Interestingly, the refolding of titin Ig domains is regulated by oxidative stress (44), which introduces yet another level of titin elasticity modulation in myocytes.

4.2.2. Titin Ig domain refolding against force produces work. If titin Ig domains shorten (refold) against a force, they generate work, a process driven by the elastic energy stored in stretched titin (117, 119). Although Ig domain unfolding–refolding is energetically costly (i.e., heat loss), domain folding does recover energy in excess of the work done by a titin spring that undergoes purely elastic recoil (117). From experimental data and theoretical considerations, it was deduced that the energy delivery by titin domain refolding is maximal at a force of ~6 pN, at which it is similar to the energy delivered by the myosin power stroke (~60 zJ) (117). To recruit this titin work in an actively contracting sarcomere, the myosin motors were suggested to provide the trigger: Engagement of the myosin motor on actin would shorten the previously stretched sarcomere, thereby lowering the force on titin and inducing domain refolding (**Figure 4*a***). If the refolding occurred at a force of 6 pN/titin, work production through the process would be optimal. If a titin domain refolding event took place simultaneously in the ~2,000 parallel titin molecules present in a typical sarcomere, and in many other sarcomeres of the Ca^{2+}-activated muscle fiber, the work produced by the fiber would measurably increase (117). This extra kick from titin thus has the potential to boost active work production in muscle.

An important aspect of this scenario is that the SL range, at which the extra kick can provide an energy boost, depends on the stiffness of the titin spring (**Figure 4*b***). Due to alternative splicing of I-band titin, cardiac and skeletal myocytes express titin springs of different length, and the sarcomeric passive length–tension curves have a different steepness. From these curves, one can deduce the force/titin at a given SL (108, 117). For instance, titin is relatively stiff in

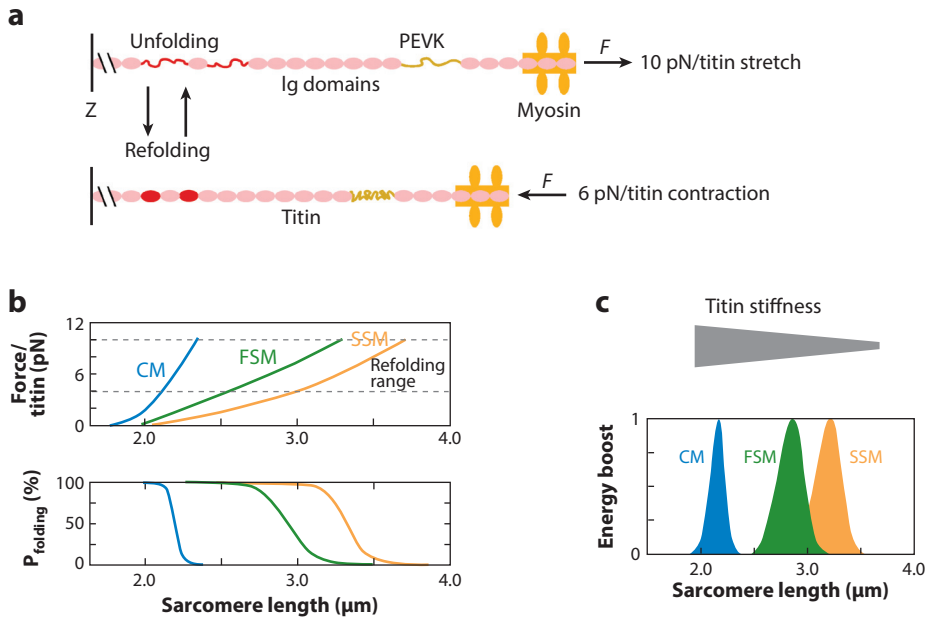


Figure 4

Unfolding–refolding of titin Ig-like domains under force as a means of elastic energy storage and delivery. (*a*) Oversimplified I-band segment of a single titin molecule in the half-sarcomere, which unfolds Ig domains under a stretch force of 10 pN (*top*) and refolds the domains against a force of 6 pN (*bottom*). In situ, the refolding may be triggered (and synchronized between different titin molecules) by the motor activity of myosin during contraction, which shortens the sarcomere and thus unloads titin. The process is accompanied by elastic recoil of the titin spring elements, including the PEVK domain. (*b*) Passive sarcomere length–force curves (*upper panel*) of three muscle types with very stiff titin springs (CM), intermediate-level titin stiffness (FSM), or compliant titin springs (SSM), scaled down to the force of a single titin molecule (117). The force range, within which previously unfolded Ig domains refold, is indicated. The lower panel shows the probability of titin Ig domain refolding over a range of physiological sarcomere lengths for the three muscle types. Panel adapted from Reference 117. (*c*) Because the mechanical energy delivered by an Ig domain folding contraction is positive for refolding against forces of 4–10 pN (a maximum of ~6 pN), the optimum energy boost from such a refolding event occurs at different sarcomere length in muscles with different titin stiffness. Abbreviations: CM, cardiac muscle; FSM, fast skeletal muscle; Ig, immunoglobulin; PEVK, element rich in proline, glutamic acid, valine, and lysine; SSM, slow skeletal muscle.

human heart (120), more compliant in fast skeletal muscle [e.g., human vastus lateralis (121)], and most compliant in slow skeletal muscle [e.g., human soleus (122)] (**Figure 4*b***, top). Because the force range within which previously unfolded titin Ig domains refold is 4–10 pN, the Ig domain folding probability shows a characteristic dependence on SL, which is unique for each myofiber type (117) (**Figure 4*b***, bottom). Therefore, the energy boost from the titin kick also occurs in a relatively narrow SL range that is specific for each muscle type (**Figure 4*c***). Remarkably, the extra kick appears to be largest within the respective physiological working ranges of myocardium (which operates at shorter SLs) and skeletal muscles (which operate at longer SLs). Thus, a principal effect of the titin-splicing diversity may be to "program" titin length and stiffness, such that the titin folding contraction occurs at the optimal working SL range of a particular muscle type.

