Titin Is a Major Human Disease Gene

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Titin is a giant multi-functional sarcomeric filament that provides passive stiffness to cardiac myocytes. At its N terminus, titin is embedded in the Z-disk of the sarcomere. The rest of the molecule is divided between an elastic I-band region, a thick filament-binding A-band region, and the M-band region where the C terminus is embedded (Figure 1A, bottom). The extensible I-band region of titin functions as a molecular spring that develops passive force during diastole when sarcomeres are stretched. This force is important for centering the A-band in the sarcomere and, together with the extracellular matrix, for defining diastolic stiffness. Other regions of titin (Z-disk, A-band, and M-band) are involved in numerous cellular processes including force-dependent signaling.

Here we discuss recently discovered post-transcriptional and post-translational modifications of titin and address their roles in acquired cardiac disease, including dilated cardiomyopathy (DCM) and heart failure with preserved ejec tion fraction (HFpEF, often termed diastolic heart failure; for the purposes of this study we restrict the term HFpEF to HF patients with left ventricular EF > 0.50 in the absence of hypertrophic cardiomyopathy or valvular, infiltrative, or pericardial disease). The review also focuses on recent work that reveals mutations in the titin gene as a major source of familial cardiomyopathies, including mutations in the spring region of titin linked to arrhythmogenic right ventricular dysplasia and mutations in the A-band region of titin responsible for ≈30% of DCM cases. These findings have given rise to the emerging view that titin gene is a major disease gene.

Titin Isoforms and Heart Disease

The extensible I-band region of titin comprises 3 distinct elements: (1) tandem Ig segments that consist of serially-linked immunoglobulin(Ig)-like domains, (2) the springlike PEVK (containing a high percentage of proline, glutamic acid, valine, and lysine residues), and (3) the N2B element with its extensible unique sequence (N2B-Us) (Figure 1A, middle). Titin is encoded by a single gene. Variable mRNA splice pathways result in distinct titin isoform classes. In cardiac muscle, 3 classes of titin isoforms are present: adult N2BA, adult N2B, and the fetal cardiac titin (FCT) isoforms (Figure 1A middle). These 3 titin isoform classes differ in their I-band region; the rest of the molecule (Z-disk, A-band, M-line localized regions) is largely identical. The tandem Ig segments can be visualized as beads on a string with folded Ig domains with a diameter of 4 to 5 nm separated by short peptide linkers. All isoforms contain a proximal tandem Ig segment (Ig1-15) and a distal tandem Ig segment (Ig84-105). The N2BA and FCT titin isoforms also contain a middle tandem Ig segment that contains a variable number of Ig domains (Figure 1A, middle). The N2B element is found in all cardiac titin isoforms. In addition to behaving as a large molecular spring, the N2B-Us is a substrate for various kinases that effect its mechanical properties (see below). Although the N2B element is found in both adult cardiac titin isoforms, the N2A element is only found in the N2BA isoform (hence its name) and FCT isoforms. Similar to the N2B element, N2A contains Ig domains and a unique sequence that binds the calpain protease p94 and proteins that 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. Like the N2B-Us, the PEVK region of titin also behaves as a molecular spring. The PEVK sequence is encoded by 114 exons, 7 of which are found in the N2B titin isoform, whereas the PEVK region of N2BA titin contains additional exons and is much larger. The additional Ig domains and PEVK sequence and the inclusion of the N2A element make the N2BA titin isoform larger than the N2B isoform (≈3.3 MDa versus 2.97 MDa). The FCT class of titin isoforms (3.5–3.6 MDa) contains the largest middle tandem Ig segment and the largest PEVK sequence of all the cardiac titin isoforms.

The force required to stretch a titin molecule depends nonlinearly on its fractional extension. At a given sarcomere length the extension of N2B and N2BA titin (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, because N2B titin has a shorter contour length (attributable to fewer Ig domains, a shorter PEVK segment, and absence of the N2A element), the fractional extension of the N2B isoform is greater. Therefore, more force is needed to stretch the N2B titin isoform—it is stiffer because it is shorter. For this reason, sarcomeres that express different titin isoforms develop levels of passive force that greatly differ (Figure 1B).

