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Cardiac Titin and Heart Disease

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Abstract

The giant sarcomeric protein titin is a key determinant of myocardial passive stiffness and stress sensitive signaling. Titin stiffness is modulated by isoform variation, phosphorylation by protein kinases and possibly oxidative stress through disulfide bond formation. Titin has also emerged as an important human disease gene. Early studies in patients with dilated cardiomyopathy (DCM) revealed shifts toward more compliant isoforms, an adaptation that offsets increases in passive stiffness based in the extracellular matrix. Similar shifts are observed in heart failure with preserved ejection fraction (HFpEF). In contrast, hypophosphorylation of PKA/G sites contributes to a net increase in cardiomyocyte resting tension in HFpEF. More recently, titin mutations have been recognized as the most common etiology of inherited DCM. In addition, some DCM-causing mutations affect RBM20, a titin splice factor. Titin mutations are a rare cause of hypertrophic cardiomyopathy (HCM) and also underlie some cases of arrhythmogenic right ventricular dysplasia. Finally, mutations of genes encoding proteins that interact with and/or bind to titin are responsible for both DCM and HCM. Targeting titin as a therapeutic strategy is in its infancy, but could potentially involve manipulation of isoforms, post-translational modifications, and up-regulation of normal protein in patients with disease causing mutations.

Keywords

titin; cardiomyopathy; myocardium; genetics; heart failure

Introduction

Titin is a giant sarcomeric filament whose first described function was provision of passive stiffness to cardiac myocytes. Over the last decade it has become apparent that titin has a number of other functions and plays a major role in heart disease.

At its N-terminus, titin is embedded in the Z-disk of the sarcomere. The rest of the protein is divided into an elastic I-band region, a thick filament-binding A-band region and the M-band region where the C-terminus is embedded (Fig. 1A, bottom)[1]. The I-band region is a molecular spring that develops passive force during diastole when sarcomeres are stretched[2].

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Together with the extracellular matrix (ECM) this force defines passive myocardial stiffness[2]. Titin is also largely responsible for elastic recoil of the cardiac myocyte[3], which contributes to diastolic suction, an important mechanism of left ventricular (LV) filling. Recoil occurs when the myocyte contracts below slack length. With relaxation the titin-based spring recoils and the sarcomere returns to slack length. Finally, titin also facilitates length-dependent activation[3], a key determinant of the Frank-Starling relation. Other regions of titin (Z-disk, A-band, and M-band) are involved in various cellular processes including force-dependent signaling[4].

In this review, we discuss post-transcriptional and post-translational modifications of titin and address their roles in acquired disease, including dilated cardiomyopathy (DCM) and heart failure with preserved ejection fraction (HFpEF) (often termed diastolic heart failure). We also focus on recent work establishing mutations in the titin gene (TTN) as a major source of familial cardiomyopathies, including those in the spring region linked to arrhythmogenic right ventricular dysplasia[5] and in the A-band region responsible for ~30% of cases of DCM[6].

Titin isoforms and heart disease

The extensible I-band region of titin is comprised of three elements: 1) tandem Ig segments consisting of serially-linked immunoglobulin (Ig)-like domains, 2) the spring like PEVK segment (with high percentages of proline, glutamic acid, valine, and lysine), and 3) the N2B element with its extensible unique sequence (N2B-Us) (Fig. 1A, middle)[7, 8]. Titin is encoded by a single gene; variable splicing results in distinct isoform classes[8]. In cardiac muscle three classes are present: adult N2BA, adult N2B, and fetal cardiac titin (FCT)(Fig. 1A middle)[8–10]. These classes differ in their I-band region; the rest of the molecule is largely identical[8]. The tandem Ig segments can be visualized as “beads on a string” with folded Ig domains(diameter 4–5 nm) separated by short peptide linkers[11]. All isoforms contain a proximal tandem Ig segment (Ig1-15) and a distal tandem Ig segment (Ig84-105)[7, 8]. N2BA and FCT isoforms also contain a middle tandem Ig segment with a variable number of Ig domains (Fig. 1A, middle)[9]. The N2B element is found in all isoforms.

