ESC European Society of Cardiology

Cardiovascular Research (2022) **118**, 2903–2918
https://doi.org/10.1093/cvr/cvab328

INVITED REVIEW

Titin (TTN): from molecule to modifications, mechanics, and medical significance

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Received 23 July 2021; editorial decision 23 September 2021; accepted 13 October 2021; online publish-ahead-of-print 18 October 2021

Abstract

The giant sarcomere protein titin is a major determinant of cardiomyocyte stiffness and contributor to cardiac strain sensing. Titin-based forces are highly regulated in health and disease, which aids in the regulation of myocardial function, including cardiac filling and output. Due to the enormous size, complexity, and malleability of the titin molecule, titin properties are also vulnerable to dysregulation, as observed in various cardiac disorders. This review provides an overview of how cardiac titin properties can be changed at a molecular level, including the role isoform diversity and post-translational modifications (acetylation, oxidation, and phosphorylation) play in regulating myocardial stiffness and contractility. We then consider how this regulation becomes unbalanced in heart disease, with an emphasis on changes in titin stiffness and protein quality control. In this context, new insights into the key pathomechanisms of human cardiomyopathy due to a truncation in the titin gene (*TTN*) are discussed. Along the way, we touch on the potential for titin to be therapeutically targeted to treat acquired or inherited cardiac conditions, such as HFpEF or *TTN*-truncation cardiomyopathy.

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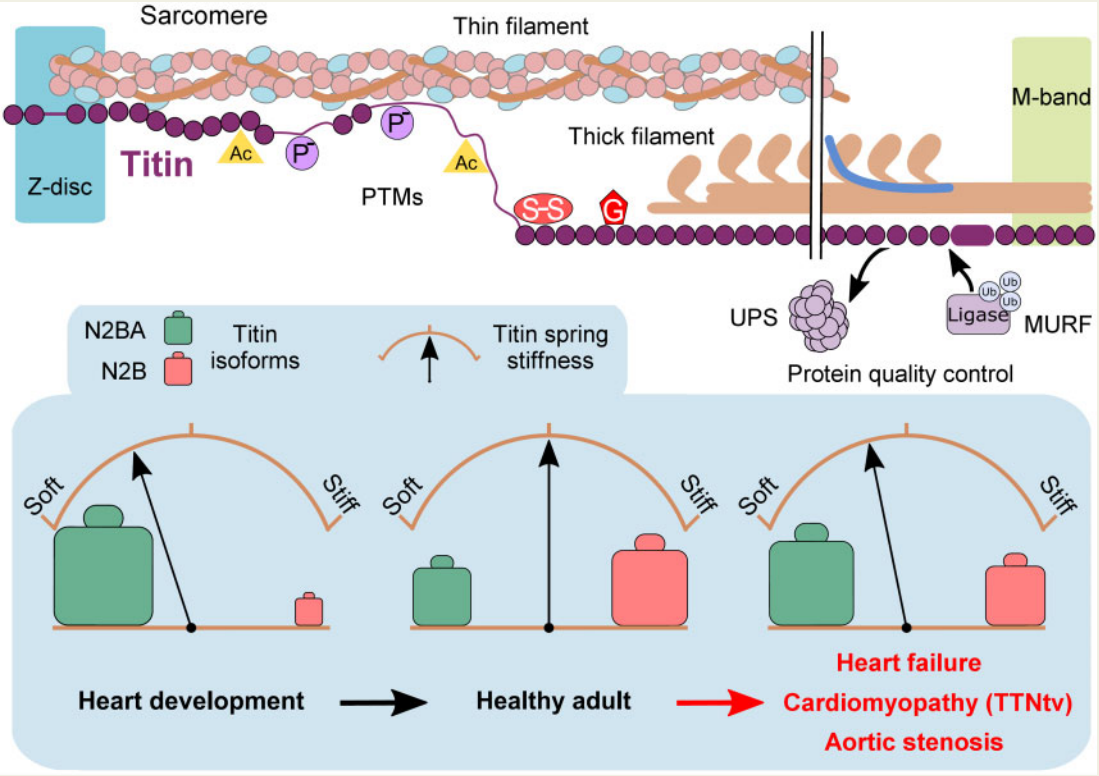
Graphical Abstract