Adult cardiac muscle coexpress N2B and N2BA cardiac titin at the level of the half sarcomere. The number of titin molecules per thick filament is likely to be constant (6 per half

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thick filament), but the expression ratio of compliant (N2BA) to stiff (N2B) titin is variable (in human control left ventricular myocardium ≈40:6018). Because of the intimate relationship between the size of the I-band region of titin and titin-based passive tension, 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-based passive tension (Figure 1B).

Variable titin expression ratios have been found in patients with cardiac disease. Patients with ischemic cardiomyopathy have been shown to express increased levels of N2BA titin that was accompanied by decreased stiffness at the myofibrillar level.18 Changes in titin isoform expression have also been found in patients with end-stage HF attributable to nonischemic DCM, where the compliant N2BA isoform was upregulated and associated with decreased passive stiffness and increased chamber compliance.19,20 The study by Nagueh et al19 also suggested a physiological benefit of this change in titin expression via correlation between the titin isoform shift and improved exercise tolerance. Upregulation of compliant titin isoforms has also been found in patients with HFrEF, a group that accounts for about half of all HF cases and is characterized by increased diastolic stiffness.21,22 An adaptive change in isoform expression toward increased expression of compliant titin isoforms also occurs in mice with pathological hypertrophy23 and rats with long-term hypothyroidism.24 Overall these studies suggest that upregulation of the more compliant N2BA titin isoform is an important compensatory adaptation to counteract the increased stiffness of the extracellular matrix.19

Mechanisms that underlie changes in titin isoform expression are not well understood, although a possible breakthrough is the recent discovery of the role of the splice factor RBM20.25 Naturally occurring RBM20 mutations in both human patients and in a rat model result in low expression levels of RBM20 and expression of large and highly compliant titin isoforms.25 It is thus possible that a reduction in expression level of RBM20 in

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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 (shown in blue and yellow) span from Z-disk (N terminus) to M-band (C terminus). Middle: Composition of extensible I-band region of the N2B, N2BA titin isoforms (found in adults), and the fetal cardiac titin isoform (FCT). Red blocks denote Ig-like domains; blue, unique sequence; and yellow, PEVK sequence. Top: Phosphorylation sites in the N2B and PEVK spring elements of titin (the same sites are present in all 3 isoforms) and their upstream signaling pathways. Highly conserved sites are shown (present in mice and man), sites in parenthesis are not conserved and are not indicated on figure. For CaMKII sites, see Hidalgo et al37,38; for PKA/PKG sites Handannii et al44 and Krueger et al45; ERK2 sites see Raskin et al46; PKC sites Hidalgo et al. Numbering based on Q8WZ42. AC indicates anelyl cyclase; ANGII, angiotensin II; ET, endothelin; GC, guanylyl cyclase; IP3, inositol triphosphate; MAPK: mitogen-activated protein kinase; and NO, nitric oxide. See text for additional abbreviations and details. B, Schematic of force-extension curves of titin isoforms and the effects of phosphorylation on passive tension. C, Schematic of effect of oxidation to form cysteine disulfide crosslinks in the N2B region of titin (inset) and its effect on passive tension.
cardiac state underlies the upregulation of compliant titin isoforms. Clearly more work is needed to understand the mechanisms that drive titin isoform expression, with a focus on RBM20, and to explore whether experimentally upregulating complaint titin isoforms can be used to ameliorate increased myocardial stiffness in HF patients.

**Post-Translational Modifications and Disease**

It is well-known that post-translational modifications of contractile and regulatory proteins greatly affect cardiac function. Recent single molecule force spectroscopy studies of titin have discovered that kinases phosphorylate the extensible region of titin (Figure 1A, top) and significantly alter the stiffness of the PEVK and N2B-Us spring elements. This allows for rapid adjustment of titin stiffness (Figure 1B) and adaptations of cardiac performance to meet hemodynamic loads.

