Biophysics of Titin in Cardiac Health and Disease

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Introduction

Titin is a gigantic multifunctional filamentous protein that spans from the Z-disk to the M-band region of the cardiac sarcomere. The elastic I-band region of titin generates passive force during sarcomere stretch that plays important roles in maintaining the structural organization of the sarcomere and that contributes greatly to diastolic stiffness. Recent work has shown that to match hemodynamic demands the mechanical properties of titin can be finely tuned through differential expression of titin isoforms and phosphorylation of titin's spring-like elements. Titin may also play a role in mechanically sensing sarcomere length changes due to its placement in the sarcomere and extensible, force-bearing I-band region. The precise ways in which titin behaves as a mechanosensor is not well established, but rapid progress is being made in our understanding of the various proteins involved in signaling pathways that interact with titin. Recent work also revealed mutations in the titin gene (TTN) as a major source of familial cardiomyopathies, including mutations in titin's spring region linked to arrhythmogenic right ventricular dysplasia and mutations in titin's A-band region responsible for ~30 % of dilated cardiomyopathy (DCM) cases. This chapter discusses the mechanical properties of titin and the role titin plays in cardiac health and disease.

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The Giant Protein Titin

Titin spans the entire half sarcomere (Fig. 1a) and is embedded in the Z-disk at its N-terminus and in the M-band at its C-terminus [1]. The rest of titin is divided between its elastic I-band region and its thick filament-binding A-band region [2]. The elastic I-band region of titin consists of three distinct elements (Fig. 1b): (1) serially linked immunoglobulin(Ig)-like domains, (2) the spring-like PEVK (containing a high percentage of proline, glutamic acid, valine, and lysine residues), and (3) the spring-like N2B element [3]. These three elements largely determine the titin-based passive force that develops when sarcomere length increases (Fig. 1c) [4]. This force is important for centering the thick filament in the sarcomere [5] and for defining diastolic stiffness [6]. This region also contributes to viscosity in cardiac myocytes via PEVK-actin interaction and possibly titin–titin interaction [7].

A fundamental determinant of titin-based stiffness is titin isoform composition. Titin is the largest protein known in nature and is encoded by a single gene [3],

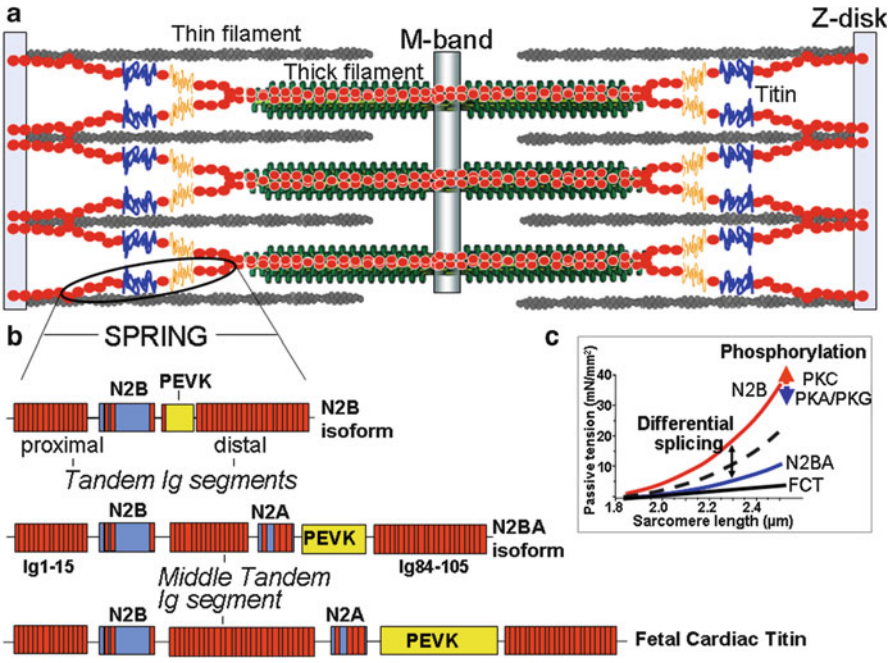

Fig. 1 (**a**) Schematic of the sarcomere. Titin spans from the Z-disk to the M-band and contains a spring-like region that generates passive force in stretched sarcomeres. (**b**) The three cardiac titin isoforms express different elastic I-band regions. The adult N2BA isoform and FCT isoform classes contain the N2A element, a middle tandem Ig segment, and a longer PEVK sequence compared to the adult N2B isoform. (**c**) Titin-based passive tension levels are determined by titin isoform composition and the phosphorylation status of the spring-like elements. The size of titin's I-band region is inversely proportional to its molecular stiffness, and phosphorylation events can either increase or decrease titin stiffness

