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The Giant Protein Titin: A Major Player in Myocardial Mechanics, Signaling, and Disease Henk L. Granzier and Siegfried Labeit

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This Review is part of a thematic series on **Myocyte Intra- and Extrasarcomeric Structural Proteins**, which includes the following articles:

The Giant Protein Titin: A Major Player in Myocardial Mechanics, Signaling, and Disease

The Dystrophin Glycoprotein Complex: Structure and Function in Cardiac and Skeletal Muscle

Cardiac Myosin Binding Protein C: Its Role in Physiology and Disease

David Kass, Editor

The Giant Protein Titin

A Major Player in Myocardial Mechanics, Signaling, and Disease

Henk L. Granzier, Siegfried Labeit

Abstract—The sarcomere contains, in addition to thin and thick filaments, a filament composed of the giant protein titin (also known as connectin). Titin molecules anchor in the Z-disc and extend to the M-line region of the sarcomere. The majority of titin's I-band region functions as a molecular spring. This spring maintains the precise structural arrangement of thick and thin filaments, and gives rise to passive muscle stiffness; an important determinant of diastolic filling. Earlier work on titin has been reviewed before. In this study, our main focus is on recent findings vis-à-vis titin's molecular spring segments in cardiac titins, including the discovery of fetal cardiac isoforms with novel spring elements. We also discuss new insights regarding the role of titin as a biomechanical sensor and signaling molecule. We will end with focusing on the rapidly growing knowledge regarding titinopathies. (*Circ Res.* 2004;94:284-295.)

Key Words: myocardial stiffness ■ contractility ■ stretch ■ connectin ■ passive stiffness

Titin forms an intrasarcomeric filament that is usually viewed as a stable structural and mechanical component of the myocardium (for earlier reviews¹⁻⁶). Here, we discuss recent work that indicates that the titin filament is dynamic both in structure and function. We focus on the significant restructuring of the titin filament that occurs in chronic cardiac diseases and during neonatal cardiac development when compliant fetal cardiac titins are replaced by stiffer adult isoforms. Furthermore, specific motifs in titin have been identified that assemble, some in response to stretch, regulatory protein complexes, including transcriptional and proteolytic regulators. We also review recent biomechanical data that indicate that titin is not a fixed spring, but that a variety of mechanisms including calcium binding can rapidly adjust its mechanical properties. Finally, we focus on whether this adaptable titin spring may tune signaling responses of the myocardium and its possible role in cardiomyopathies.

Role of Titin in Passive Myocyte Mechanics

In absence of external force, passive cardiac myocytes attain an equilibrium sarcomere length, or slack length, of ≈ 1.9 μm . Sarcomere stretch creates an opposing force, known as passive force. Passive force is largely derived from the extensible region of titin that runs from near the Z-disc to the edge of the A-band (Figure 1).⁷ This extensible region is highly flexible and in the absence of external force, thermally driven bending motions shorten the segment to a near zero end-to-end length⁸ (Figure 2B). Sarcomere stretch increases the end-to-end length and reduces the bending motions (Figures 2C and 2D). This lowers conformational entropy and gives rise to the so-called passive force (physiological levels 0 to ≈ 5 pN/molecule⁹) that pulls Z-discs toward each other.^{10,11} The extensible region of titin is held away from the Z-disc by titin's inextensible near Z-disc region (this region binds strongly to the thin filament and can therefore withstand compressive forces^{8,10}) and when the sarcomere short-

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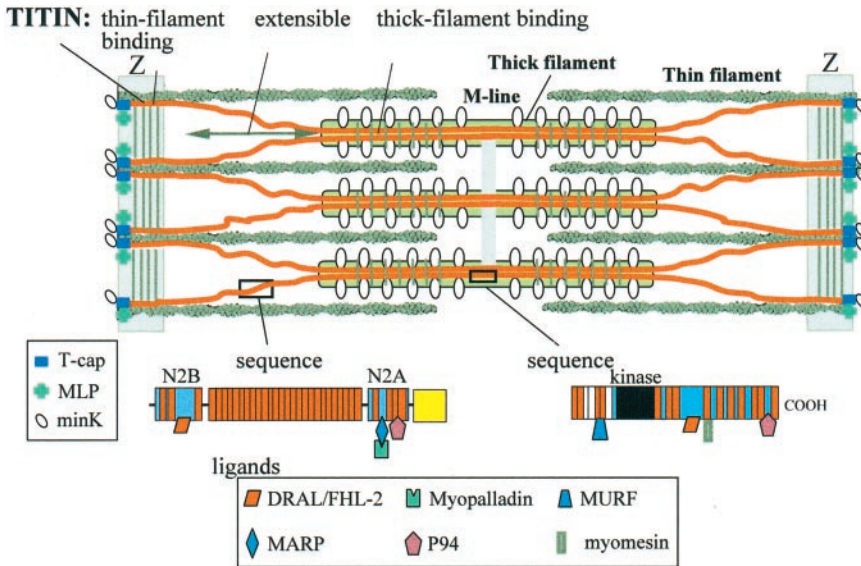


Figure 1. Layout of titin in cardiac sarcomere. Titin's N- and C-terminal regions are embedded in the Z-disc and M-line, respectively. Titin-binding ligands located in Z-disc, I-band, and M-line are also shown. Shown I-band sequence is that of N2BA cardiac titin. Vertical gray lines in Z-disc region denote α -actinin and those in A-band region MyBP-C. Figure not to scale.

ens to below the slack length, the thick filament moves into titin's near Z-disc region.^{12,13} As a result, titin's extensible region is stretched in a direction opposite of that when the sarcomere is elongated above the slack length, generating the so-called restoring force that pushes the Z-discs toward their slack length position (see Figure 2A).

Titin's extensible region is composed of tandem Ig segments [tandemly arranged immunoglobulin (Ig)-like domains], the PEVK segment [rich in proline (P), glutamate (E), valine (V) and lysine (K)], and the N2B-unique sequence [(N2B-Us)]. These segments have distinct bending rigidi-

ties,^{9,14} and as a result, stretch of slack sarcomeres (Figures 2C and 2D) initially gives rise to extension of tandem Ig segments (largely due to straightening of sequences that link Ig domains^{11,15}) followed by extension of PEVK and N2B-Us segments (which is likely due to straightening of random coil sequences^{9,14,15}). The N2B-Us is cardiac-specific, and toward the upper limit of the physiological sarcomere length its extension lowers passive force levels.¹⁵⁻¹⁷ This decreases the likelihood of Ig domain unfolding, and thereby limits energy loss due to repeated unfolding/refolding of domains during stretch/release cycles.¹⁵⁻¹⁷ The complex composition of cardiac titin's extensible region with multiple subsegments that extend at different sarcomere lengths results in a unique passive force-extension curve that is shallow close to the slack length but steeper at higher degrees of extension (Figure 2).