Keywords Heart failure • Cardiomyopathy • Sarcomere • Mechanical function • Signalling

1. Introduction

The heart needs to continually pump throughout a lifetime and meet the ever-changing requirements of the body. Some changes take place relatively slowly throughout development, ageing, and disease, while others occur almost on a beat-to-beat basis. How the heart adapts to these changes continues to be the subject of an entire research field, as many details remain incompletely understood. For example, increased ventricular filling and pressure induce stretch and stress signalling pathways within the heart, which are complex, intertwined in many ways, and challenging to study—notably *in vivo*.[1] At the level of the cardiac cells, a myriad of proteins has been identified which participate in mechanical and chemical signalling in various ways. The focus of this review is the mechanosensitive protein titin[2] in the sarcomeres, the contractile units of the cardiomyocytes (CMs). We zoom in on titin's properties as a molecular spring whose elasticity can be extensively modulated under physiological and pathophysiological conditions, resulting in larger modulations in cardiac function, such as diastolic filling or length-dependent activation (LDA), the basis of the Frank-Starling law. Titin has several mechanisms it can exploit to physiologically modulate myocardial stiffness and distensibility, including post-translational modifications (PTMs, e.g. acetylation, oxidation, and phosphorylation) and isoform switch (*Figure 1*). Other possibilities to fine-tune the mechanical properties of the cardiac sarcomere/CMs/heart involve the binding of specific titin regions to Ca^{2+}, chaperones, or protein ligands, such as actin (not reviewed here, but see Ref.[2]). However, titin's giant size and complexity leaves it vulnerable to dysregulation, which can result in the development of cardiac disease. To date, the dysregulation of titin, its binding partners, and associated signalling pathways have been implicated in various forms of heart disease, such as heart failure (HF) with preserved ejection fraction (HFpEF), HF with reduced ejection fraction (HFrEF), aortic stenosis, and ischaemic injury (*Figure 1*). Moreover, next-generation sequencing has revealed pathogenic variants in the titin gene (*TTN*) as a major cause of inherited cardiomyopathies,[3] the pathomechanisms of which have only recently become clearer,[4] as discussed further below in this review. The overall goal of our review is to explore how cardiac titin properties can be changed at a molecular level, with an emphasis on titin stiffness and protein quality control (PQC), and how this regulation becomes unbalanced in heart disease. We also highlight new insight suggesting that titin can be therapeutically targeted to help treat acquired or inherited cardiac conditions, such as HFpEF or *TTN*-truncation cardiomyopathy.

2. Structure, expression, and isoform diversity of titin

Spanning the half-sarcomere from the Z-disc to the M-band as the 'third' sarcomeric filament system (along with myosin and actin filaments), titin is well placed to assist in the regulation of both passive and, to some extent, active force development of the heart (*Figure 2A*).[5,6] The intrinsic