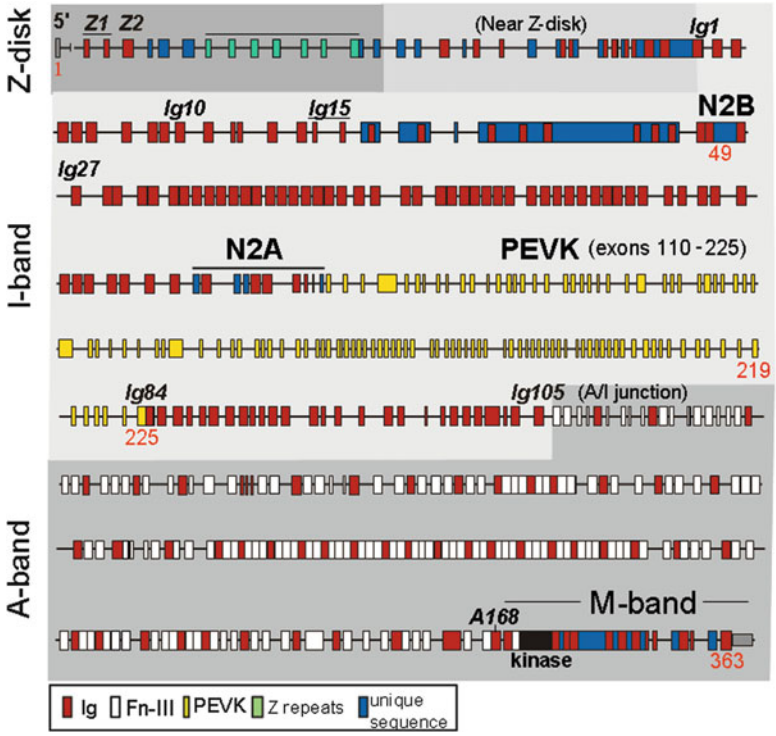

Fig. 2 The exon structure of the human titin gene (based on [3]). Each exon is represented by a box. Approximately 220 of titin's 363 exons are found in the I-band region. Titin's I-band region comprises immunoglobulin(Ig)-like domains, the PEVK element, and unique sequences. The A-band region of titin is bound to the thick filament and contains Ig and fibronectin-III (Fn-III) domains

but variable mRNA splice pathways result in distinct titin isoform classes. The human TTN contains 363 exons (Fig. 2) that code for 38,138 amino acids (4.2 MDa), although this full-length gene product is not known to be expressed in any muscle type. In cardiac muscle three classes of titin isoforms are present: adult N2BA, adult N2B, and the fetal cardiac titin (FCT) isoforms [8, 9]. Differential splicing of the I-band region of the TTN results in these three titin isoform classes; the rest of the gene (Z-disk, A-band, M-band localized regions) is largely identical. For this reason, it is common for titin isoforms to be identified by their elastic I-band regions. The I-band region of titin contains ~220 exons [3] that code for unique sequences, PEVK segments, and Ig domains. Exon 49 (~2,780 nucleotides) codes for the so-called N2B element that is found in all cardiac titin isoforms. This N2B element contains a 572 residue unique sequence (N2B-Us) flanked by Ig24/25 at its N-terminus and Ig26 and its C-terminus. In addition to behaving as a large molecular spring, the N2B-Us is a substrate for various kinases that affect its

mechanical properties [10, 11]. Therefore, the N2B-Us plays crucial roles in changing titin-based passive tension levels that may be physiologically relevant.

While the N2B element is found in both adult cardiac titin isoforms, the N2A element is found in the N2BA isoform (hence its name) and FCT isoforms (Fig. 1b). Similar to the N2B element, N2A contains Ig domains and unique sequence. The N2A region is encoded by exons 102–109 and contains four Ig domains and a ~125 residue unique sequence. The N2A element is relatively understudied, although it is a binding site for the protease calpain-3 [12] and proteins involved in signaling pathways (see section "Titin-Based Signaling"). The remaining unique sequences in the I-band region of titin belong to three novel exons (Novex I, II, and III) that are not present in the conventional titin isoforms [3].

Like the N2B-Us, the PEVK region of titin also behaves as a molecular spring that can be phosphorylated to quickly change the spring-like properties of titin. The PEVK sequence is encoded by 114 exons although most of these exons are spliced out in the cardiac titin isoforms. For example, only seven PEVK exons are found in the N2B titin isoform while the PEVK region of N2BA titin contains several additional exons [13].

The last component of titin's extensible I-band region is the serially linked immunoglobulin(Ig)-like domains. These domains are structurally very similar to each other and natively exist as a stable, folded β-barrel [14, 15]. The N2B titin isoform contains a proximal tandem Ig segment (Ig1–15) and a distal tandem Ig segment (Ig84–105). These segments can be visualized as "beads on a string." Each folded Ig domain has a diameter of 4–5 nm separated by short peptide linkers [16]. The N2BA titin isoform contains the proximal and distal tandem segments as well as a middle tandem Ig segment that contains a variable number of domains [17]. The additional Ig domains and PEVK sequence and the inclusion of the N2A element make the N2BA titin isoform larger and more compliant than the N2B isoform (~3.3 MDa vs. 2.97 MDa). The FCT class of titin isoforms (3.5–3.6 MDa, [8]) contains a larger middle tandem Ig segment than the N2BA isoform and the largest PEVK sequence of all the cardiac titin isoforms [9, 18]. An exciting new discovery regarding titin splicing is that the TTN is spliced by RNA binding motif protein 20 (Rbm20) [19]. Mutations in Rbm20 result in exceptionally large titin molecules [19] and have been linked to DCM [20, 21]. Because of the intimate relationship between the size of titin's I-band region and titin-based passive tension (see below), with larger elastic I-band regions corresponding to lower passive tension, the titin isoform expression ratio in the heart is a crucial determinant of titin stiffness. The shorter, stiffer N2B isoform accounts for 80 % of mouse titin and 60 % of human titin [22]. Differences in titin isoform expression account for cardiac passive tension variability between species (more N2B is found in small mammals that have higher heart rates [22]), and the titin isoform ratio changes in diseased states and gives rise to alterations in passive stiffness. In order to understand the interplay between pathology and titin isoform expression it is important to discuss how titin's I-band region mechanically behaves in stretched muscle.