In addition to its spring function, N2B-Us is a substrate for various kinases that affect its mechanical properties (see below). As noted, the N2B element is found in all cardiac isoforms, whereas the N2A element is only found in N2BA and FCT isoforms[7, 8]. Similar to N2B, the N2A element contains Ig domains and a unique sequence; the N2A unique sequence binds calpain protease p94 and proteins belonging to the muscle ankyrin-repeat protein family (MARPs), which relocate from the sarcomere to the nucleus to regulate transcription following mechanical stress[2]. Like N2B-Us, the PEVK region is also a molecular spring[12]. PEVK is encoded by 114 exons, seven of which are found in the N2B isoform. The PEVK region of N2BA titin contains additional exons and is much larger[13]. Inclusion of additional Ig domains, the PEVK sequence and the N2A element make the N2BA isoform larger than the N2B isoform (~3.3 MDa vs. 2.97 MDa)[8]. FCT (3.5–3.6 MDa) contains the largest middle tandem Ig segment and the largest PEVK sequence of all titin isoforms[9].

The force required to stretch titin depends non-linearly on its fractional extension[14]. At a given SL the absolute extension of N2B and N2BA titin is the same. However, since N2B titin is shorter its fractional extension is greater[15]. Therefore, more force is needed to stretch the N2B isoform, i.e., it is stiffer because it is shorter. Thus, sarcomeres that express different titin isoforms develop highly variable levels of passive tension(Fig. 1B).

Adult cardiac muscle co-expresses N2B and N2BA titin at the level of the half-sarcomere[16]. The number of molecules per thick filament appears constant (6 per half thick filament) but the expression ratio of N2BA to N2B titin is variable (in normal human LV ~40:60[17]). Because of the close relationship between the size of the I-band region and titin-based passive tension, the isoform ratio is a critical determinant of titin-based passive tension (Fig. 1B).

Altered titin isoform expression ratios have been reported in patients with heart disease. Patients with ischemic cardiomyopathy express increased amounts of N2BA titin, accompanied by reduced myofibrillar stiffness[17]. A similar shift has been found in end-stage HF due to non-ischemic cardiomyopathy, and is associated with reduced passive myocardial stiffness and increased chamber compliance[17–19]. Nagueh et al[18] suggested a physiological benefit of this change in isoforms by correlating the shift with improved exercise tolerance. Upregulation of compliant isoforms has also been found in patients with HFpEF, a group accounting for about half of all HF cases and characterized by increased diastolic stiffness[20,21]. Interestingly, an increase in expression of compliant isoforms occurs in mice with pathological hypertrophy[22] and rats with hypothyroidism[23]. Together, these studies suggest that up-regulation of the N2BA isoform is compensatory, counteracting the increased stiffness of the extracellular matrix.[18]

The mechanisms underlying changes in titin isoform expression are poorly understood. The recent discovery of the splice factor RBM20[24] is a possible breakthrough. Naturally occurring RBM20 mutations in patients and a rat model result in low RBM20 levels and expression of large, highly compliant titin isoforms[24]. Thus, reduced expression of RBM20 in cardiac disease may underlie upregulation of compliant titin isoforms. More work is needed to understand mechanisms of titin isoform expression, with a focus on RBM20 and whether experimentally upregulating compliant isoforms can ameliorate increased myocardial stiffness in HF.

Post-translational modifications and disease

Post-translational modifications (PTMs) of contractile and regulatory proteins have major effects on cardiac function. Recently, single molecule force spectroscopy has revealed that the extensible region of titin is phosphorylated (Fig. 1A, top), altering the stiffness of PEVK and N2B-Us spring elements. This allows for rapid adjustment of titin stiffness (Fig. 1B) and adaptations of cardiac performance to meet hemodynamic loads.