The PEVK spring element has been found to be phosphorylated by protein kinase C\(\alpha\) (PK\(C\alpha\)) (see Figure 1A top). PKC\(\alpha\) is activated by the \(\alpha\)-adrenergic signaling pathway, and PKC\(\alpha\), the predominant isozyme in the heart, is a key player in contractile dysfunctions and heart failure. PK\(C\alpha\) phosphorylates the PEVK element of titin which leads to increased passive tension. The primary sites of phosphorylation are 2 highly-conserved serine residues (S11878[S26] and S12022[S170]) within the constitutive PEVK element.

Phosphorylation of these conserved serine residues reduces the bending rigidity of the PEVK region, which is consistent with the increased passive tension in response to PKC phosphorylation seen at the tissue level. The link between PK\(C\alpha\), PEVK phosphorylation, and passive tension was further established by a study that showed that PK\(C\alpha\) had no effect on passive tension in mice in whom the PEVK sites were genetically removed.

The N2B element of titin is also a kinase substrate whose mechanical properties change after phosphorylation. Protein kinase A (PKA), which is stimulated by the \(\beta\)-adrenergic pathway, reduces passive tension in cardiac myocytes. PKA phosphorylation of the N2B element was shown to be inhibited by binding of the 4 and a half LIM protein 1 (FHL1) to the N2B-Us. FHL1 has previously been shown to bind to the N2B-Us and assemble a stretch sensing signalosome that consists of components of the mitogen activated signaling pathway. These new findings suggest a possible link between stretch sensing and phosphorylation-based regulation of passive stiffness. Another novel pathway involves CaMKII, a \(\gamma\) and calmodulin-dependent serine/threonine kinase that is activated by increases in cellular Ca\(^{2+}\). Four isoforms have been described (\(\alpha\), \(\beta\), \(\gamma\), and \(\delta\)), of which CaMKII\(\beta\) is the predominant isoform in the heart. Hidalgo and colleagues have shown that CaMKII\(\beta\) phosphorylates titin in skinned and intact myocardium and that the titin N2B and PEVK spring elements, but not Ig domains are phosphorylated by CaMKII\(\beta\). Furthermore, the phosphorylation sites overlap with the PKC sites (including the PKC sites S26 and S170 of the PEVK element, see Figure 1A, top).

The effect of CaMKII\(\beta\) phosphorylation of the PEVK sites is likely to be similar to that reported for PKC phosphorylation (ie, an increase in passive tension and that of phosphorylation of the N2B element a reduction in passive tension). Western blot studies with phospho-specific antibodies suggest that CaMKII\(\beta\) phosphorylation of the N2B element might be the dominant process. Considering that the ERK2 and CaMKII\(\beta\) signaling pathways play important roles in cardiac health and disease, additional research that focuses on the roles of ERK2 and CaMKII\(\beta\) phosphorylation of titin is warranted.

The mechanical properties of the N2B-Us can be altered by more than just phosphorylation status. For example, there are 6 cysteine residues in the human N2B-Us that have the potential to form disulfide bonds with one another, depending on the oxidative state within the sarcomere. A disulfide bond would reduce the contour length of the sequence and change its mechanical response to stretch. The effect of cysteine cross-linking on the mechanics of the N2B-Us was shown at both the single molecule level and the tissue level, where oxidative stress increased passive tension and hysteresis in wild-type tissue (Figure 1C) but had an attenuated effect in tissue from a mouse model where the entire N2B element was removed.
stress is elevated in HF patients and has been correlated with myocardial dysfunction.\textsuperscript{52}

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

**Titin Mutations and Disease**

Until recently, a small number of titin mutations had been documented in association with human cardiomyopathies.\textsuperscript{5,43–48} It was predicted that the difficulty of complete sequencing of such a large gene in many patients might be responsible for this dearth of identified mutations.\textsuperscript{12} Most of these mutations were associated with DCM. Associations with hypertrophic cardiomyopathy (HCM) were rare. Missense HCM mutations have included Arg740Leu, which increases titin-α actinin binding, and Ser3799Tyr, which increases four and a half LIM protein 2 (FHL2) binding.\textsuperscript{43,45} Interestingly, mutations of genes encoding proteins known to interact with titin, including myomesin,\textsuperscript{49} cardiac ankyrin repeat protein (ANKRD1),\textsuperscript{50,51} FHL2,\textsuperscript{52} and telethonin (TCAP)\textsuperscript{53} have been found to be associated with both HCM and DCM.