Extension of Titin's I-Band Region

The amount of force borne by titin's I-band region depends on sarcomere length. Since the thin and thick filaments stretch only a small amount [23, 24], changes in sarcomere length (SL) directly correspond with the end-to-end length of titin's extensible I-band region. As the SL increases above slack SL, the three elements of I-band titin extend and develop a passive restoring force that resists extension increases. However, the tandem Ig domains, PEVK, and unique sequences do not extend equally (Fig. 3). This was determined by electron microscopy performed on stretched mouse cardiac myocytes that were stained with antibodies to epitopes that flank the distinct spring-like elements of titin's I-band region [25]. Measuring the distances between epitopes as a function of SL showed that the tandem Ig domains extend at low SL ranges and that the N2B-Us and PEVK segments extend as the tandem Ig domains become taut. This behavior (early tandem Ig extension followed by PEVK and N2B unique sequence uncoiling) has also been shown in rat ventricle (mostly N2B), cow ventricle (equal N2B and N2BA), and cow atrium (mostly N2BA) [26]. The sum of the extensions of each titin I-band element is equal to the SL minus the unchanging length contributions of the thick filament and portion of titin bound to the thin filament. This fact is exemplified in a mouse model in which the N2B element of titin's I-band region is deleted and the remaining elements (tandem Ig, PEVK) compensate for this loss by extending further [27]. The increased extension of the remaining elastic elements of titin also resulted in higher passive tension [27], a phenomenon that is well described by the wormlike chain (WLC) equation: $F = \frac{k_B T}{L_p}\left(\frac{z}{L_c} + \frac{1}{4(1-z/L_c)^2} - \frac{1}{4}\right)$. This equation describes the force needed to extend a polymer as a function of persistence length (L_p) and fractional extension (z/L_c). Persistence length is a measure of bending rigidity and is inversely proportional to force, with a molecule of lower L_p (more flexible) requiring more

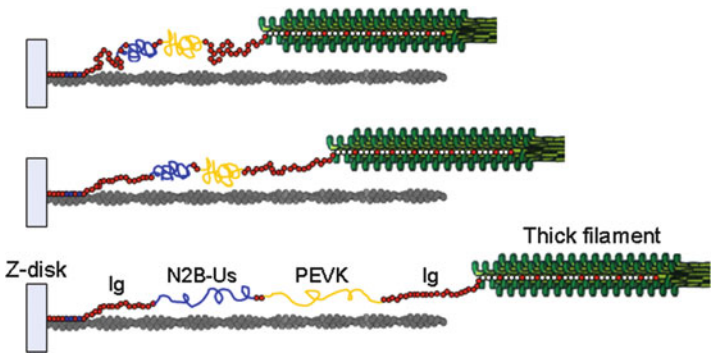

Fig. 3 The spring-like elements of titin's I-band region fractionally extend at different rates depending on sarcomere length. As the SL increases from slack, the proximal and distal Ig segments straighten out while the more flexible (entropically stiffer) N2B unique sequence and PEVK element remain compact. After the Ig segments become taut, the N2B-Us and PEVK extend

force to stretch. Fractional extension is the end-to-end length of the molecule (z) divided by the molecule's contour length (L_c), which is the distance traced along the molecular backbone. The force required to stretch a molecule depends nonlinearly on fractional extension, with significantly more force needed as its extension approaches its contour length. This last point explains why the N2B titin isoform is stiffer, i.e., requiring more force to stretch, than the N2BA titin isoform. At a given SL the extension of the I-band region of the N2B and N2BA titin isoforms (physical distance between the end of the thin filament-binding region of titin and beginning of the thick filament-binding region) is the same. However, since the N2B isoform has a shorter contour length (due to fewer Ig domains, a shorter PEVK segment, and absence of the N2A element), its fractional extension is greater. Therefore more force is needed to stretch the N2B titin isoform—it is stiffer because it is shorter. For this reason, changes in titin isoform expression change myocardial stiffness [22]. To gain a more quantitative understanding of how the N2B and N2BA isoforms differ, it is useful to model titin's I-band region as springs connected in series.

The magnitude of titin-based passive tension is a combined effect of the elements of titin's I-band region. The resistance of each element to stretch must be known in order for an accurate estimation of the entire region's stretch response. For example, consider three ideal springs ($F = -kx$) connected in series, where k is the spring constant, x is the displacement from equilibrium, and F is force. Each spring is initially at equilibrium and the length of the spring system is $x_1^0 + x_2^0 + x_3^0 = x_{\text{total}}^0$, where the zero superscript indicates equilibrium, i.e., no potential energy stored in the spring. If an external force stretches the system, force balance dictates that the tension in each spring is the same and the displacement of each spring from equilibrium is equal to F/k_i, where k_i is the spring constant of spring i. The sum of each spring's displacement from equilibrium equals the total displacement of the system from equilibrium: $\sum_i (x_i^f - x_i^0) = x_{\text{total}}^f - x_{\text{total}}^0$. The contribution of a spring to the total displacement of the system depends on its stiffness, with a compliant spring extending further than a stiff spring under the same external force. In the sarcomere, ideal Hookean springs are replaced by the entropic spring elements of titin's I-band region and the equilibrium length of the spring system is defined as the slack sarcomere length (length where force is zero). The relationship between force and extension is approximated by the WLC equation, and force balance in the system still holds. Technically, force balance is only satisfied at equilibrium, but this idea is helpful for understanding titin-based passive tension as a function of SL. In mechanical studies length changes are imposed by a motor and the restoring force response is measured with a force transducer [28, 29]. At a given SL the extension of each spring-like titin element depends on the contour length and persistence length of each element, with the tandem Ig domains, PEVK element, and N2B-Us extending different lengths such that two constraints are satisfied: (1) equal tension throughout titin's I-band region and (2) the sum of spring displacements from equilibrium equals the current SL minus the slack SL. Satisfying these constraints for wormlike chains is not trivial, but to make

things easier the wormlike chain can be inverted (approximately) such that extension is a function of L_c, L_p, and force (Eq. (6) in [30]). The extension of each titin I-band component can then be estimated as a function of force and summed, which results in the total extension of the I-band region of titin (which directly relates to SL) as function of force. Therefore, to model the force-extension characteristic of titin's I-band region, the individual properties of the distinct I-band elements need to be known. This process also allows predictions of changes in titin-based passive tension from single molecule studies of titin fragments. To directly measure the mechanical properties of titin's elastic elements, each component must be studied in isolation at the single molecule level.