Passive myocytes are not purely elastic but instead exhibit viscoelasticity, as revealed by passive force hysteresis in stretch-release cycles and by force relaxation when the passive myocyte is held at a stretched length.¹⁶ The mechanisms that underlie viscoelasticity may involve (1) dynamic interaction between titin and actin,^{18,19} (2) dynamic crosslinks in the PEVK and N2B-Us regions (either intramolecular or between adjacent molecules),^{20,21} and (3) unfolding of Ig domains.²² None of these sources, however, appear to be the sole explanation for viscoelasticity. First, hysteresis is still present in thin filament-extracted myocytes¹⁰; second, single molecule force-extension curves of the PEVK and N2B-Us display (at relatively high force) little hysteresis^{9,14}; third, there is no direct evidence for Ig domain unfolding at physiological force levels.²¹ Thus, despite its physiological relevance,²³ the exact molecular basis of titin's viscoelasticity remains to be established. Our working hypothesis is that a number of mechanisms contribute, including intra- and intermolecular events that involve the PEVK segment.

A striking characteristic of striated muscle is the exquisite structural organization with A-bands positioned centrally in the sarcomere, a feature that cannot be explained by the inherently unstable sliding filament-crossbridge model of contraction. However, the discovery of titin provided an

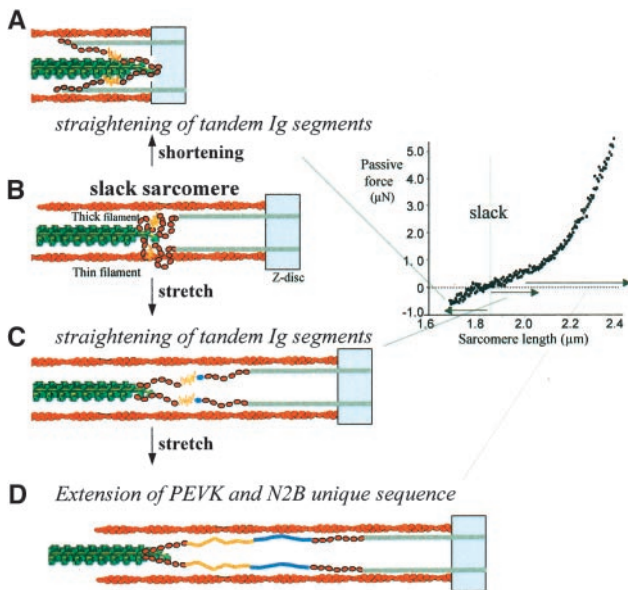


Figure 2. Mechanism of passive and restoring force generation. Titin's extensible region is in a shortened state in slack sarcomeres (B) and extends on sarcomere stretch (C and D), lowering conformational entropy and giving rise to an entropic force, known as passive force. When slack sarcomeres shorten to below the slack length (A), the thick filament moves into titin's incompressible near Z-disc region (in gray) and the extensible region now extends in a direction opposite of that during stretch, developing restoring force. Figure not to scale.

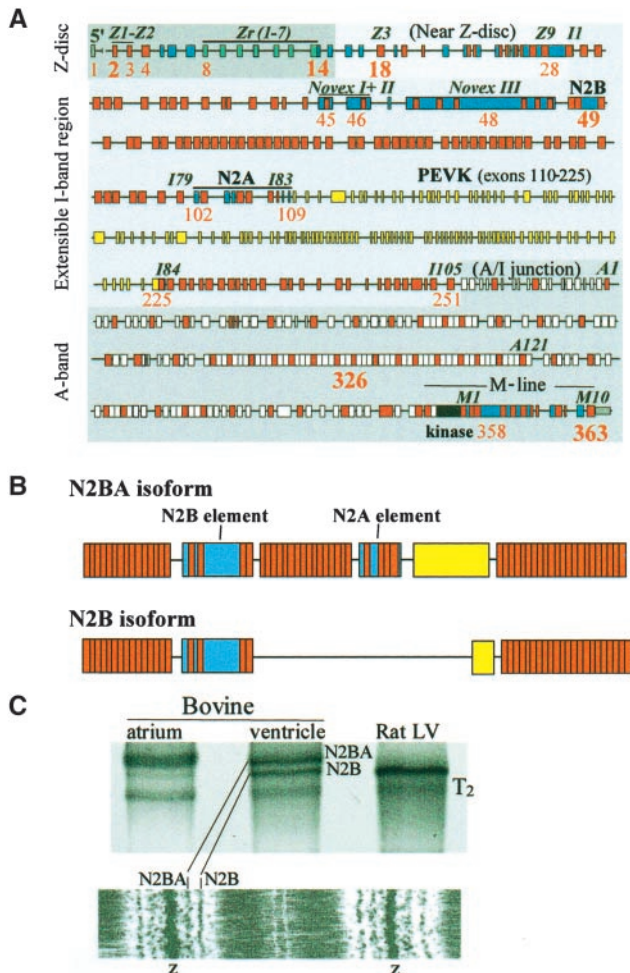


Figure 3. A, Exon-intron structure of human titin gene (based on Bang et al²⁵). Exons discussed in this review are marked in red and those involved in disease (see sections on Hereditary Titin Diseases and Acquired Titin Diseases) are shown in bold. B, Domain structure of extensible I-band region of cardiac isoforms. Number of Ig domains (red) interspersed between N2B and N2A elements varies.²⁷ C, SDS-PAGE (top) shows differences in titin isoform expression in different species, and immunoelectron micrograph (bottom) reveals coexpression of N2B and N2BA isoforms at the level of the half sarcomere (results from Trombitas et al³²). T2 is a major degradation product of full-length titin (based on Trombitas et al³²). Reproduced from Trombitas K, Wu Y, Labeit D, Labeit S, Granzier H. Cardiac titin isoforms are coexpressed in the half-sarcomere and extend independently. *Am J Physiol Heart Circ Physiol.* 2001;281:H1793–H1799, by permission of the American Physiological Society ©2001.

explanation. Because each half-thick filament (M-line to edge of A-band) contains its own set of titin filaments connecting it to the nearest Z-disc, titin's force will center the A-band within the sarcomere.²⁴ Although this force may not be sufficient to prevent translocation of the A-band during systole, when active forces are low or absent during diastole, titin is expected to rapidly reset the central A-band location. Titin's passive force also opposes sarcomere stretch and together with collagen (see later) it determines the upper limit of the physiological sarcomere length (SL) range of the heart. Titin's restoring force is likely to be a factor in setting the lower SL limit during systole and in the elastic recoil that

drives early diastolic filling. Thus, titin is important in maintaining the structural integrity of the sarcomere, setting the physiological sarcomere length range, and is a determinant of diastolic filling.