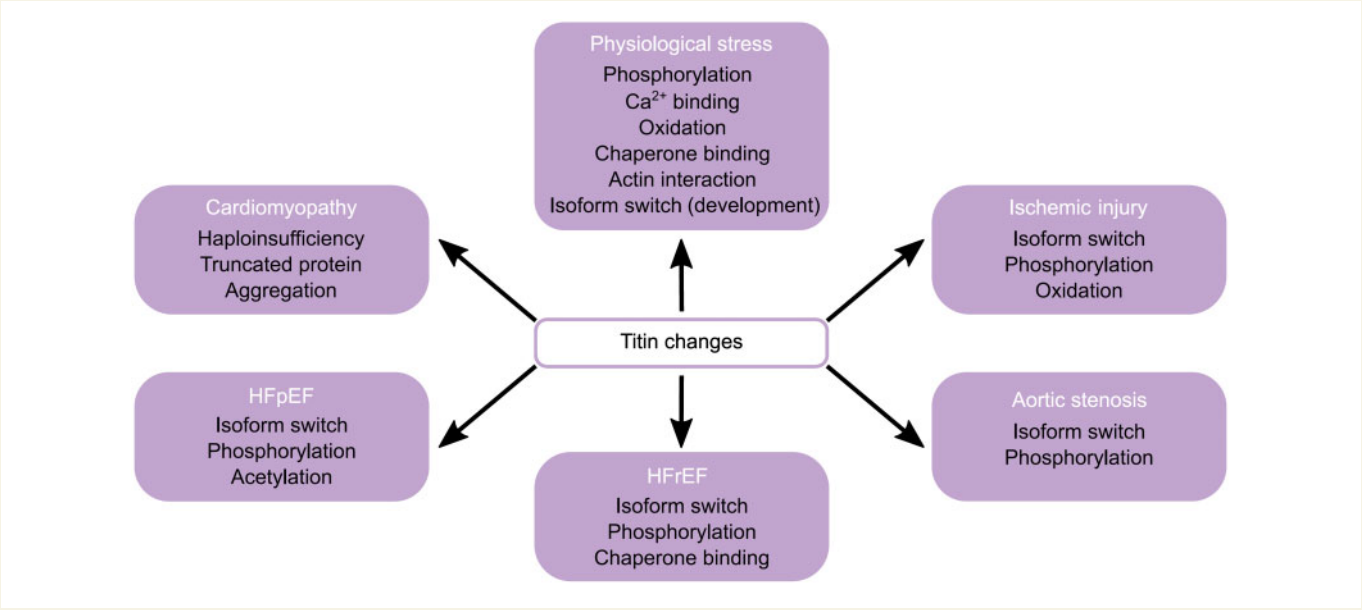


Figure 1 Known changes in cardiac titin properties under physiological stress and as a cause or consequence of heart disease.

properties of titin vary along its length, depending on the location within the sarcomere: A-band titin is bound to myosin filaments and is functionally inextensible, I-band titin lays slightly oblique between the Z-disc and the I/A-band junction and is elastic, and the ends of the titin filament are anchored at the Z-disc (via α-actinin, actin, and telethonin) and M-band (via myomesin). The specific amino acid sequences and domain structures in each region determine the structural and functional roles of these titin segments.[2] A-band titin is largely comprised of super-repeats of relatively stable fibronectin-type 3 and immunoglobulin-like (Ig) domains[7] providing a template for thick-filament assembly and A-band length,[8] while the M-band region engages in protein–protein interactions that serve structural and regulatory functions.[9,10] I-band titin is variable and contains several extensible elements, such as the PEVK-repeats (motifs rich in proline, glutamic acid, valine, and lysine), Ig-domain regions, and unique sequences, notably the N2B-unique sequence (N2Bus), enabling titin's spring-like properties.[11,12] Unique sequence elements are also present in Z-disc and M-band titin.

Part of the variability within I-band titin comes from the high degree of differential splicing that occurs in this region.[13] Of note, this continues to cause inconsistencies with the nomenclature when assigning specific domain numbers to I-band titin. Further adding to the confusion is that commonly referred-to databases for human titin, such as UniProtKB and NCBI (canonical sequence accession numbers, Q8WZ42-1 and NP_001243779.1, respectively), falsely recognize some PEVK-repeats or globular Ig domains as insertion sequences. Human *TTN* has 364 exons and the complete meta-transcript 363 exons, from which several common transcripts (isoforms) are generated (*Figure 2B*). A good overview is provided at http://cardiodb.org/titin. Because the frequency at which an exon is incorporated into the titin molecule varies greatly in the elastic I-band region, the different isoforms have different spring length and variable compliance.[2,13,14]