Mechanical Properties of Titin's Elastic Elements

Tandem Ig Segments

The majority of titin's elastic I-band region is made up of the tandem immunoglobulin(Ig)-like domains. These domains have a characteristic β-barrel structure and have been classified as members of the intermediate I-set of the immunoglobulin family [31, 32]. The tandem Ig domains of titin's I-band region can be further classified by their position within the I-band (Fig. 1). The proximal Ig segment is immediately adjacent to the actin-binding region of titin and contains Ig1–15 (Ig1 begins the I-band region of titin) and the distal Ig segment contains Ig84–105; these two Ig segments are constitutively expressed and form the entirety of the serially linked Ig segments in the N2B titin isoform. To be clear, the cardiac titin isoforms also contain three Ig domains in the N2B element and one Ig domain immediately C-terminal to the N2B element. In addition, the N2BA titin isoform contains a middle proximal Ig segment of varying length [17]. Ig domains have stable tertiary structure and the atomic coordinates of many Ig domains have been determined [14, 32–34]. Although there are some primary sequence differences between the Ig domains [35], they all seem to form β-barrels. Molecular dynamics simulations [36, 37] and single molecule force spectroscopy [38–40] have studied the unfolding behavior of Ig domains in detail and found that the average force needed to unfold an Ig domain in titin's I-band region is between 150 and 300 pN at physiological pulling speeds [39–41]. Although there is some evidence for two-step unfolding of Ig domains [42], most evidence has shown that the hydrophobic core readily disassembles once the mechanical stability of an Ig domain is compromised due to rupture between terminal β-strand networks [36, 38]. It is unclear whether Ig unfolding takes place in vivo and if unfolding is physiologically relevant. The unfolding rate under zero force of various Ig domains has been estimated between 10^{-4} and 10^{-5} domains/s [38, 39]. Unfolding rate is force dependent and can be approximated by $\alpha(F) = \alpha(0)\exp(F \cdot \Delta x / k_B T)$ [43], where F is the external force acting on the domain and Δx is the distance along the reaction coordinate from

the folded protein state to the transition barrier. The upper limit of the force experienced by the I-band region of titin is <5 pN as determined from muscle mechanics [29], thick filament density [23], and working SL data [44], and Δx has been estimated as ~0.3 nm [38, 39]. Using these values, estimates that 1 out of every 10,000–100,000 Ig domains unfold per second at the end-diastolic sarcomere length. This suggests that Ig domain unfolding is not common under physiological conditions. When an Ig domain does unfold from a stable β-barrel structure to an unfolded polypeptide random coil, the contour length of titin's I-band increases by ~30 nm (the average length of an amino acid is 0.38 nm [45] and Ig domains are approximately 90 residues). This increase in contour length relieves some of the tension in titin by reducing the fractional extension of the PEVK and N2B elements, although the reduction in passive tension that results from unfolding of one domain is small (estimated at <1 %).

Ig domains may play an important role in disease mechanisms. For example, a mutation linked to arrhythmogenic right ventricular dysplasia was recently found in the tenth Ig domain of the proximal tandem Ig segment [46]. This is the first time an Ig domain in titin's I-band region has been linked to cardiac disease, and nuclear magnetic resonance (NMR) and proteolysis assays showed that domains containing the disease-linked mutation undergo structural changes and are more prone to degradation [46]. The structural weakening of the Ig domain with the mutation was also confirmed at the single molecule level [47]. Also, numerous titin mutations that result in truncated titin molecules have been discovered in patients with DCM [48]. Most of the mutations were found in the thick filament binding, A-band portion of titin, with relatively few mutations found in titin's elastic I-band region. Under the assumption that random mutations are equally likely to occur at any spot along the TTN, the paucity of reported mutations in titin's I-band suggests that they are particularly detrimental and are less likely to be propagated within a population.

PEVK

Containing a high percentage of proline (P), glutamic acid (E), valine (V), and lysine (K) residues, the PEVK element is well described as an entropic spring. Circular dichroism experiments suggest that the PEVK element may contain polyproline II helices [49], although the propensity to form helical structures is most likely isoform dependent, with the PEVK region of the N2B titin isoform predicted to form helices at very low levels [50]. Most single molecule studies have focused on the constitutively expressed PEVK element that is the full PEVK region of the N2B titin isoform, although the N2BA titin isoform contains two polyE (E-dense regions of negative charge) and 20 PEVK repeat motifs (26–28 residues each) while the N2B titin isoform contains zero polyE regions and only five repeat motifs [51]. The PEVK region of the N2BA titin isoform is also much larger (~800 residues compared to 188 for N2B titin; the L_c of random coil polypeptides is

~0.38 nm/residue) which contributes to the larger size and increased compliance of the N2BA isoform. The high concentration of bulky proline residues and charge clusters suggests that the PEVK region does not contain large stable structures [2], and this idea is supported by single molecule stretch studies of the constitutively-expressed PEVK segment using atomic force microscopy (AFM). AFM force-extension traces do not show force peaks indicative of structural transitions when PEVK is stretched; instead, the PEVK element smoothly extends in a characteristic WLC fashion with a persistence length of ~1 nm [50, 52–54].