The Titin Gene and Differential Splicing

The human titin gene contains 363 exons (Figure 3A) that are predicted to code for a total of 38 138 amino acid residues.²⁵ Exons 1 to ≈251 are Z-disc and I-band exons, 116 of which are PEVK exons. These PEVK exons code for either conserved ≈28-residue PEVK repeats (PPAKs²⁶) or more complex glutamate (E)-rich motifs^{25,26} (see later). The A-band region of titin is encoded by exons ≈252 to 363. Notably,

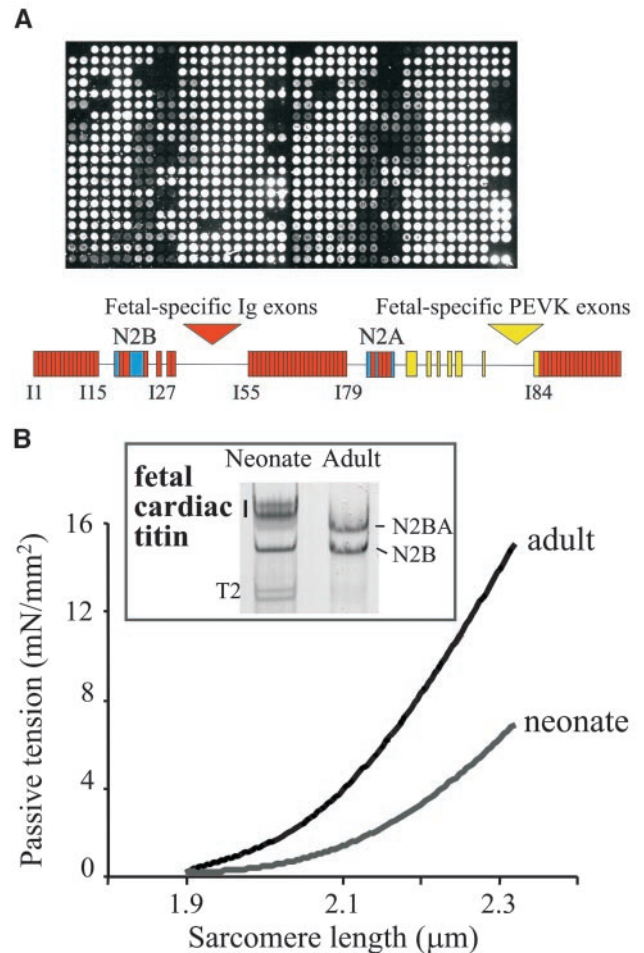


Figure 4. A, Titin exon microarray with all 363 titin exons found in the human titin gene was used to determine the exon composition of human soleus cDNA.²⁸ Array detects all 313 exons identified previously in soleus titin by sequencing.³² Thus, the array is a powerful tool for rapidly determining the exon composition of titin isoforms. Bottom, the array was used to study titin exon composition during development and this revealed a fetal titin isoform with Ig and PEVK exons that are absent in adult N2BA titin: see red and yellow insertion arrows, respectively, in domain organization of I-band region of adult N2BA titin. B, Titin-based passive tension is lower in neonatal than adult porcine myocardium. Inset, SDS-agarose gel⁹⁶ reveals high levels of fetal cardiac titin in neonatal porcine myocardium. The combined molecular mass encoded by the fetal-specific exons is ≈120 kDa, explaining why the fetal isoform can be detected as a slower migrating band (details in Lahmers et al²⁸).

exon 358 codes for a serine-threonine kinase domain with unknown physiological function.

Sarcomeric proteins are typically encoded by multiple genes that are expressed in both developmental and tissue-specific patterns. In contrast, there is only a single titin gene and multiple splice pathways in the I-band encoding region of the titin gene give rise to isoforms with distinct spring compositions.²⁷ Exon 49 (containing the N2B sequence) is excluded in skeletal muscle titins, but is present in all cardiac titin isoforms.^{25,27} Splicing of exons 49/50 to exon 219 results in the “small” 2970-kDa cardiac isoform known as N2B titin (so named because it contains the N2B element).²⁷ A second class of cardiac isoforms contains, in addition to exon 49 (N2B element), also exons 102 to 109 (coding for the N2A element), the so-called N2BA titin isoform (name reflects presence of both N2B and N2A elements). N2BA titins have a longer PEVK segment and contain additional Ig domains (Figure 3B).

We recently developed a titin exon microarray that allows all 363 titin exons of the human titin gene to be monitored simultaneously (Figure 4A) and used it to study titin in fetal, neonatal, and adult myocardium.²⁸ The titin microarray revealed fetal cardiac titins that contain additional spring elements both in the tandem Ig and PEVK region of the molecule (many of these elements have not been found in adult titins). The fetal isoform predominates in fetal and neonatal myocardium (Figure 4B, inset) and gradually disappears during postnatal development with a time course that varies in different species.²⁸ Consistent with the presence of additional spring elements in fetal cardiac titin, passive myocardium is less stiff in the neonate than in the adult (Figure 4B). The regulation of titin’s spring composition in fetal and neonatal myocardium may allow adjustment of diastolic filling behavior during development of the heart.

Stiffness Variation Due to Differential Expression of Titin Isoforms

A given sarcomere stretch results in a fractional extension of titin’s extensible segment (end-to-end length divided by the contour length) that is much higher for the N2B isoform (due to its shorter extensible segment) than for the N2BA isoform.²⁹ Thus, cardiac myocytes that express high levels of N2B titin have higher passive stiffness than those that express N2BA titin. Because high N2B expression levels are encountered in animals with high heart rates, it has been suggested²⁹ that high N2B expression allows rapid early diastolic filling (due to high restoring forces) and rapid setting of the end diastolic volume (due to the high stiffness at long SLs) when diastolic filling times are short (eg, ≈ 50 ms for the mouse).

Large mammals coexpress N2B and N2BA titins, with coexpression occurring at the level of the half-sarcomere (Figure 3C). Each half-thick filament binds 6 titin molecules³⁰ and this value appears to be constant despite widely varying coexpression ratios.²⁹ Titin’s constant stoichiometry may reflect the constant requirement for functions performed by titin’s inextensible regions, such as thick-filament length control and construction and maintenance of Z-discs and M-lines⁶ (note that the inextensible regions of isoforms are largely constitutively expressed³¹). Thus, varying the coex-

pression ratio of isoforms while keeping the stoichiometry constant does not impact critical functions performed by titin’s inextensible region, but allows development of graded passive force levels in between those of N2B- and N2BA-pure myocytes.³² This mechanism for passive stiffness modulation appears to be widely used because the coexpression ratio varies greatly in ventricular myocardium of different species,²⁹ modestly across the LV wall (in pig²⁹ and dog³³ the N2BA/N2B expression ratio is $\approx 30\%$ higher in subendocardium than in subepicardium), and greatly during heart development.²⁸ Adjustments in the coexpression ratio have been reported in various disease states (see section Acquired Titin Diseases) and may be important in pathological changes in chamber stiffness.

Rapid Adjustment Mechanisms of Titin’s Mechanics

Titin’s elasticity can be adjusted on a time scale of weeks by differential splicing of its spring elements,³⁴ and additional rapid transient adjustment mechanisms have been discovered recently. Yamasaki et al¹⁸ tested fragments representing the subdomains comprising the extensible region of cardiac N2B titin (tandem Ig segments, the N2B element, and the PEVK domain) for binding to F-actin and found that only the PEVK region bound F-actin. The functional significance of PEVK-actin interaction was investigated using both an *in vitro* motility technique and studies of passive cardiac myocyte mechanics.¹⁸ The findings indicate that, as the thin filament slides relative to titin, a dynamic interaction between the PEVK domain and F-actin retards filament sliding and, furthermore, that this interaction contributes to passive myocyte stiffness.¹⁸ The results of Kulke et al¹⁹ are in agreement with these findings. Although physiological calcium levels alone have no effect on PEVK-actin interaction,^{18,19} S100A1, a soluble calcium-binding protein found at high concentrations in the myocardium, inhibits PEVK-actin interaction in a calcium-sensitive manner.¹⁸ Thus, a dynamic interaction between titin and actin contributes to passive stiffness of the sarcomere and the interaction may vary with the physiological state of the myocardium. Stuyvers et al³⁵ demonstrated that the stiffness of rat cardiac trabeculae increases as calcium levels decay during the diastolic interval, supporting the hypothesis that a dynamic interaction between titin and actin contributes to passive stiffness of the sarcomere and that the interaction varies cyclically with the calcium level.