Recently, using several large familial registries of cardiomyopathy patients, Herman et al\textsuperscript{6} published a landmark study in which both next-generation and dideoxy sequencing were used to sequence the titin gene in a large number of patients. They focused on mutations (nonsense, frameshift, splicing, and copy number) that are likely to have an important effect on the full-length structure of titin, as opposed to single amino acid missense mutations. Their results provide a much more complete picture of the frequency and nature of titin mutations in these patients and are in accord with the idea that titin mutations are indeed much more common than previously known. Specifically, they found that ≈30% of DCM patients have mutations in the titin gene, whereas only 1% of HCM mutations were localized to titin. Of the matched subjects without evidence of heart disease, 3% also had titin mutations. The penetrance of these DCM mutations was very high in patients aged >40 years. Based on these results mutations of titin appear to be a rare cause of HCM, whereas they are by far the most common genetic cause of DCM.

The DCM-causing mutations are not randomly distributed along the titin gene; instead, the bulk of the mutations are located in the large A band region of titin that associates with the thick filament\textsuperscript{6} (Figure 2). Strikingly, there were no mutations in the Z-disk or M-band regions. The A band portion of the protein 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 the thick filament associated protein that crosslinks the thick filament with titin’s C terminus, myomesin, obscurin, protease calpain-3, myosin binding protein C, FHL2/DRAL, and muscle specific ring finger protein-1.\textsuperscript{14} Because the kinase domain in particular may play a key role in strain sensitive signaling and communications with the nucleus in conjunction with the other proteins in this region, it is intriguing to consider that these mutations may result in diverse effects on gene expression and cardiac remodeling in DCM.

Absence of mutations in the Z-disk and paucity of mutations in the I-band region could indicate that mutations in these regions do not cause DCM or HCM (the patient populations that were studied).\textsuperscript{5} Alternatively, mutations in the Z-disk and I-band regions of titin could be highly detrimental to sarcomere function and be incompatible with life, and therefore are not seen in patient populations. Truncation of titin in the Z-disk and I-band regions would result in proteins that are insufficiently long to span to the A-band region, abolishing the mechanical functions of titin. In contrast, truncations in the A-band region of titin result in mutant titins that should be able to incorporate in the Z-disk, span from Z-disk to A-band and be able to make connections to thick filament proteins. Indeed, in a mouse model that conditionally deletes the M-band exons of titin ME1 and ME2, the mutant titin does incorporate into the sarcomere and the A-band is relatively normal except for structural defects in the M-band region.\textsuperscript{55} Consistent with this, histo-pathological examination of hearts with truncated titin in the study of Herman et al\textsuperscript{6} did not suggest marked sarcomere disorganization. Furthermore, it is possible that increased production or incorporation of nonmutated titin can compensate in affected individuals, as was suggested in a mouse heterozygous for a truncation mutation in the middle of the A-band region.\textsuperscript{56} These mice appeared to have normal cardiac function and morphology until they were subjected to the stress of exposure to angiotensin II or isoproterenol. Perhaps a similar mechanism occurs in patients with A-band titin truncations who also are apparently normal until middle age when they develop DCM,\textsuperscript{6} suggesting that stresses encountered as adults act as a trigger for development of clinical disease. A similar mechanism could also underlie the sex effects that were noted, with more adverse events at earlier ages in men than in women.\textsuperscript{6}
Finally, titin mutations also appear to cause arrhythmogenic right ventricular dysplasia. In a study of 38 affected families, 7 were found to have unique titin variants, including a prominent Thr2896Ile mutation which completely segregated with the arrhythmogenic right ventricular dysplasia phenotype in a single large family. This mutation locates to the 10th Ig domain in the proximal tandem Ig. It is surprising that a single point mutation in an Ig domain leads to cardiomyopathy, but the combination of a variety of experimental techniques has suggested a hypothesis that links altered Ig10 dynamics with degradation of healthy myocardium. Nuclear magnetic resonance data and proteolysis assays have shown that Ig10 domains harboring the disease-linked mutation are structurally compromised and more prone to degradation; Atomic Force Microscopy data also show that mutant Ig10 unfolds at a lower force compared with native Ig10, which is consistent with the idea that the mutation weakens the β-barrel structure of the domain and results in a higher percentage of unfolded mutant Ig10 compared with 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 the essential force-generating mechanism of titin and likely lead to further titin degradation and possibly even apoptosis.