N2B Element

The N2B element is found in all cardiac titin isoforms but is absent in skeletal muscle. Encoded by a single exon, the human N2B element contains 926 amino acids that comprise three ~90 amino acid Ig domains (Ig24–26) interspersed by unique sequences. The largest continuous unique sequence in the N2B element is 572 residues and is located between Ig25 and Ig26; this large unique sequence is termed the N2B-Us and has been studied in detail at the single molecule level. Like the PEVK, the N2B-Us acts as an entropic spring. Using AFM, the L_p of the N2B-Us was found to be ~0.65 nm [41, 50, 52]. Thus the N2B-Us has a lower L_p than the PEVK, which, according to the WLC equation, makes the N2B-Us harder to stretch (although, physically, a lower L_p means increased local flexibility). The N2B-Us is not thought to contain stable tertiary structures because its force-extension trace is absent force peaks [41, 50, 52]. The presence of secondary structure in both the PEVK and N2B-Us cannot be excluded by AFM experiments since the force resolution is typically ~10 pN. Also like the PEVK, the structure of the N2B-Us is unknown. Because both molecules are thought to be largely intrinsically disordered, solving the structures is highly challenging. Ligand binding [41] and phosphorylation [11, 53, 55] change the mechanical properties of the PEVK and N2B-Us, but the uncertainty regarding the structures of these elastic regions precludes predicting how protein binding and posttranslational modifications (PTMs) alter the structure of these extensible proteins.

Titin Stiffness Modulation

The two primary known ways in which titin-based stiffness is altered are changes in titin isoform expression ratio and PTMs of titin's elastic I-band region. While these two mechanisms are distinct, their effects on passive tension are not independent since the effect of PTMs on titin stiffness is isoform dependent [56] due to the different degrees that titin isoforms fractionally extend as a function of sarcomere length.

Titin Isoform Composition

As detailed above and shown in Fig. 1, the N2B, N2BA, and FCT isoforms have different I-band region compositions which imparts them with different mechanical properties. Since the FCT isoforms have the largest I-band region, they are more compliant than the shorter adult titin isoforms at a given SL. According to the WLC equation, the force needed to stretch a polymer rapidly increases as the fractional extension approaches one, i.e., when the end-to-end length of the polymer (z) nears its contour length (L_c). Co-expression of titin isoforms occurs at the level of the half sarcomere [57], which means that each titin isoform has the same end-to-end length between their anchoring points in the thin and thick filament of the sarcomere at a given SL. Since the N2B and N2BA isoforms have a shorter L_c than the FCT isoform class, at a given SL stretch the N2B and N2BA isoforms are at a greater fractional extension and require more force to stretch. The compliant FCT isoforms are highly expressed during embryonic development and at birth but are replaced by the stiffer adult isoforms during the first weeks of postnatal development [8] as the newborn heart must quickly adapt to the increased hemodynamic loads required to sustain independent life. In the adult, the N2BA titin isoform is considered compliant compared to the shorter N2B isoform because the I-band region of N2BA titin is larger. The ratio of N2BA:N2B isoform expression therefore directly influences passive tension and is quite variable between species and certain disease states.

Animals with faster heart rates typically have stiffer myocardium and a lower N2BA:N2B ratio [22], which might be necessary to allow for rapid early diastolic filling and rapid setting of the end-diastolic volume that accompanies the shorter diastolic period at the fast heart rate. Of particular interest is how titin isoform composition changes in certain pathological states. For example, 2 weeks of pacing tachycardia induced DCM in dogs and was characterized by chamber dilation and increased chamber stiffness [58]. In this study, the N2BA:N2B ratio differed in the subendocardium and subepicardium between the control and paced hearts, although the gradient difference did not reach significance. A similar study that paced dogs for 4 weeks showed that the ratio of N2BA to N2B titin in the left ventricle was significantly reduced from 1.01 in controls to 0.80 in the paced group [59]. This up-regulation of the stiffer N2B isoform was also accompanied by increased titin-based passive tension. Pressure overload imposed on a hypertensive rat model showed a reduction in N2BA titin expression [60] and is consistent with hypertensive rat cardiomyocyte studies that found elevated levels of passive tension [61, 62].

Variable titin expression ratios have also been found in human patients with cardiac disease. Patients with coronary artery disease (CAD) have been shown to express increased levels of N2BA titin that was accompanied by decreased stiffness at the myofibril level [63]. This did not correspond to decreased left ventricular wall stiffness, however, and was explained by increased expression of collagen, an extracellular matrix (ECM) protein that plays an important role in passive tension, and desmin, a cytoskeletal protein that is thought to contribute to ventricular wall stiffness [64]. The change in titin isoform expression in human CAD patients has

been suggested to be a compensatory mechanism to counteract the increased ECM-based stiffness associated with the disease [65], which contrasts the animal model studies mentioned above in which titin stiffness contributed to the pathology. However, human patients are usually treated only after chronic cardiac stress has already led to the pathological state, which makes it unclear whether changes in titin expression contribute to the pathology or respond to the pathology. Changes in titin isoform expression have also been found in patients with end-stage heart failure due to DCM where the compliant N2BA isoform was up-regulated and associated with decreased passive stiffness and increased chamber compliance [66]. The same study also suggested a physiological benefit of this change in titin expression via correlation between the titin isoform shift and improved exercise tolerance. This suggests that up-regulation of the more compliant N2BA titin isoform may be a beneficial compensatory adaptation [66].

Posttranslational Modifications

It is well known that PTMs of contractile and regulatory proteins greatly affect cardiac function, and recent research has shown that titin is also modified by various kinases that lead to changes in titin-based passive tension. The mechanical properties of the tandem Ig segments, PEVK sequence, and N2B element together determine titin-based passive tension, and therefore changes in the properties of these three spring-like elements affect titin stiffness and myocardial stiffness. Single molecule force spectroscopy experiments have studied the elastic properties of titin fragments in isolation and have discovered that kinase phosphorylation significantly alters the stiffness of the PEVK and N2B-Us. This allows for rapid adjustment of titin stiffness and quick adaptations of cardiac performance to meet hemodynamic needs.