Titin-actin interaction experiments have focused on the PEVK segment found in the cardiac N2B isoform,^{18,19} and whether the PEVK segment of N2BA titin also interacts with actin remains to be established. Considering that PEVK-actin interaction includes an electrostatic component¹⁸ and that the cardiac N2B PEVK has charge characteristics different from those of other PEVK isoforms,¹⁸ differences in actin-binding properties are to be expected. Indeed, a recombinant PEVK fragment from skeletal muscle titin does not bind actin under physiological conditions.¹⁸ Furthermore, experiments by Campbell et al³⁶ on passive myocardium of a species that expresses high levels of N2BA titin, did not provide evidence for a dynamic PEVK-actin interaction. Thus, titin’s ability to

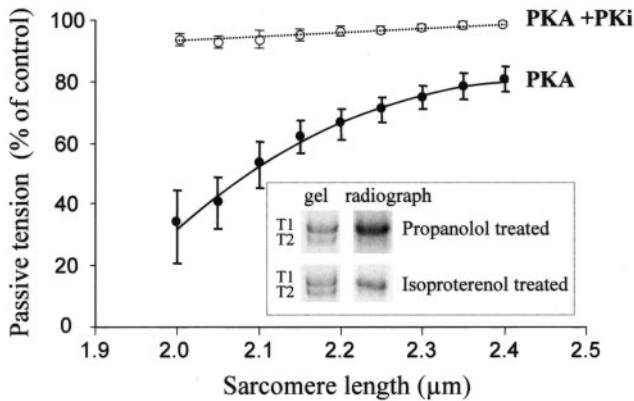


Figure 5. Passive tension after PKA treatment as a function of SL. Cells treated with PKA or PKA+PKi (PKA-specific inhibitor). PKA reduces passive tension with a magnitude that is inversely related to SL. Inset, SDS-PAGE and corresponding autoradiograph of intact cells that were treated with propanolol or isoproterenol, followed by skinning and incubation with [γ - 32 P]ATP in presence of PKA (for details, see Yamasaki et al³⁷). β -Adrenergic stimulation of intact cells enhances titin phosphorylation. Increased phosphorylation in intact cells is detected by reduced 32 P incorporation in skinned cells. Note that T1 (intact molecule) is phosphorylated but not T2 (A-band segment of titin) (based on Yamasaki et al³⁷). Reproduced from Yamasaki R, Wu Y, McNabb M, Greaser M, Labeit S, Granzier H. Protein kinase A phosphorylates titin's cardiac-specific N2B domain and reduces passive tension in rat cardiac myocytes. *Circ Res*. 2002;90:1181–1188, by permission of the American Heart Association ©2002.

bind actin may be most pronounced in sarcomeres that express high levels of N2B titin.

It was recently shown that the N2B unique sequence is a substrate for protein kinase A (PKA).³⁷ Thus, as with the well-characterized myofibrillar PKA substrates MyBP-C and TnI, titin also contains a PKA-responsive domain expressed only in cardiac muscle. Interestingly, PKA-based titin phosphorylation reduces passive tension of cardiac myocytes (Figure 5). This may be explained by assuming that phosphorylation increases the length of the N2B element, possibly due to destabilizing native structures (for details, see Yamasaki et al³⁷). Back-phosphorylation assays revealed that β -adrenergic agonists stimulate, whereas antagonists depress, titin phosphorylation (Figure 5, inset). Thus, the PKA responsive element of cardiac titins may allow modulation of diastolic function. We speculate that when β -adrenergic stimulation enhances the heart beat frequency and speed of contraction and relaxation, the reduced titin force resulting from N2B phosphorylation allows for more complete ventricular filling.

The direct effect of calcium on the force-extension curve of the PEVK segment was also recently studied.³⁸ Recombinant PEVK fragments were used in which the two conserved PEVK elements (E-rich motifs and PEVK repeats) were represented. Mechanical experiments were performed at the level of the single molecule. Results revealed that calcium-induced conformational changes in the E-rich motif (exon 129) increase the flexibility of the molecule, as reflected by the reduced persistence length.³⁸ To test whether these findings have physiological significance, the effect of calcium on titin-based tension was studied in single skeletal

muscle fibers (mouse soleus was chosen because it contains 9 E-rich motifs). Results revealed that titin-based tension measured during both a rapid and slow stretch is increased by calcium.³⁸ This increase is consistent with the calcium-induced reduction in persistence length of single molecules (for details, see Kellermayer et al³⁹). Additional titin-based force in the presence of calcium may play a role in maintaining the central A-band location in the sarcomere, especially at the beginning of activation when nonuniformity of active force development may be high.⁴⁰

Differential splicing events control the PEVK segment composition, with the N2B cardiac titin isoform containing solely PEVK repeats and N2BA cardiac and skeletal muscle titin isoforms containing a variable number of PEVK repeats and E-rich motifs.^{25,27,31,41} We predict that N2B cardiac titin is not calcium responsive (absence of E-rich motifs), whereas N2BA cardiac titin is calcium responsive (E-rich motifs are present). Thus, by splicing in certain PEVK exons and excluding others, unique molecules may be constructed, some of which have mechanical properties that are calcium sensitive and can thereby adapt to the physiological state of the cell.

How these various rapid adjustment mechanisms of titin's mechanical behavior are represented in the various titin isoforms and their physiological significance requires further investigation. Because the N2B element is included in all cardiac titin isoforms, its phosphorylation and ensuing extension is predicted to reduce passive stiffness of both N2B and N2BA titins, but with a much larger magnitude in the N2B isoform. (The extensible region is shortest in N2B titin and the phosphorylation-induced extension of the N2B element will therefore have the greatest impact on the fractional extension of the extensible region of this isoform). Thus, β -adrenergic stimulation is predicted to have the largest effect on diastolic stiffness when N2B expression dominates. Titin's two other rapid adjustment mechanisms (actin binding and calcium binding) involve the PEVK region, and due to differential splicing of this region, these adjustment mechanisms will not be equally represented in different isoforms. As discussed earlier, the N2B PEVK may not bind calcium (E-rich exons are absent), but instead may interact with actin, giving rise to elevated passive stiffness that can be diminished with Ca/S100. The N2BA PEVK, on the other hand, may not interact with actin but instead increase passive stiffness by binding of calcium.