For the titin kick to be physiologically relevant and aid active contraction, several prerequisites must be met. First, the sarcomere in the muscle must be stretched high enough for the

force/titin to reach at least 4 pN. Because of the long working SL range of rodent and human skeletal muscles observed in vivo (see above), this criterion appears to be fulfilled, at least for some muscles. Second, Ig domain refolding in myocytes must be fast enough to prevent titin from going slack during contraction in light of the rapid actomyosin cycle activity (123). Although this issue remains unsettled, the unfolding–refolding steps detected in myofibrils occurred on the sub-100-ms timescale (117). Moreover, myocytes are crowded with molecular chaperones, some of which target the titin springs (120, 124, 125) and could increase the Ig domain folding rate over that measured in vitro. Another point is that the properties of titin as an entropic spring permit it to equilibrate with any force in the sarcomere at a speed that is much faster than the fastest muscle contraction (98, 99, 126). Hence, titin is unlikely to go slack during active contraction. Third, titin Ig domain folding must occur in a synchronized manner in the myriad of parallel titin molecules present in a myofiber. This point has not yet been addressed experimentally. However, if myosin head engagement and the onset of muscle shortening are triggers for titin domain refolding, the folding could by synchronized via the synchrony in actomyosin contraction (117). This way, titin, actin, and myosin would work cooperatively in the sarcomere to maximize work production. In conclusion, the novel concept of titin-based power output in muscle poses a testable hypothesis that, if proven correct, would add significantly to our understanding of muscle contraction.

4.3. The Hypothesis of Force Enhancement via Ca^{2+}-Dependent Titin Stiffening

A topic of considerable recent interest has been the potential role played by titin in eccentric muscle contractions, i.e., when a muscle is stretched during activation (127–129). The classical sliding-filament theory of muscle contraction fails to describe certain features of eccentric contractions, including residual force enhancement (RFE) (130). This occurs when an activated muscle is stretched and then held at the stretched length until all transient force response has ceased; now the isometric steady-state force at the stretched length is greater than the isometric steady-state force at the same length for a purely isometric contraction (131) (**Figure 5*a***). Principal candidate mechanisms of RFE include increased SL inhomogeneity in actively stretched muscle and altered stiffness of titin (127–129). The evidence for the latter is summarized below, and current interpretations are critically discussed.

4.3.1. The role of Ca^{2+} binding in the titin PEVK element. Contraction of striated muscle is initiated by Ca^{2+} binding to troponin C. However, Ca^{2+} also binds to the elastic I-band region of titin (132), specifically the PEVK segment, which increases the stiffness of this spring element (133) (**Figure 5*b*,*c***). This effect occurs in the differentially spliced, but not the constitutively expressed, subsegment of PEVK (134). Critical amino acid residues within the PEVK element, which mediate the Ca^{2+} binding, appear to be negatively charged glutamates in so-called E-rich motifs, as their presence was required for the calcium effect to occur (133). In human titin, the differentially spliced PEVK region has an isoelectric point (P_i) of 5.1 (negative net charge), whereas the P_i of the constitutively expressed bit of PEVK is 8.8 (positive net charge) (**Figure 5*b***). Thus, the binding of Ca^{2+} might enhance intramolecular attraction and compactness in the negatively charged PEVK subsegment, which would be expected to increase titin-based stiffness. This mechanism could explain the increased tension and stiffness of a noncross-bridge structure during Ca^{2+} influx in active skeletal muscle (135–139). If titin indeed represents this structure (which requires further validation), it is conceivable that a titin-based stiffening mechanism plays a central role in RFE. However, the effect of Ca^{2+} on titin stiffness is much too small

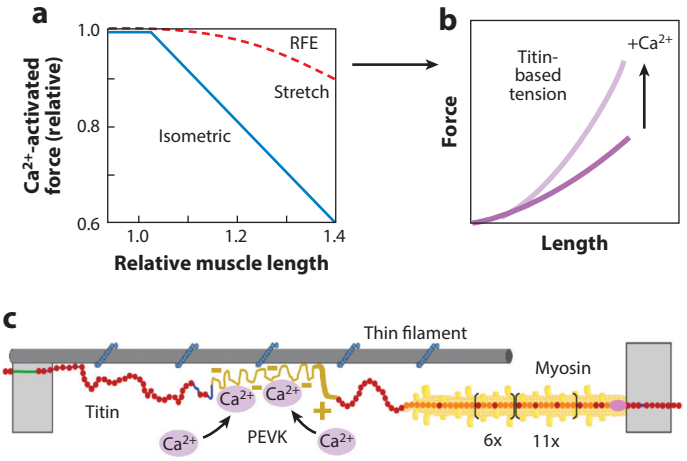


Figure 5

Suggested contribution of titin to RFE in actively stretched muscle. (*a*) Isometric steady-state length–force relationship of a representative muscle fiber stretched during Ca^{2+}-triggered activation (*dashed red curve*), which shows RFE compared to the isometric steady-state force at the same lengths for a purely isometric contraction (*solid blue line*). (*b*) A proportion of RFE may be due to titin stiffening through Ca^{2+} binding. (*c*) Schematic of a half-sarcomere, in which the constitutive part of titin's PEVK element (*thick yellow line*) is shown as binding more strongly than the differentially spliced PEVK part (*thin yellow line*) to the thin actin filament. Ca^{2+} binds to the differentially spliced PEVK subsegment, which stiffens titin somewhat, but weakens the PEVK–actin interaction. +/− symbols (*yellow*) indicate the net charge of the PEVK subsegments. Abbreviations: PEVK, element rich in proline, glutamic acid, valine, and lysine; RFE, residual force enhancement.

to account for the full magnitude of RFE (133, 140). Reports of large increases in the titin-based stiffness of overstretched skeletal myofibrils when Ca^{2+} is present (141, 142) have been criticized for their lack of technical soundness (129). Notwithstanding this discussion, it seems clear that Ca^{2+}-dependent PEVK stiffening explains only a minor portion of the extra force in actively stretched muscle.

4.3.2. The role of titin–actin interactions. Additional titin-related effects in RFE have been tentatively proposed. A current idea considers that specific titin spring elements could interact with actin during eccentric muscle activity when Ca^{2+} is elevated. As a result, the effective titin spring would be shorter, and titin could generate increased passive tension during and after eccentric stretching (142–144). Moreover, a "winding filament" hypothesis has been put forth, which suggests that titin, following activation by Ca^{2+} influx, is wound upon the thin filaments due to the cross-bridge activity (which twists the actin filaments somewhat) (128, 145). It was speculated that a major site of interaction with actin might be the N2A region of titin. However, these proposals are not backed by experimental findings.