PEVK Phosphorylation

Although AFM traces suggest that the PEVK element does not contain stable structures, it does not seem to be purely a random coil since phosphorylation of PEVK by protein kinase C (PKC) changes its physical properties. PKC is activated by the α1-adrenergic signaling pathway that is a key mediator of physiological and pathological adaptation. PKC is involved in numerous cellular processes including regulation of cystolic calcium (Ca^{2+}), myofilament Ca^{2+} sensitivity, and cardiac contractility [67]. It was found that PKCα, the predominant isozyme in the heart and a key player in contractile dysfunction and heart failure [68, 69], phosphorylates the PEVK element of titin and leads to increased passive tension [70]. This passive tension increase was reversed by introduction of the dephosphorylating agent protein phosphatase-1 (PP1), which suggests that PKCα

phosphorylation directly affects titin stiffness. The primary sites of phosphorylation were found to be two highly conserved serine residues (S26 and S170) within the constitutive PEVK element. The PEVK sequence adjacent to these phosphorylation sites is also highly conserved across species [70], which suggests that the PEVK has some structure because pure random coils would likely permit primary sequence drift. Phosphorylation of these conserved serine residues reduces the L_p of the PEVK element (from ~1 to 0.67 nm) [53], which is consistent with the increased passive tension seen at the tissue level [70]. Mutation of these conserved serines to alanine diminished the PKC effect and also reduced the L_p of PEVK to ~0.55 nm in the absence of PKC phosphorylation [53]. This further supports the idea of PEVK structure because if it was a pure random coil the L_p would not be significantly altered by point mutations. The link between PKCα, PEVK phosphorylation, and passive tension was further established by a study that showed that PKCα had no effect on passive tension in mice that had the constitutive PEVK element genetically removed [71]. The combination of techniques including single molecule force measurements and novel mouse models has established that posttranslation modifications of titin via PKC directly influence titin-based passive tension. The mechanisms by which phosphorylation and mutation change the PEVK's mechanical properties are unclear due to the limited information regarding its structure and require further study.

N2B-Us Phosphorylation

The N2B element of titin is also a kinase substrate that experiences changes in its mechanical properties following phosphorylation. Protein kinase A (PKA), which is stimulated by the β-adrenergic pathway, reduces passive tension in cardiac myocytes [10]. Phosphorylation assays and immunoelectron microscopy showed that PKA targets the large unique sequence in the N2B element (N2B-Us) [10]. The effect of PKA on passive tension is increased in the N2B titin isoform compared to N2BA, presumably due to the N2B isoform having a larger fractional extension at a given SL [56]. PKA also reduces passive tension in human cardiac fibers [72], with a more pronounced effect present when PP1 dephosphorylation was performed prior to PKA treatment, which shows that basal levels of phosphorylation play an important role in determining passive tension levels.

Similar to PKA, protein kinase G, which is cGMP-dependent, phosphorylates the N2B-Us and reduces passive tension, and the PKG phosphorylation site is also a residue targeted by PKA (Fig. 4) [11]. The effect of PKG on the passive tension of skinned fiber bundles from human donor hearts has been studied and a significant reduction in passive tension following PP1 treatment was found. Single molecule data suggests that PKG phosphorylation increases the L_p of the N2B-Us, which is consistent with the reduced passive tension measured in muscle mechanics experiments [11]. More work is needed to confirm this single molecule result due to the limited data collected in the initial study. Recently, the serine/threonine

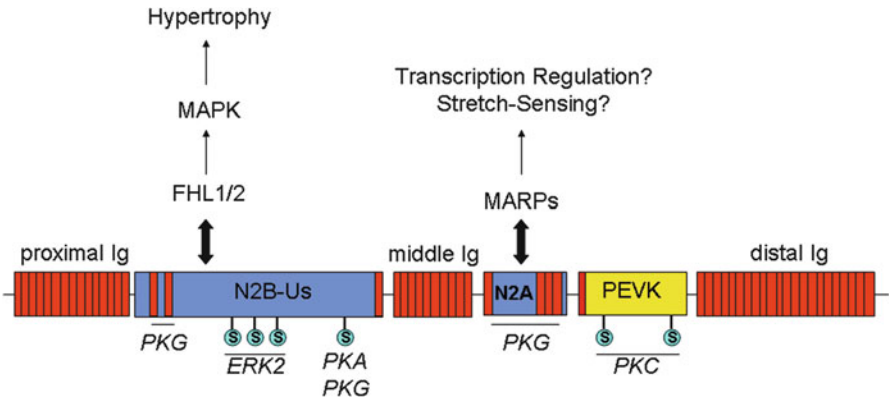

Fig. 4 Schematic of titin's elastic I-band region showing kinase phosphorylation sites and interactions between titin and proteins involved in signaling pathways. PKG phosphorylates the N2A element and the Ig24-unique sequence-Ig25 region of the N2B element, but site-specific information is unknown. The N2A and N2B unique sequences are the regions of titin most likely to be involved in cell signaling

kinase ERK2 has been shown to phosphorylate the N2B-Us at multiple residues [73], although the effect that this has on the mechanical properties of titin is unknown. ERK2 is involved in the MAPK pathway [74] (see section "Titin-Based Signaling"), a key player in hypertrophy signaling [75], which suggests interplay between the phosphorylation status of titin and the trophic state of the heart.

The mechanical properties of the N2B-Us can be altered by more than just phosphorylation status. For example, there are six cysteine residues in the human N2B-Us that have the potential to form disulfide bonds with one another depending on the oxidative state of the sarcomere. A disulfide bond would reduce the contour length of the N2B-Us and change its mechanical response to stretch. AFM experiments carried out in the presence and absence of the reducing agent DTT show that disulfide bonds can drastically decrease the L_c of the N2B-Us, which is predicted to significantly affect titin-based passive tension [76]. The effect of cysteine cross-linking on the mechanics of the N2B-Us was also shown at the tissue level where oxidative stress increased passive tension and hysteresis in wildtype tissue but had an attenuated effect in tissue from a mouse model where the entire N2B element was removed [29]. The study between oxidative conditions and changes in passive tension is especially interesting considering that oxidative stress is elevated in heart failure patients and has been correlated with myocardial dysfunction [77].