The principal cardiac isoforms are the Z-disc-anchored N2B, N2BA, and Novex-3 variants,[15] as well as the recently identified C-terminal Cronos isoform driven by an internal promoter (*Figure 2B*).[16] On a typical Coomassie-stained titin-protein gel of human heart tissue (*Figure 2C*), these isoforms appear together with a proteolytic fragment, T2 (2.4 MDa). The two most common cardiac isoforms, N2B (3 MDa) and N2BA (3.2–3.8 MDa), determine titin extensibility and myofibrillar passive stiffness.[12,17] The N2BA isoforms exist in many splice variants that have different I-band length. The N2B isoform (named after the N2B element coded by *TTN* exon 49) is shorter and less compliant than the N2BA isoforms, as its spring segment has only 6 PEVK-repeats, no 'middle'-Ig domains, and no N2A element (*Figure 2B*).[12–14] The short isoform Novex-3 (~650 kDa), which splices-in exon 48 (thus introducing a stop), does not appear to be relevant for titin stiffness; however, the exon 48-encoded Novex-3 region may have a role as a structural and regulatory element, e.g. through its interaction with obscurin.[15] Cronos (2.3 MDa; close in size to the T2 fragment) is more highly expressed in developing CMs than in adult hearts,[18] where it makes up only ~10% of the total titin-protein pool (human adult hearts).[4] In human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), Cronos has been shown to enable partial sarcomere formation in the absence of full-length titin.[18] However, Cronos failed to rescue cardiac sarcomerogenesis in adult mice lacking Z-disc-anchored titin.[19] Moreover, Cronos transcript and protein content were found to be unaltered in end-stage failing hearts from dilated cardiomyopathy (DCM) patients vs. non-failing human donor hearts.[4]

2.1 Titin splicing regulation and mechanical consequences

The titin N2B vs. N2BA isoform expression pattern is mediated, at least in part, by the splicing factor RNA-binding motif protein-20 (RBM20).[20] RBM20 suppresses the splicing-in of I-band titin exons,[21,22] thereby promoting expression of the shorter, stiffer N2B isoform (*Figure 2D*, inset).[20,23] RBM20 is also regulated by titin splicing itself: spliced-out regions of *TTN* can form functional motifs of circular RNA, such as cTTN1, which regulate the activity of RBM20 and that of another splicing