A high prevalence of titin mutations was also recently reported by Golbus et al., who analyzed the 1000 Genomes cohort. A cumulative frequency of titin indels of 9% was found, with just >5% of the general population having an 18-bp in-frame deletion in the PEVK region of titin. As suggested by the authors, the discovered titin variants might not cause diseases 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. Thus, titin may be an important disease gene not only because it causes diseases on its own but also because it may modify the phenotype of mutations in other genes.

Future Directions

Therapeutic modalities targeting titin are at present largely theoretical, but the previous discussion suggests some possibilities for consideration. Increasing the expression of compliant N2BA titin or inducing the production of even more compliant fetal isoforms in disease states where myocardial passive stiffness is increased (for example HFpEF) offers the possibility of improving diastolic compliance and relieving symptoms. As discussed earlier, such an approach might be possible by manipulation of splicing of titin through reducing the expression or activity of RBM20. A potential negative effect of increasing compliant titin 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 respect to diastolic function. This also underscores the possibility that limiting fibrosis by manipulation of the extracellular matrix might also provide a means to improve compliance.

Post-translational modifications offer additional possibilities. B-Adrenergic blockers have a well-established role in the treatment of ischemic and nonischemic DCM. Although there is no evidence-based rationale, many patients with HFpEF also receive β-adrenergic blocking drugs despite the fact that they could reduce phosphorylation of the PKA/PKG sites of titin and further increase myocardial passive stiffness. Although chronic administration of catecholamines that increase PKA activity and heart rate cannot be recommended in patients with heart failure, it is possible that β-blockers should be avoided in HFpEF patients because of their effect on titin. It is also at least possible that drugs that increase PKA activity without causing major changes in heart rate (eg, phosphodiesterase inhibitors such as milrinone) could be beneficial in HFpEF. Similarly, interventions that improve endothelial function in HFpEF with resultant increased PKG activity might also be useful. The results of the soon to be completed RELAX trial of sildenafil in HFpEF will be of great interest in this regard, especially in light of the recent finding that sildenafil improves diastolic left ventricular distensibility and increases titin phosphorylation in the dog. Correspondingly, if phosphorylation of the PKCα sites of titin is increased in HF, this would increase passive stiffness and suggest the possibility of interventions that reduce PKC activity. Endothelial activation, which is present in both heart failure and pulmonary hypertension, augments PKC activity and could also 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, but nothing is known as yet about whether and how it phosphorylates titin. Finally, as mentioned earlier it might be possible that diastolic stiffness can be reduced through reduction of oxidative stress–induced disulfide bonds.

Treating patients with titin truncation mutations will undoubtedly be challenging. In line with the observations that such mutations do not necessarily cause functional or morphological changes, it is possible that gene therapy or other approaches designed to increase production of nonmutated titin could delay or abolish the development of DCM. If the idea that stresses encountered later in life result in induction of DCM is correct, perhaps patients at risk for DCM could be treated prophylactically with drugs such as angiotensin-converting enzyme inhibitors or β-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 increasingly recognized as contributors to the pathophysiology of acquired heart disease. Most recently, as predicted based on its size and critical functions, mutations of titin have emerged as a major cause of DCM. The rapidly increasing in-depth knowledge of titin and how it is modified in disease provides novel avenues for developing molecular therapeutics.
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References


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