The PEVK spring element is phosphorylated by protein kinase Cα (PKCα)(Fig. 1A top). PKC is activated by the α1-adrenergic signaling pathway. PKCα, the predominant isozyme in heart, is a key player in contractile dysfunction and heart failure[25,26]; PKCα phosphorylation of the PEVK element leads to *increased* passive tension.[27] The primary

phosphorylation sites are two highly-conserved serines (S26 and S170).[27] Phosphorylation of these residues reduces the bending rigidity of the PEVK region[28], which is consistent with increased passive tension in response to PKC phosphorylation[27]. The link between PKCα, PEVK phosphorylation and passive tension was further established by a study showing that PKCα had no effect on passive tension in mice with genetically removed PEVK sites[29].

The N2B element is also a kinase substrate. Both protein kinase A (PKA), stimulated by the β-adrenergic pathway, and PKG, a cGMP-dependent kinase that is part of signaling cascades initiated by nitric oxide (NO) and natriuretic peptides (NPs), phosphorylate S469 within the unique sequence of the N2B element and *reduce* passive tension in cardiac myocytes[30,31,32](Fig. 1A,B). Amore pronounced effect is observed when PP1 de-phosphorylation is performed prior to PKA treatment; thus, basal phosphorylation level plays an important role in determining passive tension.

Whether basal PKA/PKG phosphorylation of titin is altered in cardiac disease has been addressed in several studies. Comparing end-stage DCM patients with non-failing donor hearts revealed a trend towards reduced basal phosphorylation of PKA sites.[32] A study employing endomyocardial biopsies also provided evidence for hypo-phosphorylation in patients with HFpEF and DCM; mechanical experiments revealed increased passive tension of cardiac myocytes that was partially normalized after PKA/PKG treatment.[20] However, passive tension was not fully normalized by PKA phosphorylation (by itself, the aforementioned shift toward N2BA titin in HFpEF would be expected to result in *lower* passive tension than in controls[20,21]). Higher passive tension despite normalization of PKA/PKG phosphorylation levels could be explained by a change in basal phosphorylation of PKCα sites in the PEVK spring, a possibility that remains to be investigated. This idea was supported by a study in mice with increased after-load induced heart failure where PEVK S26 was hyper-phoshorylated relative to sham controls and PP1 treatment normalized phosphorylation level as well as passive tension.[22]

A recently discovered phosphorylation pathway involves extracellular-signal-regulated kinase-2 (ERK2), which phosphorylates N2B-Us at 3 conserved serines[33](see Fig. 1A, top). It was surmised that ERK2 phosphorylation lowers titin-based passive tension, but experimental evidence for this is still required. Furthermore, ERK2 phosphorylation is inhibited by binding of the 4 and a half LIM protein 1 (FHL1) to the N2B-Us.[33] FHL1 was previously shown to bind to N2B-Us and assemble a stretch sensing signalosome consisting of components of the mitogen activated signaling pathway.[34] These findings suggest a link between stretch sensing and phosphorylation based regulation of passive stiffness. Another pathway involves CaMKII, a Ca^{2+}/calmodulin dependent serine/threonine kinase activated by increases in $[Ca^{2+}]_{in}$. Four isoforms are described (α, β, γ, and δ); CaMKIIδ is predominant in heart.[35] Hidalgo and colleagues showed that CaMKIIδ phosphorylates N2B and PEVK spring elements but not Ig domains in skinned and intact myocardium.[36,37] This find was confirmed and extended by Hamdani et al.[38] Furthermore, Hidalgo et al. showed that the phosphorylation sites overlap PKC sites (including S26 and S170 of the PEVK element, see Fig. 1A, top).[37] The effect of CaMKIIδ phosphorylation of PEVK is likely similar to that reported for PKC, i.e., increased passive tension, while phosphorylation of the

N2B element likely reduces passive tension. Western blot studies with phospho-specific antibodies suggest that N2B phosphorylation is dominant[37] a conclusion consistent with the passive tension reduction seen upon CaMKII phosphorylation of skinned myocytes[38]. Considering that the ERK2 and CaMKIIδ signaling pathways play important roles in health and disease[35,39], additional research is warranted focusing on ERK2 and CaMKII phosphorylation of titin.