Ample evidence indeed suggests that the mechanical properties of the myocytes are affected by interaction between titin and the actin filaments (82, 146, 147–150). As regards the titin spring region, in vitro binding assays showed that the PEVK segment, but not the Ig domains or N2Bus, binds actin and that this interaction is much stronger for the constitutively expressed than the differentially spliced PEVK subsegment (147–149, 151) (**Figure 5*b***). Furthermore, recombinant PEVK fragments tethered the actin filaments in the actin–myosin in vitro motility assay, which slowed down actin–filament sliding (147–149). Again, the effect was larger for the constitutively

expressed PEVK than for the differentially spliced subsegment (148, 149). The interaction was found to impose a viscoelastic load on the passively stretched sarcomere and presumably also the actively contracting sarcomere (147, 150).

Importantly, the PEVK–actin binding propensity was modest and further diminished by physiological levels of Ca^{2+} (147, 149). The presence of Ca^{2+} reduced the tethering of actin filaments by PEVK in the in vitro motility assay and partially recovered the speed of actin–filament sliding over myosin in some studies (147, 149), whereas it had no effect on the actin–titin interaction in others (148, 150). These observations did not confirm an earlier finding of increased interaction between actin filaments and isolated native titin in the presence of Ca^{2+} (146). Furthermore, two reports suggested that the PEVK–actin interaction is weakened by Ca^{2+}/S100A1 (148, 150), but an independent study found it to be unaffected by Ca^{2+}/S100 and Ca^{2+}/calmodulin (147). No experimental data are available suggesting that the N2A element of titin binds actin or that the titin filaments are wound around the actin filaments. In the absence of such evidence, the idea of a Ca^{2+}-mediated strengthening of the actin–titin interaction in RFE and eccentric contraction remains largely irreconcilable with published findings. In summary, titin stiffening via Ca^{2+} binding to the PEVK element is a possible contributor to RFE, whereas suggestions about other contributions from titin that are triggered by Ca^{2+} are highly speculative.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

TTN is established beyond doubt as a major disease gene, and titin protein is critically important for scaffolding functions in the sarcomere, myocyte elasticity, stretch-dependent tension development, and intracellular mechanosensitivity. The main theme of this review is that titin has also emerged as a modulator of active muscle contraction. In all of the distinct active mechanisms discussed above, titin stiffness plays a crucial role. Therefore, it is clear that alterations in titin stiffness affect the active contractile properties of muscle. Some principles about how titin-based stiffness can be tuned in (cardio)myocytes have been noted above. These include titin–isoform switching in development and heart disease, alterations in the phosphorylation and oxidation state of I-band titin in healthy and diseased hearts, and chaperone binding to the titin springs (42, 49, 50).

A direction of future research will be to investigate in more detail how titin modifications affect the active contractile properties of cardiac and skeletal muscle cells under normal and disease conditions. In past studies, increased titin stiffness has often been interpreted as being detrimental to diastolic heart function (49), but much less is known about whether increased or decreased titin stiffness benefits or impedes active contraction. To help approach these issues, various experimental tools are available. Rodent models with reduced titin stiffness due to mutation in the *RBM20* splicing factor have been generated (35, 36, 75, 90), and novel genome editing technology has already been used to mutate titin (59, 152). Shortening/truncation of titin filaments in animal models or stem cell–derived CMs is a path taken recently (21, 56, 59, 81, 152, 153), as is the pharmacological manipulation of signaling networks involved in titin phosphorylation to modify titin stiffness (154). One area worth exploring in more detail as a "handle" to alter titin stiffness may be the redox state of the myocytes (43, 44) and the chaperone expression level or activity (120). It would also be useful to develop a molecular tool that could be applied to specifically cut the titin springs without affecting other myocyte proteins to study the effect of acute titin stiffness elimination on active contraction. Finally, all approaches aimed at better understanding titin's role as a regulator of active contraction would require more atomic detail of the sarcomeric ultrastructure, particularly in the A-band. Future studies on titin as a protein that links the passive and active properties of the sarcomere are likely to provide significant physiological insight with potential implications for novel therapeutic options in heart and muscle diseases.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Thanks to Gabriele Reimus for help with figure preparations. I apologize to the authors of many original papers relevant to this review, which could not be cited due to space constrictions; instead, I sometimes cited overview articles. This work was supported by German Research Foundation grant SFB1002 (TPA08) and through the ERA-CVD EU program (MINOTAUR).

LITERATURE CITED

1. Hill AV. 1938. The heat of shortening and dynamic constants of muscles. *Proc. R. Soc. B* 126:136–95
2. Campbell KS. 2011. Impact of myocyte strain on cardiac myofilament activation. *Pflügers Arch*. 462(1):3–14
3. McDonald KS. 2011. The interdependence of Ca^{2+} activation, sarcomere length, and power output in the heart. *Pflügers Arch*. 462(1):61–67
4. Fukuda N, Terui T, Ohtsuki I, Ishiwata S, Kurihara S. 2009. Titin and troponin: central players in the Frank-Starling mechanism of the heart. *Curr. Cardiol. Rev.* 5(2):119–24
5. de Tombe PP, Mateja RD, Tachampa K, Ait Mou Y, Farman GP, Irving TC. 2010. Myofilament length dependent activation. *J. Mol. Cell. Cardiol.* 48(5):851–58
6. Bullard B, Pastore A. 2011. Regulating the contraction of insect flight muscle. *J. Muscle Res. Cell Motil.* 32(4–5):303–13
7. Steiger GJ. 1971. Stretch activation and myogenic oscillation of isolated contractile structures of heart muscle. *Pflügers Arch*. 330(4):347–61
8. Kulke M, Neagoe C, Kolmerer B, Minajeva A, Hinssen H, et al. 2001. Kettin, a major source of myofibrillar stiffness in *Drosophila* indirect flight muscle. *J. Cell Biol.* 154(5):1045–57
9. Bullard B, Linke WA, Leonard K. 2002. Varieties of elastic protein in invertebrate muscles. *J. Muscle Res. Cell Motil.* 23(5–6):435–47
10. Krzic U, Rybin V, Leonard KR, Linke WA, Bullard B. 2010. Regulation of oscillatory contraction in insect flight muscle by troponin. *J. Mol. Biol.* 397(1):110–18
11. Perz-Edwards RJ, Irving TC, Baumann BA, Gore D, Hutchinson DC, et al. 2011. X-ray diffraction evidence for myosin-troponin connections and tropomyosin movement during stretch activation of insect flight muscle. *PNAS* 108(1):120–25
12. Bang ML, Centner T, Fornoff F, Geach AJ, Gotthardt M, et al. 2001. The complete gene sequence of titin, expression of an unusual approximately 700-kDa titin isoform, and its interaction with obscurin identify a novel Z-line to I-band linking system. *Circ. Res.* 89(11):1065–72
13. Zou J, Tran D, Baalbaki M, Tang LF, Poon A, et al. 2015. An internal promoter underlies the difference in disease severity between N- and C-terminal truncation mutations of titin in zebrafish. *eLife* 4:e09406
14. Freiburg A, Trombitas K, Hell W, Cazorla O, Fougerousse F, et al. 2000. Series of exon-skipping events in the elastic spring region of titin as the structural basis for myofibrillar elastic diversity. *Circ. Res.* 86(11):1114–21
15. Miller MK, Granzier H, Ehler E, Gregorio CC. 2004. The sensitive giant: the role of titin-based stretch sensing complexes in the heart. *Trends Cell Biol*. 14(3):119–26
16. Linke WA. 2008. Sense and stretchability: the role of titin and titin-associated proteins in myocardial stress-sensing and mechanical dysfunction. *Cardiovasc. Res.* 77(4):637–48
17. Gautel M, Djinović-Carugo K. 2016. The sarcomeric cytoskeleton: from molecules to motion. *J. Exp. Biol.* 219(Pt. 2):135–45
18. Labeit S, Gautel M, Lakey A, Trinick J. 1992. Towards a molecular understanding of titin. *EMBO J.* 11(5):1711–16