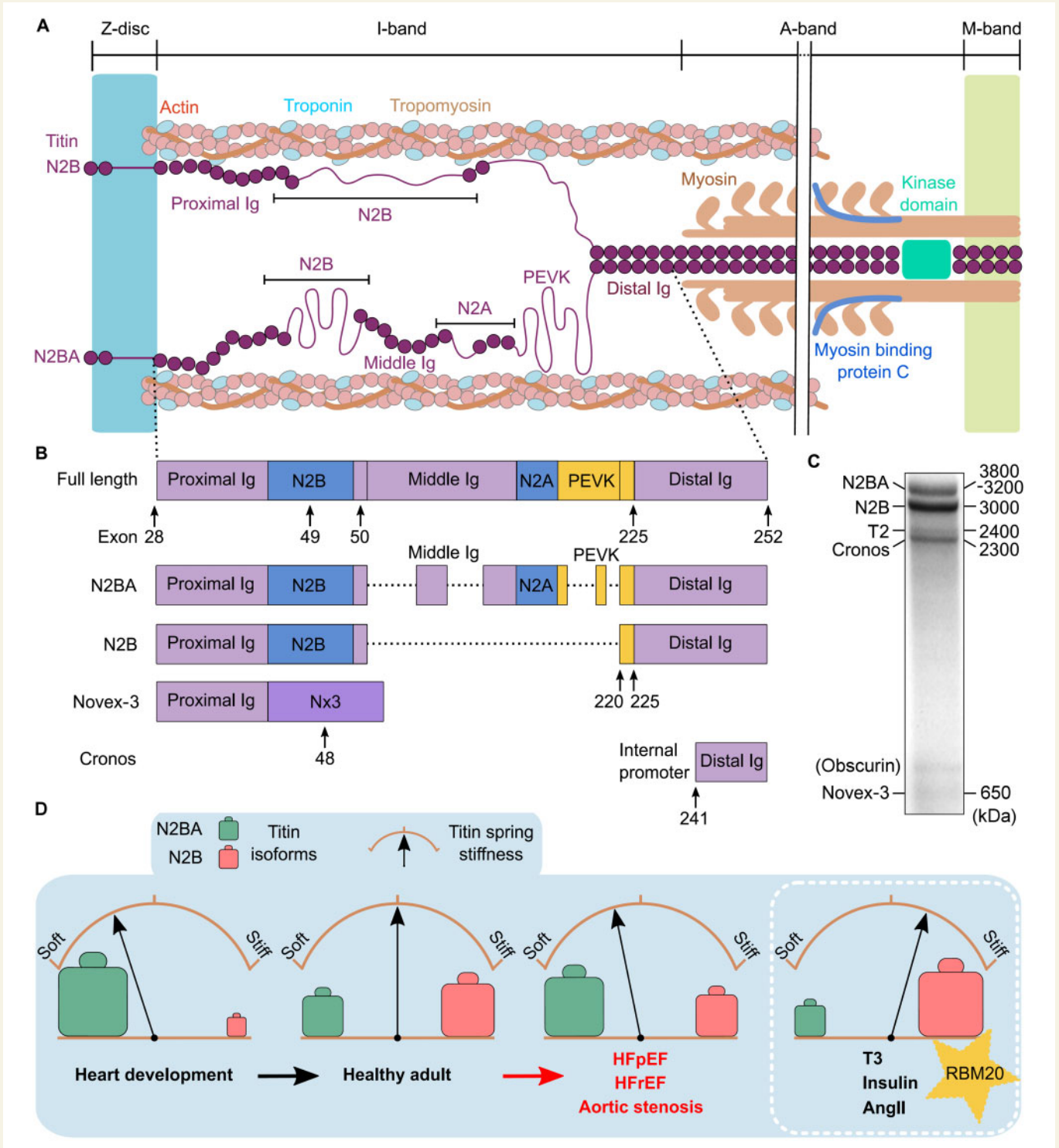


Figure 2 Titin isoforms in cardiac muscle, isoform switch, and consequences for titin-based stiffness. (*A*) Architecture of cardiac titin N2B and N2BA isoforms in the half-sarcomere. Note the abbreviated A-band region (double-line break). (*B*) Titin isoforms ('Full length' is a theoretical variant); only main I-band segments are shown. Dotted lines indicate alternative splicing. (*C*) Titin species separated on a typical Coomassie-stained titin gel (1.8% SDS–PAGE) of adult human heart tissue. (*D*) The proportion of N2BA (green) vs. N2B (red) titin isoforms can change under numerous conditions. This isoform switch determines a change in titin spring stiffness. AngII, angiotensin II; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; Ig, immunoglobulin-like domain; PEVK, segment rich in proline (P), glutamic acid (E), valine (V), and lysine (K) residues; RBM20, RNA-binding motif protein 20; T3, thyroid hormone.

factor, SRSF10.[24] RMB20-deficient rats or mice have been found to produce aberrantly large N2BA-isoforms and no N2B, resulting in reduced myocardial passive stiffness and dilated ventricles.[20,21,23] In humans, RBM20 pathogenic variants are associated with the expression of oversized N2BA isoforms,[4,20,25] greater titin compliance,[26] and irregular calcium homeostasis[27] leading to the development of DCM.[20,26] Interestingly, both insulin and thyroid hormone, T3, modulate RBM20 expression and activity pathways.[28,29] This could explain why T3[30,31] and insulin[32] (and also Angiotensin II) promote the expression of the N2B titin isoform, e.g. during heart development.[30] Long and compliant, foetal N2BA titin isoforms render titin-based stiffness very low in the embryonic heart, but a transition to shorter and less stretchable (adult) N2BA isoforms and N2B in the pre-/perinatal period greatly increases this stiffness (*Figure 2D*). Thus, long-term cardiac stiffness regulation can be achieved by changing the relative abundance of the N2BA and N2B isoforms, known as isoform switching.