The mechanical properties of N2B-Us can be altered by more than just phosphorylation. For example, the six cysteine residues in human N2B-Us have the potential to form disulfide bonds with one another, depending on oxidative state. A disulfide bond would reduce the contour length of the sequence and change its mechanical response to stretch. The effect of cysteine cross-linking was shown at both the single molecule and tissue level[40], where oxidative stress increased passive tension and hysteresis in wild-type tissue[40](Fig. 1D) but had an attenuated effect in a mouse where the N2B element was removed.[41] Studies of oxidative conditions and changes in passive tension are important since oxidative stress is elevated in HF and correlated with myocardial dysfunction.[42]

In summary, titin-based myocardial stiffness is determined by isoform composition and phosphorylation of titin's elastic I-band, with different kinases affecting titin elasticity in disparate ways. Comprehensive studies of isoform expression and phosphorylation are mandatory for determining the mechanisms by which titin stiffness changes during disease.

Titin mutations and disease

Early work revealed a small number of titin mutations in association with human cardiomyopathies.[5,43–48] We predicted that the difficulty of complete sequencing of such a large gene might be responsible for this dearth of mutations.[3] Most of the initially identified mutations were associated with DCM. Associations with hypertrophic cardiomyopathy (HCM)were rare, and included Arg740Leu, which increases titin-α actinin binding, and Ser3799Tyr, which increases four and a half LIM protein 2 (FHL2) binding.[43,45] Interestingly, mutations of genes encoding proteins that *interact* with titin, including myomesin[49], cardiac ankyrin repeat protein (ANKRD-1)[50,51], FHL2[52] and telethonin (TCAP)[53] are associated with both HCM and DCM.

Recently, using Herman et al[6] published a landmark study in which both next-generation and dideoxy sequencing were employed to sequence titin in large numbers of patients with non-ischemic DCM. They focused on mutations (nonsense, frameshift, splicing, copy number) that are likely to affect titin's full-length structure. Their results provide a much more complete picture of the frequency and nature of titin mutations and are in accord with the idea that they are indeed more common than previously understood. Specifically, they found that ~30% of DCM patients have titin mutations, whereas only 1% of HCM mutations were localized to titin. 3% of matched subjects without heart disease also had titin mutations. The penetrance of titin DCM mutations was very high in patients older than 40 years. Thus, titin mutations appear to be a rare cause of HCM (as previously thought) whereas they are by far the most common genetic cause of DCM.

Titin DCM mutations are not randomly distributed along the gene; the bulk are located in the large A band region that associates with the thick filament[6]. There were no mutations in the Z-disk or M-band regions. The A band portion of titin is thought to be critical for biomechanical sensing and signaling and contains the titin kinase domain as well as binding/interacting sites for a number of key proteins, including myomesin, the thick filament associated protein that crosslinks the thick filament with titin's C terminus, obscurin, protease calpain-3, myosin binding protein C, FHL2/DRAL, and muscle specific ring finger protein-1.[54] Since the kinase domain may play a key role in strain sensitive signaling and communications with the nucleus in conjunction with other proteins in this region, it is intriguing to consider that these mutations may result in diverse effects on gene expression and cardiac remodeling.

Absence of mutations in the Z-disk and a paucity of mutations in the I-band region could indicate that such mutations do not cause DCM or HCM (the patient populations studied[6]). Alternatively, mutations in the Z-disk and I-band regions of titin could be detrimental to sarcomere function and incompatible with life, and therefore not encountered in patient populations. Truncation in the Z-disk and I-band regions would result in proteins that cannot span to the A-band region, abolishing titin's mechanical functions. In contrast, truncations in the A-band region result in mutants that should be able to be incorporated in the Z-disk, span from Z-disk to A-band and connect to thick filament proteins. Indeed, in a mouse model that conditionally deletes titin's M-band exons MEx1 and MEx2, the mutant titin does incorporate into the sarcomere and the A-band is relatively normal except for structural defects in the M-band region.[55] Consistent with this, histo-pathologic examination of hearts with truncated titin did not suggest marked sarcomere disorganization[6]. It is also possible that increased production and/or incorporation of non-mutated titin can compensate in affected individuals, as suggested in a mouse heterozygous for a truncation mutation in the A-band region[56]. These mice appeared to have normal cardiac function and morphology until exposed to angiotensin II or isoproterenol. A similar mechanism could occur in patients with A-band truncations who are apparently normal until middle age when they develop DCM[6], suggesting that stresses encountered as adults trigger development of clinical disease. A similar mechanism could also underlie gender effects, with more adverse events at earlier ages in men than in women[6].