Since titin-based stiffness at the tissue level is determined by titin isoform composition and the phosphorylation state of titin's elastic I-band, with different kinases affecting titin elasticity is disparate ways, comprehensive studies of titin isoform expression and phosphorylation status is ideal for determining the mechanisms through which titin stiffness changes during acute and chronic stress.

Titin-Based Viscosity

Studying purified fragments of titin's elastic I-band region at the single molecule level is an ideal way to determine the properties of the tandem Ig, PEVK, and N2B elements in isolation. However, the cell is a protein-dense environment and interactions between the large myofilaments of the sarcomere can lead to intracellular viscosity, which increases the force needed to stretch the muscle since viscosity, which is a frictional force, opposes motion. Whenever sarcomere length is changing, viscosity is present and must be overcome [78].

Due to the high density and close proximity of actin, myosin, and titin in the sarcomere, it is not surprising that viscous interactions occur between these myofilaments. The main source of titin-based viscosity in the cell arises from interaction between the PEVK element of titin and the actin-based thin filaments. This protein–protein interaction opposes filament sliding and has been studied with single molecule spectroscopy [79], in vitro motility assays [54, 80], and myocyte mechanics [7, 80]. Integrative studies have also been performed on a mouse model in which the constitutive PEVK element of titin is deleted (exons 219–225) [7]. This study showed that removal of the PEVK element reduced viscosity by 60 % in myocytes, 50 % in muscle fibers, and 30–40 % in intact isolated hearts due to decreased PEVK-actin interaction. The affinity between PEVK and actin seems to be an electrostatic effect between actin, which is negatively charged [81], and PEVK. The constitutively-expressed PEVK element contains five basic (p*I* 9–10) PEVK repeat motifs that are each ~28 residues [51]. Therefore, at physiological pH levels the PEVK element has a net positive charge which may drive the electrostatic PEVK-actin interaction. This interaction is dependent on ionic strength [79, 80, 82] as well as lattice spacing [7], with increased ionic strength and increased lattice spacing both reducing PEVK-actin interaction. These experimental observations are consistent with the hypothesis that an electrostatic force drives PEVK-actin binding since Coulomb's Law of Electrostatics states that electrostatic force is proportional to the product of the two point charges (higher ionic strength effectively shields the PEVK from actin) and inversely proportional to the square of the distance between the two charges. The interaction between PEVK and actin has been well studied for the PEVK element of the N2B titin isoform, but the dynamics between actin and the additional PEVK sequence of the N2BA titin isoform are unknown.

Viscous force is speed dependent, and in cardiac cells the speed at which the myofilaments slide past each other is directly related to heart rate. For this reason, titin-based viscosity is predicted to be higher in small animals with high heart rates compared to humans. On the other hand, viscous forces may play a physiologically more relevant role in animals that experience a wider range of heart rates. For example, the human heart rate can triple during exercise while the murine heart rate changes only slightly. Although the magnitude of viscosity may be larger in small animals, it is not anticipated that this viscous force changes much as small animals are stressed. An important factor to consider when discussing viscosity is that

viscosity is greatest after a cell or tissue has been at rest, i.e., not in dynamic equilibrium. Since the heart is always beating, viscosity experiments are most relevant if a physiological stretch protocol is administered, and indeed it has been shown that repeated stretches decrease the viscous contribution [4, 29], although a significant level of steady-state viscosity remains.

Titin-Based Signaling

How do changes in hemodynamic load lead to altered titin isoform expression? Does titin act as a mechanosensor? The answers to these intriguing questions likely lie in the elucidation of titin's involvement in signaling pathways. Since titin spans the entire half sarcomere and interacts with other proteins in the Z-disk, I-band, A-band, and M-band regions of the sarcomere, titin's role in stretch-sensing and signaling pathways may be very complex, but exciting results have already been realized.

Z-Disk Signaling

The protein-dense Z-disk region of the sarcomere mechanically connects adjacent sarcomeres and helps maintain the highly ordered myofilament lattice structure. The Z-disk undergoes strain when tension develops in the sarcomere as evidenced by changes in Z-disk thin filament lattice spacing as a function of active tension [83] and passive tension [23]. Two Ig domains at titin's N-terminus (Z1 and Z2, Fig. 2) embed titin in the Z-disk and strongly bind to T-cap (also called telethonin) and α-actinin. T-cap connects two titin molecules from the same half sarcomere in a sandwiched structure [84] via strong β-strand cross-linking [85, 86]. A possible mechanism for titin-based signaling involves T-cap's interaction with muscle LIM protein (MLP), a nuclear regulator of myogenic differentiation [87] that also promotes myogenesis [88]. Cardiac myocytes subject to stretch express the well-known stretch response markers brain natriuretic peptide and atrial natriuretic factor, although this response to stretch is absent in MLP deficient mice [89]. MLP mutations associated with mechanical stress signaling deficiency have been linked to hypertrophic cardiomyopathy (HCM) [89, 90], and mutations in T-cap have been linked to HCM and DCM with the pathological phenotype suggested to arise from compromised binding between T-cap, titin, and other Z-disk proteins [91, 92]. Many of the sarcomeric proteins localized in the protein-dense Z-disk of the sarcomere have been implicated in complex signaling pathways, and titin may play a key biomechanical stress sensor function.