The overall picture that emerges is that expressing high levels of N2B titin results in high baseline passive stiffness due to the short extensible region of this isoform, augmented by titin-actin interaction, and that phosphorylation and Ca/S100 can lower this high baseline stiffness. In contrast, expressing high levels of N2BA results in a low baseline passive stiffness and calcium binding can elevate this low stiffness. These hypotheses are depicted in Figure 6. Future work is required to more fully elucidate the array of rapid adjustment mechanisms represented in titin's various isoforms as well as to assess their full physiological role in the heart.

Relative Contribution of Titin to Passive Myocardial Tension

In addition to titin, the extracellular matrix protein collagen also contributes to passive myocardial tension. Toward the upper

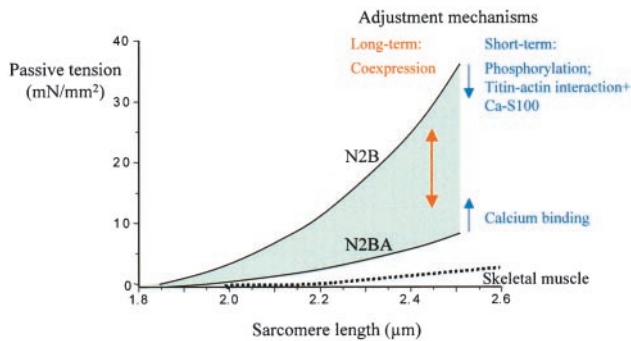


Figure 6. Passive tension/sarcomere length relations of cardiac myocytes that predominately express N2B titin and those that predominately express N2BA titin. Coexpression of titin isoforms at variable ratios allows intermediate passive tensions (double-headed arrow) as a long-term passive stiffness adjustment mechanism. We postulate that short-term adjustment mechanisms differentially impact the isoforms, decreasing stiffness of N2B titin and increasing stiffness of N2BA titin.

limit of the SL range with reversible passive myocardial mechanics, titin and collagen contribute about equally to passive tension, whereas at shorter SLs, titin's contribution dominates⁴² (Figures 7A through 7C). Furthermore, when titin-based passive tension is high due to high expression levels of N2B titin, collagen-based passive tension is high as well, suggesting that titin and collagen vary in a coordinated fashion⁴² (Figures 7A through 7C).

Considering that collagen develops low passive tension at short to intermediate SLs and high tension at long SLs, collagen's most important mechanical role may be limiting the end-diastolic volume of the heart. Titin-based tension increases more gradually with SL and contributes also to passive tension at short-to-intermediate SLs, where collagen contributes little. At lengths below the slack SL, titin develops restoring force and thus can contribute to the elastic recoil that underlies early diastolic filling. The high passive stiffness provided by both collagen and titin in species that express high levels of N2B titin may allow rapid early diastolic filling (due to titin's high restoring force) and stable determination of the end-diastolic volume (due to titin's and collagen's passive tension). Thus, collagen and titin are both important in determining the diastolic myocardial stiffness and appear to play complementary roles tuned to the operating environment of contraction and relaxation cycles at different heart rates.

Effect of Titin on Active Force Development

Titin is usually regarded as playing no role in active force development. However, recent studies suggest that titin may affect the SL-dependent increase of Ca^{2+} sensitivity of active force, an important factor in the Frank-Starling mechanism of the heart. Measuring force-pCa relations of cardiac myocytes at different levels of passive force, achieved by modulating the prehistory of stretch, has revealed that Ca^{2+} sensitivity increases in proportion to titin-based passive force.⁴³ Furthermore, titin degradation with a mild trypsin treatment was shown to decrease Ca^{2+} -activated maximal force of rat trabeculae at SLs longer than the slack length,⁴⁴ and to depress the SL-dependent increase in Ca^{2+} sensitivity of rat cardiac myocytes.⁴⁵ Finally, a compar-

ison of length-dependent activation in myocardial specimens with different passive force levels due to differences in titin isoform expression revealed that the SL-dependent increases in Ca^{2+} sensitivity of force and maximal active force were most pronounced in myocardium with high titin-based passive force.⁴⁶

Titin may affect active force by altering the likelihood of actomyosin interaction via either modulating interfilament lattice spacing or straining the thick filament and thereby influencing cross-bridge mobility (for a detailed discussion, see Cazorla et al⁴³ and Fukuda et al⁴⁴). The notion that titin's A-band region may affect actomyosin interaction is supported by the observation that addition of A-band titin fragments to skinned cardiac myocytes increased calcium sensitivity in slack sarcomeres but not in stretched sarcomeres.⁴⁷ This suggests that in slack sarcomeres, titin has an inhibitory effect on crossbridge interaction that can be alleviated by either increasing sarcomere length or by adding A-band titin fragments.⁴⁷ The proposal that titin can inhibit crossbridge interaction with a higher degree of inhibition in slack sarcomeres than in stretched sarcomeres was recently extended by Helmes et al.⁴⁸ These authors proposed that titin's restoring force, generated below slack length, further inhibits actomyosin interaction and that this may be a factor in rapidly abolishing contraction early in diastole. The proposition that titin can depress contractility in muscle shortened below slack length and enhance contractility when stretched beyond slack warrants further experimental testing. In summary, several lines of evidence suggest that titin is not just a passive spring but that it may also play a role in the Frank-Starling mechanism of the contracting heart.

Role in Signaling: Titin-Based Protein Complexes as Biomechanical Sensors

Recent studies identified many titin ligands that were hitherto unknown components of the sarcomere (Figure 1 and Table). Interestingly, many of these ligands not only bind titin but are also found in the nucleus (see later), suggesting that they are part of interaction pathways between titin and the nucleus. Furthermore, protein binding sites are not randomly distributed along the titin filament but instead seem restricted to "hot spots": one found in and near the Z-disc, another in the central I-band region, and a third in the M-line region of the molecule.

Titin-Associated Z-Disc Proteins: Structural and Signaling Functions

Immunoelectron microscopy has demonstrated that the titin filament fully penetrates the Z-disc,⁴⁹ thereby placing the titin-capping protein T-cap (or telethonin⁵⁰) toward the periphery of the Z-disc lattice of the adjacent sarcomere.⁴⁹ T-cap functions as a versatile adaptor protein that links signaling and structural molecules to titin (Table). T-cap interacts with the cytoplasmic domains of two membrane-associated proteins: the potassium channel subunit minK/isk⁵¹ and the small ankyrin-1 (sANK1),⁵² found in the T-tubules and the sarcoplasmic reticulum (SR), respectively. Possibly, T-cap's interaction with minK anchors the T-tubules close to the Z-disc region of the sarcomere and may regulate potassium channel function in response to myocyte stretch. sANK1 is a transmembrane protein of the SR and its