19. Whiting A, Wardale J, Trinick J. 1989. Does titin regulate the length of muscle thick filaments? *J. Mol. Biol.* 205(1):263–68
20. Tskhovrebova L, Bennett P, Gautel M, Trinick J. 2015. Titin ruler hypothesis not refuted. *PNAS* 112(11):E1172
21. Granzier HL, Hutchinson KR, Tonino P, Methawasin M, Li FW, et al. 2014. Deleting titin's I-band/A-band junction reveals critical roles for titin in biomechanical sensing and cardiac function. *PNAS* 111(40):14589–94
22. Gautel M. 2011. The sarcomeric cytoskeleton: who picks up the strain? *Curr. Opin. Cell Biol.* 23(1):39–46
23. Zacharchenko T, von Castelmur E, Rigden DJ, Mayans O. 2015. Structural advances on titin: towards an atomic understanding of multi-domain functions in myofilament mechanics and scaffolding. *Biochem. Soc. Trans.* 43(5):850–55
24. Labeit S, Kolmerer B. 1995. Titins: giant proteins in charge of muscle ultrastructure and elasticity. *Science* 270(5234):293–96
25. Linke WA, Ivemeyer M, Mundel P, Stockmeier MR, Kolmerer B. 1998. Nature of PEVK-titin elasticity in skeletal muscle. *PNAS* 795(14):8052–57
26. Linke WA, Rudy DE, Centner T, Gautel M, Witt C, et al. 1999. I-band titin in cardiac muscle is a three-element molecular spring and is critical for maintaining thin filament structure. *J. Cell Biol.* 146(3):631–44
27. Opitz CA, Leake MC, Makarenko I, Benes V, Linke WA. 2004. Developmentally regulated switching of titin size alters myofibrillar stiffness in the perinatal heart. *Circ. Res.* 94(7):967–75
28. Lahmers S, Wu Y, Call DR, Labeit S, Granzier H. 2004. Developmental control of titin isoform expression and passive stiffness in fetal and neonatal myocardium. *Circ. Res.* 94(4):505–13
29. Warren CM, Krzesinski PR, Campbell KS, Moss RL, Greaser ML. 2004. Titin isoform changes in rat myocardium during development. *Mech. Dev.* 121(11):1301–12
30. Cazorla O, Freiburg A, Helmes M, Centner T, McNabb M, et al. 2000. Differential expression of cardiac titin isoforms and modulation of cellular stiffness. *Circ. Res.* 86(1):59–67
31. Neagoe C, Opitz CA, Makarenko I, Linke WA. 2003. Gigantic variety: expression patterns of titin isoforms in striated muscles and consequences for myofibrillar passive stiffness. *J. Muscle Res. Cell Motil.* 24(2–3):175–79
32. Neagoe C, Kulke M, del Monte F, Gwathmey JK, de Tombe PP, et al. 2002. Titin isoform switch in ischemic human heart disease. *Circulation* 106(11):1333–41
33. Prado LG, Makarenko I, Andresen C, Krüger M, Opitz CA, Linke WA. 2005. Isoform diversity of giant proteins in relation to passive and active contractile properties of rabbit skeletal muscles. *J. Gen. Physiol.* 126(5):461–80
34. Ottenheijm CAC, Knottnerus AM, Buck D, Luo X, Greer K, et al. 2009. Tuning passive mechanics through differential splicing of titin during skeletal muscle development. *Biophys. J.* 97(8):2277–86
35. Guo W, Schafer S, Greaser ML, Radke MH, Liss M, et al. 2012. *RBM20*, a gene for hereditary cardiomyopathy, regulates titin splicing. *Nat. Med.* 18(5):766–73
36. Methawasin M, Hutchinson KR, Lee EJ, Smith JE 3rd, Saripalli C, et al. 2014. Experimentally increasing titin compliance in a novel mouse model attenuates the Frank-Starling mechanism but has a beneficial effect on diastole. *Circulation* 129(19):1924–36
37. Krüger M, Sachse C, Zimmermann WH, Eschenhagen T, Klede S, Linke WA. 2008. Thyroid hormone regulates developmental titin isoform transitions via the phosphatidylinositol-3-kinase/AKT pathway. *Circ. Res.* 102(4):439–47
38. Krüger M, Babicz K, Frieling-Salewsky M, Linke WA. 2010. Insulin signaling regulates cardiac titin properties in heart development and diabetic cardiomyopathy. *J. Mol. Cell. Cardiol.* 48(5):910–16
39. Zhu C, Yin Z, Ren J, McCormick RJ, Ford SP, Guo W. 2015. RBM20 is an essential factor for thyroid hormone-regulated titin isoform transition. *J. Mol. Cell Biol.* 7(1):88–90
40. Linke WA, Fernandez JM. 2002. Cardiac titin: molecular basis of elasticity and cellular contribution to elastic and viscous stiffness components in myocardium. *J. Muscle Res. Cell Motil.* 23(5–6):483–97
41. Granzier H, Labeit S. 2007. Structure–function relations of the giant elastic protein titin in striated and smooth muscle cells. *Muscle Nerve* 36(6):740–55