I-Band Signaling

Except for PEVK interacting with the thin filament, the elastic I-band region of titin is thought to be unbound in the sarcomere. Because of this, the spring-like elements of titin's I-band directly bear the force that develops as sarcomere length changes. The N2B element found in all cardiac titin isoforms interacts with two members of the four-and-a-half-LIM domain protein family (Fig. 4), FHL1 [74] and FHL2 [93]. FHL1 interacts with the N2B element and is suggested to form a stretch-sensing complex downstream of G-protein-coupled receptor signaling [74]. This FHL1/N2B complex also associates with members of the MAPK signaling pathway, which are involved in hypertrophy signaling [75]. Mouse models have been used to determine the role that FHL1/N2B interaction plays in vivo. FHL1 knockout (KO) mice showed a decreased hypertrophic response and beneficial functional response to transverse aortic constriction (TAC)-induced pressure overload compared to control mice [74]. This suggests that FHL1 hypertrophy signaling responds to increased afterload, although it does not elucidate how load is being sensed. To investigate this question, mouse models missing segments of titin have been utilized. In the N2B KO mouse in which the entire N2B element has been removed, FHL2 levels were significantly reduced and cardiac atrophy was present [27]. On the other hand, when the constitutively-expressed PEVK sequence was removed (PEVK KO), which leads to increased strain placed on the rest of titin's I-band region including the N2B element, FHL1/2 were up-regulated and accompanied by cardiac hypertrophy [94]. It has been suggested that tension acting on the N2B element increases its strain and makes accessible FHL binding sites. Subsequent FHL binding may lead to assembly of a signaling complex that induces a hypertrophic response. This hypothesis is consistent with the elimination of FHL binding sites and atrophy found in the N2B KO as well as the proposed strain-induced increase of FHL binding sites and hypertrophy found in the PEVK KO.

The N2A element, which contains a ~90 residue unique sequence flanked by Ig80–83, has also been implicated in stretch-sensing pathways. The unique sequence between Ig80 and Ig81 binds to the cardiac ankyrin-repeat protein [95], diabetes-related ankyrin-repeat protein (DARP), and ankyrin-repeat domain-protein-2 (Ankrd2) [96]. These proteins belong to the muscle ankyrin-repeat protein family (MARPs), which relocate from the I-band of the sarcomere to the nucleus to regulate transcription following mechanical stress [97]. The N2A element has been proposed to form a stretch-sensing complex that involves MARPs, myopalladin, and calpain-3. In rat cardiomyocytes, externally applied stretch induced differential localization of CARP and DARP, including increased DARP levels at the intercalated disks [96]. Although not much is known about the structure of the unique sequence found in the N2A element, it is possible that it undergoes structural transitions while under strain that change its binding affinity for proteins involved in cellular pathways, similar to the proposed N2B/FHL binding mechanism.

M-Band Signaling

The region of titin bound to the thick filament near the middle of the sarcomere, the M-band region of titin, has also been implicated in signaling processes, although the strain experienced by the M-band region of titin is less than the I-band region of titin since titin is bound to the thick filament at the M-band and the thick filament is only slightly compliant [23]. The most studied domain of titin's M-band region is titin's lone catalytic domain, titin kinase (TK). TK has interested researchers because of its potential to act as a direct biological force sensor via stress-dependent phosphorylation. The crystal structure of TK has been solved [98] and shows that the C-terminal tail of TK blocks its catalytic site. This suggests that TK activity may be force dependent, with the C-terminal being displaced from its inhibitory location when external stress pulls on the termini of TK. Single molecule AFM experiments [99] and molecular dynamics simulations [100] have supported the idea that the regulatory C-terminal tail can be forcibly removed to allow solvent access to TK's ATP-binding site. It has been shown that TK can phosphorylate T-cap [98], but more evidence is needed to link TK function at the M-band with T-cap, which is embedded in the Z-disk. Nbr1 and p62, zinc-finger proteins that have been found to act as scaffolds for signalosome assembly [101], are TK substrates [102] which suggests that force-dependent TK activation may be involved in signaling. Deletion of TK results in cardiomyopathy and death in neonatal mice [103], and a conditional mouse model in which TK was removed in adult mice showed severe cardiac hypertrophy and congestive heart failure [104]. These studies show that TK may play a role in force-dependent signaling and cardiac adaptation.

Next to TK in the M-band are titin domains A168–A170 (two Ig domains and one fibronectin type 3 domain) (Fig. 2) that bind to MURF-1 [105, 106], a ubiquitin ligase that targets muscle proteins for degradation. The possible link between titin, MURF, and cellular response involves MURF-1 binding to glucocorticoid modulatory element binding protein-1 (GMEB-1), which regulates transcription [105]. The titin/MURF interaction may regulate myofibril turnover and the trophic state of the heart, although a MURF-1 KO mouse did not show a difference in the level of titin ubiquitination [107]. On the other hand, MURF-1/MURF-2 double KO mice develop extreme cardiac hypertrophy [108], which suggests that MURFs regulate myogenesis of the heart, although the role that titin plays in the process is unclear.

Summary and Future Direction

Since the discovery of titin over 35 years ago [109, 110], scientists have learned a great deal about the various roles that titin plays in striated muscle. Research has shown that titin is the elastic myofilament of the sarcomere that generates passive

restoring forces that are crucial for sarcomere organization and determining myocardial stiffness. Evidence is accumulating that titin functions as mechanosensor that plays a role in hypertrophy singling. The three spring-like elements of titin's I-band region act together to determine titin-based passive tension, but specific phosphorylation events in a single spring-like element can significantly change how the collection behaves as a whole. Titin's involvement in cardiac dysfunction is also coming to light as disease-linked titin mutations are being discovered at an accelerating rate. Nonetheless, many aspects of titin remain unknown. For example, it is unclear how titin is properly assembled in the sarcomere, and the mechanisms responsible for this are likely complex due to titin's enormous size and extension. It has been shown that titin isoform expression ratios can change, but the mechanosensing machinery and cellular response pathways that breakdown titin bound in the sarcomere and integrate newly synthesized titin require further investigation. Novel breakthroughs in the titin field, such as the discovery of the Rbm20 titin splicing pathway [19], will continue to be realized as the extraordinary biology, chemistry, and physics of this giant protein continue to be revealed.

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