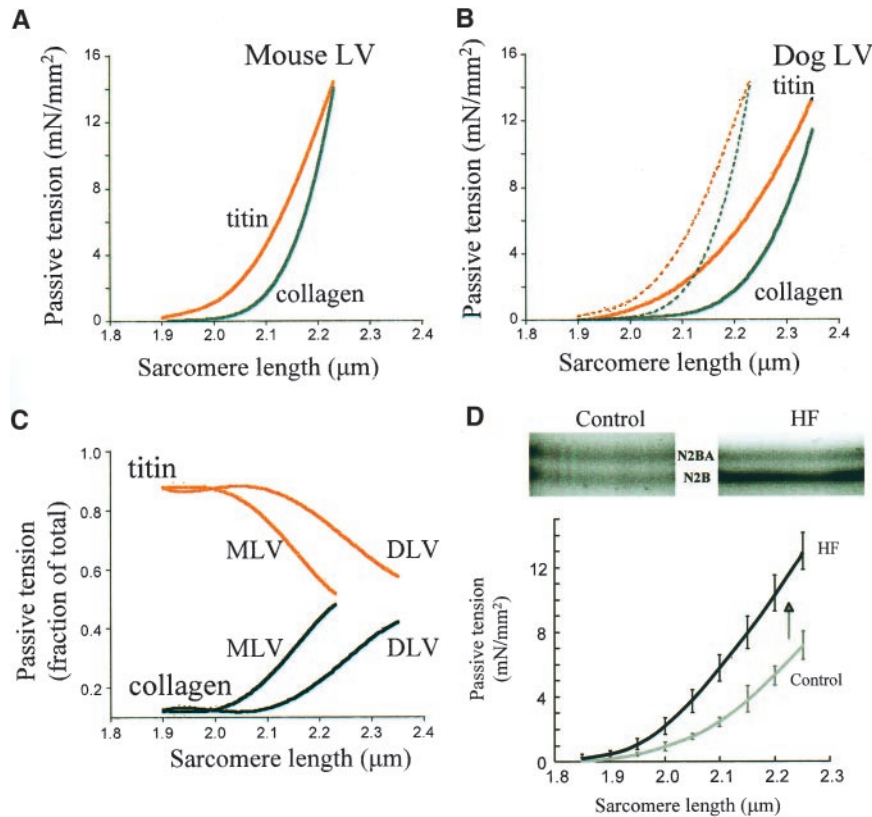


Figure 7. A through C, Contribution of titin and collagen to passive myocardial tension of mouse LV (MLV) and dog LV (DLV). A, Absolute tensions of MLV. B, Absolute tensions of DLV (broken lines reproduce MLV tensions). Both collagen-based and titin-based tensions are much higher in MLV. C, Tensions as fraction of total tension. Within the SL range studied, titin is the main contributor to passive tension (results from Wu et al^{34,42}). Reproduced from Wu Y, Bell SP, Trombitas K, Witt CC, Labeit S, LeWinter MM, Granzier H. Changes in titin isoform expression in pacing-induced cardiac failure give rise to increased passive muscle stiffness. *Circulation*. 2002;106:1384–1389, and Wu Y, Cazorla O, Labeit D, Labeit S, Granzier H. Changes in titin and collagen underlie diastolic stiffness diversity of cardiac muscle. *J Mol Cell Cardiol*. 2000;32:2151–2162, by permission of the American Heart Association ©2002 and Elsevier ©2000, respectively. D, SDS-PAGE of left ventricular myocardium of control dog and of dog with heart failure (HF) due to 4-week rapid pacing. N2B titin is upregulated in HF animal. Bottom, Titin-based passive tension–sarcomere length relations in control and HF animals. Titin-based passive tension is significantly elevated in HF animals (based on Wu et al³⁴). Reproduced from Wu Y, Bell SP, Trombitas K, Witt CC, Labeit S, LeWinter MM, Granzier H. Changes in titin isoform expression in pacing-induced cardiac failure give rise to increased passive muscle stiffness. *Circulation*. 2002;106:1384–1389, by permission of the American Heart Association ©2002.

interaction with titin's N-terminus may position the SR around the Z-disc of each sarcomere.⁵² A further role for titin in organizing the SR is suggested by the interaction between titin's near Z-disc domains Z9-Z10 and obscurin.⁵³ Obscurin contains signaling domains^{25,53,54} and interacts with the ankyrin isoform 1.5; this ankyrin isoform appears to link the SR to the sarcomere and to regulate ryanodine receptor distribution in the SR.⁵⁵ These interactions with titin's Z-disc region may be involved in positioning the SR and T-tubular membrane systems in close proximity to the I-band region of the sarcomere. Furthermore, it also ensures that these membrane systems move with the Z-disc, thereby preventing excessive strains, which possibly could tear membranes when the sarcomeres shorten during contraction. Thus, during contraction-relaxation cycles, titin maintains the structural organization of the sarcomere (by keeping the A-band in a central location, see above) as well as the organization of the SR and T-tubular systems.

T-cap also interacts with calsarcin-3⁹⁵ and the muscle growth factor myostatin. In C2C12 cells, T-cap inhibits myostatin secretion and myogenic differentiation.⁵⁶ A similar

role for T-cap is also suggested by its interaction with muscle LIM protein (MLP, also known as CsRP3), an essential nuclear regulator of myogenic differentiation.⁵⁷ Nuclear MLP is thought to play a role in promoting myogenesis through an interaction with the MEF family transcription factor, MyoD, enhancing the ability of MyoD to bind DNA.⁵⁸ Interestingly, stretching of cultured cardiac myocytes induces expression of the well-known stretch response markers, brain natriuretic peptide (BNP), and atrial natriuretic factor (ANF), but this response is absent in MLP^{-/-} mouse cardiac myocytes,⁵⁹ suggesting that MLP could be involved in stretch sensing.

The exact stretch sensing mechanism is unclear. It is worthwhile to highlight that the Z-disc forms an intricate lattice⁶⁰ and that the Z-disc lattice spacing (spacing between thin filaments in the Z-disc) is not static, but instead is responsive to both actomyosin-based tension⁶¹ (transmitted to the Z-disc via the thin filaments) and titin-based tension (Granzier and Irving, unpublished data, 2004). Considering the high number of Z-disc proteins, it seems possible that a reduced Z-disc lattice spacing after sarcomere stretch impacts

Titin-Binding Ligands, Binding Partners, and Proposed Roles

Ligand	Binds to Titin Domains (Exons)	Additional Binding Partner(s) of Ligand	Proposed Role(s)
T-cap/teletonin ⁴⁹	Z1-Z2 (2–4)	MLP, ⁵⁹ myostatin ⁵⁶ isk/minK, ⁵¹ sANK ^{–52} Calsarcins ⁹⁵	Regulation of gene expression; (differentiation) and stretch sensing Structural organization of SR and T-tubular system; regulating K ⁺ channel function? ZASP-dependent signaling?
sANK ^{–52}	Z1-Z2 (2–4)	T-cap	Structural organization of the SR
Obscurin ^{25,53}	Z9–10 (28)	Ankyrin-1.5 Novex-3 titin	Structural organization of the SR Stretch-dependent signaling?
$\alpha\beta$ -Crystallin ⁶⁶	N2B (49–50?)		Chaperone for protein refolding; control of differentiation-apoptosis?
DRAL/FLH-2 ⁶⁵	N2B (49) M-line titin is-2 (358)	MM-CK, AK, PFK	Compartmentalization of metabolic enzymes
CARP ⁶⁸	Ig80–81 (103/104)	YB-1	Regulation of gene expression
Ankrd2, DARP ⁶⁸	Ig80–81 (103/104)	Myopalladin ⁶⁹	Cytokine-like response to stress, ankrd2: stretch-dependent? DARP: metabolic? Sarcomeric integrity
p94/calpain-3 ⁶⁷	Ig82–83 (105–108) Is-7 (362) ⁷⁸		Protein degradation
MURF-1 ^{80,81}	A168–170 (357–358)	MURF-2 MURF-3 GMEB1	Regulation of gene expression; muscle trophic versus atrophy