42. Linke WA, Hamdani N. 2014. Gigantic business: titin properties and function through thick and thin. *Circ. Res.* 114(6):1052–68
43. Grützner A, Garcia-Manyes S, Kötter S, Badilla CL, Fernandez JM, Linke WA. 2009. Modulation of titin-based stiffness by disulfide bonding in the cardiac titin N2-B unique sequence. *Biophys. J.* 97(3):825–34
44. Alegre-Cebollada J, Kosuri P, Giganti D, Eckels E, Rivas-Pardo JA, et al. 2014. S-glutathionylation of cryptic cysteines enhances titin elasticity by blocking protein folding. *Cell* 156(6):1235–46
45. Yamasaki R, Wu Y, McNabb M, Greaser M, Labeit S, Granzier H. 2002. Protein kinase A phosphorylates titin's cardiac-specific N2B domain and reduces passive tension in rat cardiac myocytes. *Circ. Res.* 90(11):1181–88
46. Krüger M, Kötter S, Grützner A, Lang P, Andresen C, et al. 2009. Protein kinase G modulates human myocardial passive stiffness by phosphorylation of the titin springs. *Circ. Res.* 104(1):87–94
47. Hidalgo CG, Hudson B, Bogomolovas J, Zhu Y, Anderson B, et al. 2009. PKC phosphorylation of titin's PEVK element: a novel and conserved pathway for modulating myocardial stiffness. *Circ. Res.* 105(7):631–8
48. Hamdani N, Krysiak J, Kreusser MM, Neef S, dos Remedios CG, et al. 2013. Crucial role for Ca^{2+}/calmodulin-dependent protein kinase-II in regulating diastolic stress of normal and failing hearts via titin phosphorylation. *Circ. Res.* 112(4):664–74
49. Hamdani N, Herwig M, Linke WA. 2017. Tampering with springs: phosphorylation of titin affecting the mechanical function of cardiomyocytes. *Biophys. Rev.* 9(3):225–37
50. Beckendorf L, Linke WA. 2015. Emerging importance of oxidative stress in regulating striated muscle elasticity. *J. Muscle Res. Cell Motil.* 36(1):25–36
51. Makarenko I, Opitz CA, Leake MC, Neagoe C, Kulke M, et al. 2004. Passive stiffness changes caused by upregulation of compliant titin isoforms in human dilated cardiomyopathy hearts. *Circ. Res.* 95(7):708–16
52. Nagueh SF, Shah G, Wu Y, Torre-Amione G, King NM, et al. 2004. Altered titin expression, myocardial stiffness, and left ventricular function in patients with dilated cardiomyopathy. *Circulation* 110(2):155–62
53. Borbély A, Falcao-Pires I, van Heerebeek L, Hamdani N, Édes I, et al. 2009. Hypophosphorylation of the stiff N2B titin isoform raises cardiomyocyte resting tension in failing human myocardium. *Circ. Res.* 104(6):780–6
54. Herman DS, Lam L, Taylor MR, Wang L, Teekakirikul P, et al. 2012. Truncations of titin causing dilated cardiomyopathy. *N. Engl. J. Med.* 366(7):619–28
55. Roberts AM, Ware JS, Herman DS, Schafer S, Baksi J, et al. 2015. Integrated allelic, transcriptional, and phenomic dissection of the cardiac effects of titin truncations in health and disease. *Sci. Transl. Med.* 7(270):270ra6
56. Schafer S, de Marvao A, Adami E, Fiedler LR, Ng B, et al. 2017. Titin-truncating variants affect heart function in disease cohorts and the general population. *Nat. Genet.* 49(1):46–53
57. Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, et al. 2016. Analysis of protein-coding genetic variation in 60,706 humans. *Nature* 536(7616):285–91
58. Shih YH, Dvornikov AV, Zhu P, Ma X, Kim M, et al. 2016. Exon- and contraction-dependent functions of titin in sarcomere assembly. *Development* 143(24):4713–22
59. Hinson JT, Chopra A, Nafissi N, Polacheck WJ, Benson CC, et al. 2015. Titin mutations in iPS cells define sarcomere insufficiency as a cause of dilated cardiomyopathy. *Science* 349(6251):982–86
60. Ware JS, Li J, Mazaika E, Yasso CM, DeSouza T, et al. 2016. Shared genetic predisposition in peripartum and dilated cardiomyopathies. *N. Engl. J. Med.* 374(3):233–41
61. Satoh M, Takahashi M, Sakamoto T, Hiroe M, Marumo F, Kimura A. 1999. Structural analysis of the titin gene in hypertrophic cardiomyopathy: identification of a novel disease gene. *Biochem. Biophys. Res. Commun.* 262(2):411–17
62. Peled Y, Gramlich M, Yoskovitz G, Feinberg MS, Afek A, et al. 2014. Titin mutation in familial restrictive cardiomyopathy. *Int. J. Cardiol.* 171(1):24–30
63. Taylor M, Graw S, Sinagra G, Barnes C, Slavov D, et al. 2011. Genetic variation in titin in arrhythmogenic right ventricular cardiomyopathy-overlap syndromes. *Circulation* 124(8):876–85
64. Udd B, Vihola A, Sarparanta J, Richard I, Hackman P. 2005. Titinopathies and extension of the M-line mutation phenotype beyond distal myopathy and LGMD2J. *Neurology* 64(4):636–42