Titin mutations also appear to cause arrhythmogenic right ventricular dysplasia (ARVD)[5], a disease characterized by right ventricular dysfunction and ventricular arrhythmias. In a study of 38 affected families[5], seven were found to have unique titin variants, including a Thr2896Ile mutation which completely segregated with the ARVD phenotype in a single large family. This mutation locates to the 10th Ig domain in the proximal tandem Ig. It is perhaps surprising that a single point mutation in an Ig domain leads to cardiomyopathy, but a variety of experimental techniques suggest a hypothesis that links altered Ig10 dynamics to degradation of healthy myocardium. Nuclear magnetic resonance (NMR) studies and proteolysis assays show that Ig10 domains harboring the disease-linked mutation are structurally compromised and more prone to degradation [5]; AFM data show that mutant Ig10 unfolds at a lower force compared to native Ig10[6], consistent with the idea that the mutation weakens the domain's β-barrel structure and results in a higher percentage of unfolded mutant compared to native Ig10. This propensity to exist in an unfolded structure

combined with the increased rate of degradation suggests that the mutation leads to cleaved titins, which would abolish titin's force-generating mechanism and likely lead to further degradation and possibly even apoptosis.

A high prevalence of titin mutations was also recently reported by Golbus et al[57], who analyzed the '1000 Genomes' cohort. A cumulative frequency of titin in dels of 9% was found, with just over 5% of the general population having a 18bp in-frame deletion in the PEVK region.[57] As suggested[56], the discovered titin variants might not cause disease on their own but may modify the phenotype of mutations in other genes. If correct this would be an important consideration for future genetic testing and study of genotype-phenotype relationships.

Future Directions

Therapeutic modalities targeting titin are at present largely theoretical, but the previous discussion suggests possibilities. Increasing expression of compliant N2BA titin or inducing production of even more compliant fetal isoforms in disease states where myocardial passive stiffness is increased (e.g., HFpEF), offers the possibility of improving diastolic compliance and relieving symptoms. As discussed earlier, such an approach might be possible by manipulation of titin splicing through reducing the expression or activity of RBM20.[24] A potential negative effect of increasing compliant isoforms, however, is a reduction in titin-dependent diastolic recoil with impairment of early diastolic filling. Thus, it will be important to experimentally determine whether the net effect of such an intervention is positive with respective to diastolic function.

Post-translational modifications offer additional possibilities. Beta-adrenergic blockers have a well-established role in treating ischemic and non-ischemic DCM. Although there is at present no evidence-based rationale, many patients with HFpEF also receive such drugs despite the fact that they could reduce phosphorylation of titin's PKA/PKG sites and further increase myocardial passive stiffness. While chronic administration of catecholamines that increase PKA activity and heart rate cannot be recommended in HF patients, it is possible that beta-blockers should be avoided in HFpEF because of their effect on titin. It is also possible that drugs that increase PKA activity without causing major changes in heart rate could be beneficial in HFpEF. Similarly, interventions that improve endothelial function in HFpEF by increasing PKG activity might also be useful. Unfortunately, the recently completed RELAX trial of sildenafil in HFpEF[58] was negative despite the fact that the drug improved diastolic distensibility and increased titin phosphorylation in a dog model and has other, non-titin mediated effects that might improve diastolic function.[59] Correspondingly, if phosphorylation of titin's PKCα sites is increased in HF, this would increase passive stiffness and suggests the utility of interventions that reduce PKC activity. Endothelial activation, present in both HF and pulmonary hypertension[60], augments PKC activity and could contribute to increased diastolic stiffness and thus be a target for therapeutic intervention. It will also be of interest to study the effects of isoforms other than PKCα on titin. For example, PKCβ is markedly upregulated in end-stage DCM and causes myofilament dysfunction[61], but nothing is known as yet about whether it phosphorylates

titin. Finally, as mentioned earlier it might be possible to improve diastolic stiffness through reduction of oxidative stress-induced disulfide bonds.