the binding affinity of T-cap’s binding partners (perhaps due to steric hindrance). The Z-disc is also connected to the sarcolemma, at T-tubules (see earlier) and costamers (some of these interactions involve MLP). Furthermore, as the width of the myocyte changes during contraction (the cell functions as a constant volume system), and as transmural pressures change, tension transmitted from the sarcolemma to the Z-disc will be altered and this may also change binding affinities for Z-disc–based proteins within the altered Z-disc lattice. This hypothesis is consistent with the redistribution of MLP to nuclei in response to pressure overload in mice.⁶² Exposure to nonphysiological biomechanical signals contributes significantly to the progression of diseases such as DCM and HCM (see reviews^{63,64}), and current insights point to a role for the Z-disc region of titin and its various ligands in strain/stress sensing.

Central I-Band–Based Signaling

The N2B and N2A elements, located in the central I-band region, constitute hotspots for interactions with signaling molecules. The cardiac-specific N2B element interacts with a member of the LIM protein family known as DRAL/FHL-2.⁶⁵ DRAL/FHL-2 in turn binds the metabolic enzymes creatine kinase (MM-CK), adenylate kinase (AK), and phosphofructokinase (PFK).⁶⁵ Thus, the N2B element may play a role in the compartmentalization of metabolic enzymes, ensuring generation of ATP close to the overlap region of the sarcomere, where high levels of ATP are consumed during contraction.

The N2B element also interacts with $\alpha\beta$ -crystallin,⁶⁶ a member of the small heat shock protein family that functions as chaperones that maintain the folded state of proteins. In heart muscle, $\alpha\beta$ -crystallin participates in the ischemic stress

signaling response and possibly protects titin from structural damage under conditions of heightened vulnerability.

The N2A element is found in both skeletal muscle titins and cardiac N2BA titins. Two of its Ig domains (Ig82/83) interact with the calpain protease P94, also known as calpain-3.⁶⁷ P94 is involved in protein degradation, and binding to titin is thought to regulate P94 activity and fine-tune its functions.⁶⁷ A recent search for additional N2A ligands identified a tyrosine-rich binding motif between Ig80 and Ig81 that interacts with a conserved motif present in the three homologous muscle ankyrin repeat proteins: cardiac ankyrin repeat protein (CARP), and the two closely related proteins, ankrd2 (or Arpp) and diabetes ankyrin repeat protein (DARP).⁶⁸ CARP also interacts with myopalladin, a \approx 145-kDa protein found in the sarcomere and nucleus.⁶⁹ All three ankyrin repeat proteins were identified previously by their cytokine-like induction after cardiac injury and muscle denervation (CARP),^{70–72} skeletal muscle stretch (ankrd2/Arpp),⁷³ or during recovery after metabolic challenge (DARP),⁷⁴ suggesting that they are part of muscle stress response pathways. These pathways can be turned on rapidly as suggested by the observation in cultured cardiac myocytes that CARP’s expression level and localization in the cell are responsive to brief (\approx 90 minutes) periods of physiological levels of stretch.⁶⁸

Remarkably, all I-band ligands of titin and their associated binding partners are also found in the nucleus where they participate in transcriptional and cell cycle regulation. Notably, CARP exerts transcriptional control by interacting with the transcriptional regulator YB-1.⁷⁵ It seems likely that this dual localization (I-band and nucleus) is not a mere coincidence but instead reflects a dual function for these proteins:

being part of a titin-based stretch sensing complex in the I-band and regulating transcription in the nucleus. Furthermore, such dual localization may also provide a communication pathway between the I-band and nucleus that links stretch sensing to gene expression. For example, an increased titin-binding affinity (due to sarcomere stretch?) of a certain titin ligand would be expected to reduce the nuclear pool of the ligand, and this in turn could initiate changes in gene expression. Clearly much more work is needed to establish mechanisms of titin-based stretch sensing and the ensuing signaling pathways. The findings of CARP upregulation in numerous *in vivo* models of pressure and volume overload⁷⁶ highlight the clinical importance of this work.

M-Line–Based Signaling

The M-line region of titin contains a conserved serine/threonine kinase domain, phosphorylation motifs,⁷⁷ a second binding site for P94,⁷⁸ a second binding site for DRAL/FHL-2,⁶⁵ possibly additional binding sites for T-cap and obscurin,^{53,79} and a binding site for the RING finger protein MURF-1.⁸⁰ Analogous to the Z-disc protein T-cap, MURF-1 is a multifunctional adaptor protein that may function in the regulation of gene expression (MURF-1 interacts with the steroid regulated transcriptional activator GMEB1, see McElhinny et al⁸¹) and in protein turnover by acting as an ubiquitin ligase.⁸² MURF-1 interacts with the two titin Ig domains, A169/170, located at the M-line periphery, and can form heterodimers with the homologous proteins MURF-2 and MURF-3.⁸⁰ Both MURF-2 and MURF-3 also bind microtubules and this binding seems to regulate microtubular stability.^{83,84}

Mouse Models Indicate Crosstalk Between Signaling Hotspots

Several mouse models with genetic alterations that affect titin-based signaling have become available recently. As briefly discussed earlier, a MLP KO mouse model revealed impairment in sarcomere stretch sensing, suggesting that this could be at the basis of the dilated cardiomyopathy (DCM) phenotype of these animals, a conclusion supported by the findings of a MLP mutation in a subset of human patients with DCM.⁵⁹

The molecular mechanisms of I-band–based signaling have been studied in the naturally occurring *mdm* (muscular dystrophy with myositis) mouse. In this mouse, 4 small titin exons coding for 83 amino acids are excised from the N2A element, resulting in death at about 6 to 8 weeks of age.⁸⁵ A recent gene expression study⁸⁶ revealed striking early changes in a subset of genes, including MLP, CARP, and MURF-1, showing that the deletion of this small portion of the N2A element simultaneously affects titin ligands in the Z-disc (MLP), I-band (CARP), and M-line (MURF-1).

For the study of M-line–based titin signaling, two mouse models have become available. Bodine et al⁸² inactivated the *MURF-1* gene and showed that this increases the resistance of skeletal muscles to muscular atrophy. This is consistent with a striking up-regulation of MURF-1 in atrophied muscle (due to denervating muscles or exposure to zero-gravity), suggesting an important role for MURF-1 in regulating protein turnover.^{81,82} Gotthardt and colleagues⁸⁷ directly modified the M-line region of the mouse titin gene using a conditional

knockout approach that deleted the M-line exons 358/359 (which encode the kinase domain and MURF-1 binding site). Excision during late embryonic development (using the MCK promoter) allows the mice to survive at birth, but causes progressive myopathy, resulting in death at 5 weeks. Myopathic changes include pale M-lines devoid of MURF-1 and upregulation of CARP and novel genes. Upregulation of CARP in the kinase KO model and of MURF-1 and MLP in the *mdm* model suggests crosstalk between the three signaling hot spots along the titin filament. Interestingly, several *in vitro* studies suggest that the same titin ligand may interact with titin at different locations (Table): P94 and DRAL/FLH-2 bind to titin's N2A element and the M-line region^{65,78}; T-cap/telethonin interacts with titin's N-terminus⁴⁹ and is also phosphorylated *in vitro* by titin's kinase domain, which is located at titin's C-terminus⁷⁹; and finally, MURF-3 and obscurin have been detected both within the Z-disc and the M-line regions of the sarcomere.^{53,83} These *in vitro* findings together with those from the mouse models warrant further studies on crosstalk between physically distinct titin-based protein complexes, and how it may be involved in muscular dystrophy.