65. Ohlsson M, Hedberg C, Brådvik B, Lindberg C, Tajsharghi H, et al. 2012. Hereditary myopathy with early respiratory failure associated with a mutation in A-band titin. *Brain* 135(Pt. 6):1682–94
66. Pfeffer G, Elliott HR, Griffin H, Barresi R, Miller J, et al. 2012. Titin mutation segregates with hereditary myopathy with early respiratory failure. *Brain* 135(Pt. 6):1695–13
67. Ceyhan-Birsoy O, Agrawal PB, Hidalgo C, Schmitz-Abe K, DeChene ET, et al. 2013. Recessive truncating titin gene, *TTN*, mutations presenting as centronuclear myopathy. *Neurology* 81(14):1205–14
68. Horowits R, Kempner ES, Bisher ME, Podolsky RJ. 1986. A physiological role for titin and nebulin in skeletal muscle. *Nature* 323(6084):160–64
69. Horowits R, Podolsky RJ. 1987. The positional stability of thick filaments in activated skeletal muscle depends on sarcomere length: evidence for the role of titin filaments. *J. Cell Biol.* 105(5):2217–23
70. Konhilas JP, Irving TC, de Tombe PP. 2002. Length-dependent activation in three striated muscle types of the rat. *J. Physiol.* 544(Pt. 1):225–36
71. Zhang X, Kampourakis T, Yan Z, Sevrieva I, Irving M, Sun Y-B. 2017. Distinct contributions of the thin and thick filaments to length-dependent activation in heart muscle. *eLife* 6:e24081
72. Kobirumaki-Shimozawa F, Inoue T, Shintani SA, Oyama K, Terui T, et al. 2014. Cardiac thin filament regulation and the Frank-Starling mechanism. *J. Physiol. Sci.* 64(4):221–32
73. Linari M, Brunello E, Reconditi M, Fusi L, Caremani M, et al. 2015. Force generation by skeletal muscle is controlled by mechanosensing in myosin filaments. *Nature* 528(7581):276–79
74. Fusi L, Brunello E, Yan Z, Irving M. 2016. Thick filament mechano-sensing is a calcium-independent regulatory mechanism in skeletal muscle. *Nat. Commun.* 7:13281
75. Ait-Mou Y, Hsu K, Farman GP, Kumar M, Greaser ML, et al. 2016. Titin strain contributes to the Frank-Starling law of the heart by structural rearrangements of both thin- and thick-filament proteins. *PNAS* 113(8):2306–11
76. Kumar M, Govindan S, Zhang M, Khairallah RJ, Martin JL, et al. 2015. Cardiac myosin-binding protein C and troponin-I phosphorylation independently modulate myofilament length-dependent activation. *J. Biol. Chem.* 290(49):29241–49
77. Mamidi R, Gresham KS, Stelzer JE. 2014. Length-dependent changes in contractile dynamics are blunted due to cardiac myosin binding protein-C ablation. *Front. Physiol.* 5:461
78. Kampourakis T, Sun YB, Irving M. 2016. Myosin light chain phosphorylation enhances contraction of heart muscle via structural changes in both thick and thin filaments. *PNAS* 113(21):E3039–47
79. Cazorla O, Wu Y, Irving TC, Granzier H. 2001. Titin-based modulation of calcium sensitivity of active tension in mouse skinned cardiac myocytes. *Circ. Res.* 88(10):1028–35
80. Fukuda N, Sasaki D, Ishiwata S, Kurihara S. 2001. Length dependence of tension generation in rat skinned cardiac muscle: role of titin in the Frank-Starling mechanism of the heart. *Circulation* 104(14):1639–45
81. Lee E-J, Peng J, Radke M, Gotthardt M, Granzier HL. 2010. Calcium sensitivity and the Frank-Starling mechanism of the heart are increased in titin N2B region-deficient mice. *J. Mol. Cell. Cardiol.* 49(3):449–58
82. Linke WA, Ivemeyer M, Labeit S, Hinssen H, Rüegg JC, Gautel M. 1997. Actin-titin interaction in cardiac myofibrils: probing a physiological role. *Biophys. J.* 73(2):905–19
83. Irving T, Wu Y, Bekyarova T, Farman GP, Fukuda N, Granzier H. 2011. Thick-filament strain and interfilament spacing in passive muscle: effect of titin-based passive tension. *Biophys. J.* 100(6):1499–508
84. Godt RE, Maughan DW. 1981. Influence of osmotic compression on calcium activation and tension in skinned muscle fibers of the rabbit. *Pflügers Arch.* 391(4):334–37
85. McDonald KS, Moss RL. 1995. Osmotic compression of single cardiac myocytes eliminates the reduction in Ca^{2+} sensitivity of tension at short sarcomere length. *Circ. Res.* 77(1):199–205
86. Fuchs F, Smith SH. 2001. Calcium, cross-bridges, and the Frank-Starling relationship. *News Physiol. Sci.* 16:5–10
87. Konhilas JP, Irving TC, de Tombe PP. 2002. Myofilament calcium sensitivity in skinned rat cardiac trabeculae: role of interfilament spacing. *Circ. Res.* 90(1):59–65
88. Li Y, Lang P, Linke WA. 2016. Titin stiffness modifies the force-generating region of muscle sarcomeres. *Sci. Rep.* 6:24492