Treating patients with titin truncation mutations will be a major challenge. In line with observations that such mutations do not necessarily cause major morphological changes, it is possible that gene therapy or other approaches designed to increase production of non-mutated titin could delay or abolish development of DCM. If the idea that stresses encountered later in life induce DCM is correct[6], perhaps patients at risk for titin-mediated DCM could be treated prophylactically with drugs such as angiotensin converting enzyme inhibitors or beta-blockers.

In summary, titin is a major determinant of myocardial and ventricular function and plays a key role in nuclear signaling in response to mechanical stress. Isoform switching and changes in post-translational modification, especially phosphorylation, are recognized as contributors to the pathophysiology of acquired heart disease. Most recently, as predicted based on its size and critical functions, titin mutations have emerged as a major cause of DCM. Rapidly increasing in-depth knowledge of titin and how it is modified in disease provide novel avenues for developing molecular therapeutics.

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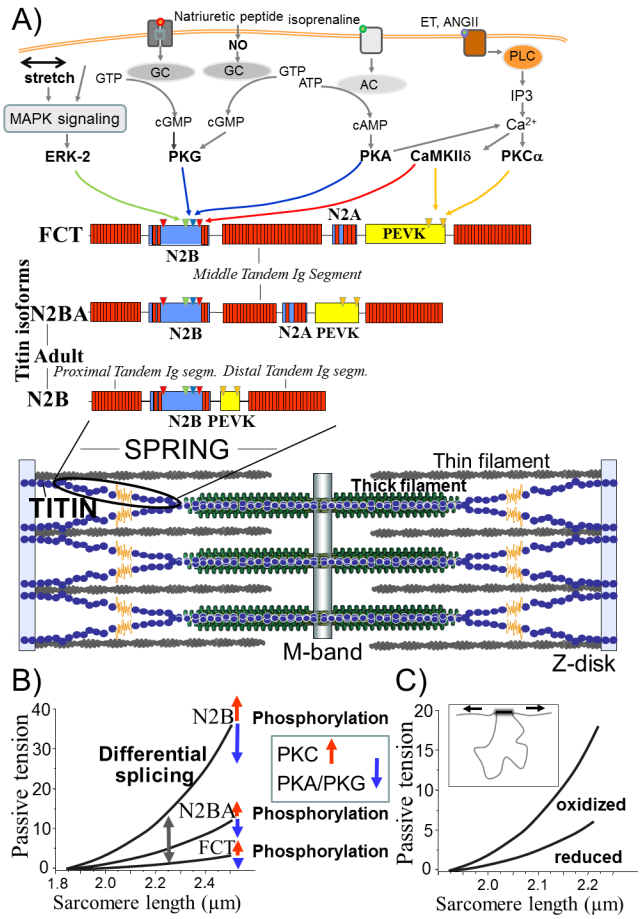

Figure 1. Schematic of titin in the sarcomere (A) and mechanisms for modifying titin-based passive tension (B and C)

A) Bottom: Single titin molecules (blue and yellow) span from Z-disk (N-terminus) to M-band (C-terminus). Middle: Composition of extensible I-band region of N2B, N2BA titin isoforms (found in adults) and fetal cardiac titin isoform (FCT). Red blocks denote Ig-like domains, blue, unique sequence and yellow, PEVK sequence. Top: Phosphorylation sites in titin's spring region (present in all 3 isoforms) and their upstream signaling pathways. Abbreviations: MAPK: mitogen-activated protein kinase; GC: guanylyl cyclase; AC: anelylyl cyclase; NO: nitric oxide; ET: endothelin; ANGII: angiotensin II; IP3: inositol triphosphate. See text for additional abbreviations and details. **B)** Schematic of force-extension curves of titin isoforms and effects of phosphorylation on passive tension. **C)** Schematic effect of oxidation to form cysteine disulfide crosslinks in the N2B region of titin (inset) and effects on passive tension.