Why Multiple Titin–Based Signaling Pathways?

As we discussed earlier (see section “Titin-Associated Z-disc Proteins...”), the Z-disc–based titin ligands may receive biomechanical input not just from titin, but also from actomyosin-based tension, and tensions that are communicated via the sarcolemma (that can be derived from cell shortening or pressure exerted on the LV wall). Thus, the Z-disc ligands are likely to integrate a variety of biomechanical signals. Considering that the thick filament backbone can be slightly stretched by both actomyosin-based tension^{88,89} and titin-based passive tension,^{89a} titin's M-line signaling complex is likely to receive mechanical input from titin and actomyosin interaction. Finally, the I-band region of titin is independent of actomyosin-based force and mechanical input to the I-band sensing complex is likely to be purely titin-based. Thus, the Z-disc, M-line, and I-band signaling hotspots along titin are expected to all sense titin-based tension- (which reflects sarcomere stretch), the Z-disc and M-line to sense in addition also actomyosin-based tension, and finally, the Z-disc sensing complex to also receive input from tensions communicated via the sarcolemma. Thus, titin's multiple signaling pathways may allow sensing of a variety of signals with graded responses.

Hereditary Titin Diseases

Due to its enormous size and multiple functions, titin is a prominent target for mutations that give rise to muscle disease. Knowledge of titin's genomic structure²⁵ has facilitated the search for titin mutations (for primer pairs and PCR conditions to survey all 363 titin exons, see Gerull et al⁹⁰ and www.titin-oligos.de; for exons with identified mutations, see Figure 3). Sequencing the entire titin gene in DCM families⁹⁰ revealed DCM-causing mutations in titin exons 18 and 326. The titin mutation in exon 326 is predicted to cause a frameshift resulting in truncated titin (≈ 2000 kDa). Western blot analysis revealed that a truncated titin is indeed expressed in skeletal muscle but of a size smaller than expected

(≈ 1100 kDa), suggesting that the truncated protein is sensitive to proteolysis. Exon 18 encodes an Ig domain and the identified mutation in this exon is predicted to disrupt its native structure,⁹⁰ underscoring the importance of maintaining Ig domains in their folded state. The skeletal muscular dystrophy TMD (tibialis muscular dystrophy) is a genetic muscle disease of dominant inheritance (common in Finland, but also found elsewhere). Sequencing of the entire titin gene in TMD families⁹¹ revealed a mutation in exon 363. This exon codes for an Ig domain and its mutation is predicted to destabilize the folded state of this domain. Finally, a recent large sequencing project⁹² identified mutations in exons 2 and 14 (Z-disc) and 49 (N2B element) in HCM patients. Protein–protein interaction studies showed that the two identified missense mutations in exons 2 and 14 lower titin’s affinity for T-cap and α -actinin, respectively, implicating impairment/perturbation of the interaction between Z-disc titin and its ligands as a potential disease mechanism.⁹² Although these sequencing studies are recent and few, and thus, the frequency of genetic titin diseases remains to be established, the work by Kimura and colleagues⁹² raises the possibility that up to several percent of all cardiac diseases include titin mutations.

Most titin mutations identified thus far are in regions of titin that are expressed in all striated muscles. Surprisingly, not all muscles are affected identical. For example, exon 362 is expressed in all muscles, but the TMD mutation in this exon affects selectively only the extensor muscles of the frontal leg compartment, whereas adjacent muscles are spared.⁹¹ Similarly, the two mutations identified in DCM are expressed in all titin isoforms but there is no clinically detectable phenotype in skeletal muscle.⁹⁰ These muscle type–specific phenotypes underscore the need to study titin’s multiple functions in different muscles, under both normal and pathological conditions.

Acquired Titin Diseases

To understand how titin responds to chronic mechanical challenge of the heart, a canine tachycardia-induced model of DCM has been used in which rapid pacing results in chamber dilation and elevated chamber stiffness.³³ Two weeks of pacing gives rise to an exaggerated transmural titin isoform ratio gradient³³ and 4 weeks of pacing results in elevated N2B titin expression and downregulation of N2BA titin, accompanied by increased titin-based passive stiffness³⁴ (Figure 7D). A recent study of the spontaneously hypertensive rat model (SHR) has shown a reduced expression of N2BA titin in response to pressure overload, consistent with elevated passive stiffness of SHR cardiac myocytes,⁹³ whereas in patients with CAD (coronary artery disease), an increase of the more compliant N2BA cardiac titin isoform occurs.⁹⁴ Thus, processing of the titin pre-mRNA is subject to subtle regulatory mechanisms that control entry to either N2B or N2BA splice pathways, and a range of adjustments can occur, leading to either increased or decreased passive stiffness. Considering that in the canine rapid pacing model, titin and collagen-based passive stiffness change in an apparently coordinated fashion,³⁴ it will be interesting to learn whether regulation of titin-based myocardial stiffness (ie, N2B/N2BA isoform type composition) and collagen-based stiffness (ie,

collagen content and type I/III composition) can be coordinated by common upstream signaling pathways.

Summary

Progress in understanding the breadth of titin’s functions has been rapid during the last several years. Several titin-based mechanisms of passive stiffness adjustment that operate on different time scales have been discovered. Understanding how these mechanisms are represented in different titin isoforms and their full physiological significance is an outstanding challenge. Titin-based stiffness adjustments have been reported to occur in various muscle diseases and, furthermore, several disease-causing titin mutations have been discovered. Recent work also indicates that titin may influence active force development by depressing contractility in muscle shortened below slack length and enhancing contractility when stretched beyond slack. In addition, titin may play a role in protein metabolism (by regulating ubiquitination and calpain activity), compartmentalization of metabolic enzymes (binding of DRAL/FHL-2), and positioning of the membrane systems of the T-tubules and sarcoplasmic reticulum (binding of sANK1 and obscurin). Titin may also act as a sarcomere stretch sensor that underlies length-dependent signaling processes in the cardiac myocyte. The last few years have seen great progress in establishing many of the players involved in titin-based sensing and signaling and has identified Z-disc, I-band and M-line signaling hotspots in titin. This work provides a sound basis for proposing detailed hypotheses for how titin may sense stretch and initiate signaling. The various mouse models that have become available with genetic alterations that affect titin-based signaling, will allow testing of a range of hypotheses. It may be anticipated that this will lead to a deeper understanding of the multiple roles of titin in muscle function and disease.

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