89. Farman GP, Gore D, Allen E, Schoenfelt K, Irving TC, de Tombe PP. 2011. Myosin head orientation: a structural determinant for the Frank-Starling relationship. *Am. J. Physiol. Heart Circ. Physiol.* 300(6):H2155–60
90. Patel JR, Pleitner JM, Moss RL, Greaser ML. 2012. Magnitude of length-dependent changes in contractile properties varies with titin isoform in rat ventricles. *Am. J. Physiol. Heart Circ. Physiol.* 302(3):H697–708
91. Terui T, Sodnomtseren M, Matsuba D, Udaka J, Ishiwata S, et al. 2008. Troponin and titin coordinately regulate length-dependent activation in skinned porcine ventricular muscle. *J. Gen. Physiol.* 131(3):275–83
92. Beqqali A, Bollen IAE, Rasmussen TB, van den Hoogenhof MM, van Deutekom HWM, et al. 2016. A mutation in the glutamate-rich region of RNA-binding motif protein 20 causes dilated cardiomyopathy through missplicing of titin and impaired Frank-Starling mechanism. *Cardiovasc. Res.* 112(1):452–63
93. Pfuhl M, Gautel M. 2012. Structure, interactions and function of the N-terminus of cardiac myosin binding protein C (MyBP-C): who does what, with what, and to whom? *J. Muscle Res. Cell Motil.* 33(1):83–94
94. Sadayappan S, de Tombe PP. 2014. Cardiac myosin binding protein-C as a central target of cardiac sarcomere signaling: a special mini review series. *Pflügers Arch.* 466(2):195–200
95. Shaffer JF, Kensler RW, Harris SP. 2009. The myosin-binding protein C motif binds to F-actin in a phosphorylation-sensitive manner. *J. Biol. Chem.* 284(18):12318–27
96. Kampourakis T, Yan Z, Gautel M, Sun Y-B, Irving M. 2014. Myosin binding protein-C activates thin filaments and inhibits thick filaments in heart muscle cells. *PNAS* 111(52):18763–68
97. Mun JY, Previs MJ, Yu HY, Gulick J, Tobacman LS, et al. 2014. Myosin-binding protein C displaces tropomyosin to activate cardiac thin filaments and governs their speed by an independent mechanism. *PNAS* 111(6):2170–75
98. Minajeva A, Neagoe C, Kulke M, Linke WA. 2002. Titin-based contribution to shortening velocity of rabbit skeletal myofibrils. *J. Physiol.* 540(Pt. 1):177–88
99. Opitz CA, Kulke M, Leake MC, Neagoe C, Hinssen H, et al. 2003. Damped elastic recoil of the titin spring in myofibrils of human myocardium. *PNAS* 100(22):12688–93
100. Edman KA. 1979. The velocity of unloaded shortening and its relation to sarcomere length and isometric force in vertebrate muscle fibres. *J. Physiol.* 291:143–59
101. Claflin DR, Morgan DL, Julian FJ. 1989. Effects of passive tension on unloaded shortening speed of frog single muscle fibers. *Biophys. J.* 56(5):967–77
102. de Tombe PP, ter Keurs HE. 1992. An internal viscous element limits velocity of sarcomere shortening in mammalian myocardium. *J. Physiol.* 454:619–42
103. Sweitzer NK, Moss RL. 1993. Determinants of loaded shortening velocity in single cardiac myocytes permeabilized with alpha-hemolysin. *Circ. Res.* 73(6):1150–62
104. Llewellyn ME, Barretto RPJ, Delp SL, Schnitzer MJ. 2008. Minimally invasive high-speed imaging of sarcomere contractile dynamics in mice and humans. *Nature* 454(7205):784–88
105. Cromie MJ, Sanchez GN, Schnitzer MJ, Delp SL. 2013. Sarcomere lengths in human extensor carpi radialis brevis measured by microendoscopy. *Muscle Nerve* 48(2):286–92
106. Moo EK, Fortuna R, Sibole SC, Abusara Z, Herzog W. 2016. *In vivo* sarcomere lengths and sarcomere elongations are not uniform across an intact muscle. *Front. Physiol.* 7:187
107. Linke WA, Ivemeyer M, Mundel P, Stockmeier MR, Kolmerer B. 1998. Nature of PEVK-titin elasticity in skeletal muscle. *PNAS* 795(14):8052–57
108. Linke WA, Stockmeier MR, Ivemeyer M, Hosser H, Mundel P. 1998. Characterizing titin's I-band Ig domain region as an entropic spring. *J. Cell Sci.* 111(Pt. 11):1567–74
109. Rief M, Gautel M, Oesterhelt F, Fernandez JM, Gaub HE. 1997. Reversible unfolding of individual titin immunoglobulin domains by AFM. *Science* 276(5315):1109–12
110. Li H, Linke WA, Oberhauser AF, Carrion-Vazquez M, Kerkvliet JG, et al. 2002. Reverse engineering of the giant muscle protein titin. *Nature* 418(6901):998–1002
111. Kellermayer MS, Smith SB, Granzier HL, Bustamante C. 1997. Folding-unfolding transitions in single titin molecules characterized with laser tweezers. *Science* 276(5315):1112–16

112. Tskhovrebova L, Trinick J, Sleep JA, Simmons RM. 1997. Elasticity and unfolding of single molecules of the giant muscle protein titin. *Nature* 387(6630):308–12
113. Erickson HP. 1994. Reversible unfolding of fibronectin type III and immunoglobulin domains provides the structural basis for stretch and elasticity of titin and fibronectin. *PNAS* 91(21):10114–18
114. Minajeva A, Kulke M, Fernandez JM, Linke WA. 2001. Unfolding of titin domains explains the viscoelastic behavior of skeletal myofibrils. *Biophys. J.* 80(3):1442–51
115. Sotomayor M, Schulten K. 2007. Single-molecule experiments in vitro and in silico. *Science* 316(5828):1144–48
116. Linke WA, Granzier H. 1998. A spring tale: new facts on titin elasticity. *Biophys. J.* 75(6):2613–14
117. Rivas-Pardo JA, Eckels EC, Popa I, Kosuri P, Linke WA, Fernández JM. 2016. Work done by titin protein folding assists muscle contraction. *Cell Rep*. 14(6):1339–47
118. Mártonfalvi Z, Bianco P, Linari M, Caremani M, Nagy A, et al. 2014. Low-force transitions in single titin molecules reflect a memory of contractile history. *J. Cell Sci.* 127(Pt. 4):858–70
119. Mártonfalvi Z, Bianco P, Naftz K, Ferenczy GG, Kellermayer M. 2017. Force generation by titin folding. *Protein Sci*. 26(7):1380–90
120. Kötter S, Unger A, Hamdani N, Lang P, Vorgerd M, et al. 2014. Human myocytes are protected from titin aggregation-induced stiffening by small heat shock proteins. *J. Cell Biol.* 204(2):187–202
121. Olsson MC, Krüger M, Meyer LH, Ahnlund L, Gransberg L, et al. 2006. Fibre type-specific increase in passive muscle tension in spinal cord-injured subjects with spasticity. *J. Physiol.* 577(Pt. 1):339–52
122. Trombitas K, Wu Y, McNabb M, Greaser M, Kellermayer MS, et al. 2003. Molecular basis of passive stress relaxation in human soleus fibers: assessment of the role of immunoglobulin-like domain unfolding. *Biophys. J.* 85(5):3142–53
123. Bianco P, Reconditi M, Piazzesi G, Lombardi V. 2016. Is muscle powered by springs or motors? *J. Muscle Res. Cell Motil.* 37(4–5):165–67
124. Bullard B, Ferguson C, Minajeva A, Leake MC, Gautel M, et al. 2004. Association of the chaperone αB-crystallin with titin in heart muscle. *J. Biol. Chem.* 279(9):7917–24
125. Donlin LT, Andresen C, Just S, Rudensky E, Pappas CT, et al. 2012. Smyd2 controls cytoplasmic lysine methylation of Hsp90 and myofilament organization. *Genes Dev*. 26(2):114–19
126. Berkovich R, Hermans RI, Popa I, Stirnemann G, Garcia-Manyes S, et al. 2012. Rate limit of protein elastic response is tether dependent. *PNAS* 109(36):14416–21
127. Herzog W, Schappacher G, DuVall M, Leonard TR, Herzog JA. 2016. Residual force enhancement following eccentric contractions: a new mechanism involving titin. *Physiology* 31(4):300–12
128. Lindstedt S, Nishikawa K. 2017. Huxleys' missing filament: form and function of titin in vertebrate striated muscle. *Annu. Rev. Physiol.* 79:145–66
129. Rassier DE. 2017. Sarcomere mechanics in striated muscles: from molecules to sarcomeres to cells. *Am. J. Physiol. Cell Physiol.* 313(2):C134–45
130. Edman KAP, Elzinga G, Noble MIM. 1982. Residual force enhancement after stretch of contracting frog single muscle fibers. *J. Gen. Physiol.* 80(5):769–84
131. Abbott BC, Aubert XM. 1952. The force exerted by active striated muscle during and after change of length. *J. Physiol.* 117:77–86
132. Tatsumi R, Maeda K, Hattori A, Takahashi K. 2001. Calcium binding to an elastic portion of connectin/titin filaments. *J. Muscle Res. Cell Motil.* 22(2):149–62
133. Labeit D, Watanabe K, Witt C, Fujita H, Wu Y, et al. 2003. Calcium-dependent molecular spring elements in the giant protein titin. *PNAS* 100(23):13716–21
134. Fujita H, Labeit D, Gerull B, Labeit S, Granzier HL. 2004. Titin isoform-dependent effect of calcium on passive myocardial tension. *Am. J. Physiol. Heart Circ. Physiol.* 287(6):H2528–34
135. Bagni MA, Cecchi G, Colombini B, Colomo F. 2002. A non-cross-bridge stiffness in activated frog muscle fibers. *Biophys. J.* 82(6):3118–27
136. Bagni MA, Colombini B, Geiger P, Berlinguer Palmini R, Cecchi G. 2004. Non-cross-bridge calcium-dependent stiffness in frog muscle fibers. *Am. J. Physiol. Cell Physiol.* 286(6):C1353–57
137. Campbell KS, Moss RL. 2002. History-dependent mechanical properties of permeabilized rat soleus muscle fibers. *Biophys. J.* 82(2):929–43

138. Nocella M, Cecchi G, Bagni MA, Colombini B. 2014. Force enhancement after stretch in mammalian muscle fiber: no evidence of cross-bridge involvement. *Am. J. Physiol. Cell Physiol.* 307(12):C1123–29
139. Rassier DE, Leite FS, Nocella M, Cornachione AS, Colombini B, Bagni MA. 2015. Non-crossbridge forces in activated striated muscles: a titin dependent mechanism of regulation? *J. Muscle Res. Cell Motil.* 36(1):37–45
140. Campbell SG, Campbell KS. 2011. Mechanisms of residual force enhancement in skeletal muscle: insights from experiments and mathematical models. *Biophys. Rev.* 3(4):199–207
141. Leonard TR, Herzog W. 2010. Regulation of muscle force in the absence of actin-myosin-based cross-bridge interaction. *Am. J. Physiol. Cell Physiol.* 299(1):C14–20
142. Powers K, Schappacher-Tilp G, Jinha A, Leonard T, Nishikawa K, Herzog W. 2014. Titin force is enhanced in actively stretched skeletal muscle. *J. Exp. Biol.* 217(Pt. 20):3629–36
143. Schappacher-Tilp G, Leonard T, Desch G, Herzog W. 2015. A novel three-filament model of force generation in eccentric contraction of skeletal muscles. *PLOS ONE* 10(3):e0117634
144. Heidlauf T, Klotz T, Rode C, Altan E, Bleiler C, et al. 2016. A multi-scale continuum model of skeletal muscle mechanics predicting force enhancement based on actin-titin interaction. *Biomech. Model. Mechanobiol.* 15(6):1423–37
145. Nishikawa KC, Monroy JA, Uyeno TE, Yeo SH, Pai DK, Lindstedt SL. 2012. Is titin a 'winding filament'? A new twist on muscle contraction. *Proc. R. Soc. B* 279(1730):981–90
146. Kellermayer MS, Granzier HL. 1996. Calcium-dependent inhibition of in vitro thin-filament motility by native titin. *FEBS Lett.* 380(3):281–86
147. Kulke M, Fujita-Becker S, Rostkova E, Neagoe C, Labeit D, et al. 2001. Interaction between PEVK-titin and actin filaments: origin of a viscous force component in cardiac myofibrils. *Circ. Res.* 89(10):874–81
148. Yamasaki R, Berri M, Wu Y, Trombitás K, McNabb M, et al. 2001. Titin-actin interaction in mouse myocardium: passive tension modulation and its regulation by calcium/S100A1. *Biophys. J.* 81(4):2297–313
149. Linke WA, Kulke M, Li H, Fujita-Becker S, Neagoe C, et al. 2002. PEVK domain of titin: an entropic spring with actin-binding properties. *J. Struct. Biol.* 137(1–2):194–205
150. Fukushima H, Chung CS, Granzier H. 2010. Titin-Isoform dependence of titin-actin interaction and its regulation by S100A1/Ca^{2+} in skinned myocardium. *J. Biomed. Biotechnol.* 2010:727239
151. Nagy A, Cacciafesta P, Grama L, Kengyel A, Málnási-Csizmadia A, Kellermayer MS. 2004. Differential actin binding along the PEVK domain of skeletal muscle titin. *J. Cell Sci.* 117:5781–89
152. Gramlich M, Pane LS, Zhou Q, Chen Z, Murgia M, et al. 2015. Antisense-mediated exon skipping: a therapeutic strategy for titin-based dilated cardiomyopathy. *EMBO Mol. Med.* 7(5):562–76
153. Granzier HL, Radke MH, Peng J, Westermann D, Nelson OL, et al. 2009. Truncation of titin's elastic PEVK region leads to cardiomyopathy with diastolic dysfunction. *Circ. Res.* 105(6):557–64
154. Bishu K, Hamdani N, Mohammed SF, Kruger M, Ohtani T, et al. 2011. Sildenafil and B-type natriuretic peptide acutely phosphorylate titin and improve diastolic distensibility in vivo. *Circulation* 124(25):2882–91