2.2 Titin–isoform switch in heart disease

Titin–isoform transitions also regulate titin-based stiffness in heart disease. A healthy adult human heart (left ventricle) has an N2BA: N2B ratio of 30:70 to 40:60,[33] and this ratio remains relatively constant during normal ageing.[34] Isoform switching occurs in many forms of HF, including HFrEF (e.g. DCM or chronic ischaemic heart disease), HFpEF, and aortic stenosis (*Figure 2D*). Various studies have found that relatively more N2BA and less N2B isoform is present in failing vs. non-failing human hearts, such as in HFrEF,[4,33,35,36] HFpEF,[37] and aortic stenosis.[37,38] An increased proportion of N2BA is correlated with an increased end-diastolic volume[35] and lower titin-based passive stiffness (*Figure 2D*).[33,35,36] However, earlier studies reported a reduced N2BA:N2B ratio in aortic stenosis[39] and HFpEF patients,[40] whereas no change in titin–isoform composition was found in human hypertrophic cardiomyopathy and sometimes even in human DCM or aortic stenosis.[41–44] Conversely, the N2BA:N2B ratio has sometimes (e.g. Refs45,46) but not consistently been found to be reduced in animal models of HF. These discrepancies may be related to the stage of the heart disease during which the cardiac muscle samples were studied, the type of HF syndrome, species characteristics, heart chamber-specific differences,[47] and/or methodological limitations. It is still incompletely understood whether titin–isoform switch causes or compensates for cardiac stiffness changes that occur during disease progression.

Our knowledge of the influence titin–isoform switch can have on cardiac stiffness has made it a potential therapeutic target for the treatment of HF. Successful attempts have been made to modulate the titin–isoform composition in animal models by reducing RBM20 expression, increasing the N2BA to N2B ratio and lowering cardiac stiffness.[23,48,49] However, RBM20 is responsible for the splicing of many other cardiac proteins and the reduction of RBM20 will have consequences for the function of at least some of them, including crucial Ca^{2+}-handling proteins.[27,50] The lack of titin specificity therefore limits the therapeutic scope of RBM20 inhibition. Regardless, targeting titin–isoform switching might improve prognosis and symptoms of some HF patients, potentially making it a therapeutic approach in the future.

3. Cardiac titin stiffness as a regulator of active contraction

The mechanical properties of titin not only determine a significant portion of CM passive stiffness but also regulate active contraction.[6] A prime example is LDA, which is characterized by an immediate increase in the Ca^{2+} sensitivity of the myofilaments with a CM stretch. A consistent observation is the correlation between I-band titin length or titin-based stiffness and LDA: CMs with a short/stiff titin spring show a larger increase in myofilament Ca^{2+} sensitivity upon cell stretching than those with a long/soft titin spring.[51,52] Similarly, RBM20-deficient rat CMs expressing long and very compliant N2BA titin have a blunted LDA,[53–55] whereas mouse CMs lacking extensible I-band titin regions show increased titin-based stiffness and improved LDA compared to wildtype (wt) CMs.[56] Therefore, the titin–isoform switch towards more compliant N2BA present in failing hearts[33] is expected to reduce LDA and (via the Frank–Starling mechanism) cardiac output. These and other observations have suggested that stretch effects mediated by titin cause alterations to both thick- and thin-filament properties; however, the underlying molecular mechanisms remain a matter of debate.[6,57–64] Titin also has the potential to more directly support the work output of the contractile system by storing and releasing elastic energy via Ig-domain unfolding–refolding transitions, which occur under physiological (low) stretch forces.[6,65,66] While the magnitude of this contribution remains to be tested *in vivo*, the mechanism may be relevant to synchronize the mechanical activities of the actomyosin and titin systems.[6,66,67] Collectively, available data leave no doubt that the mechanical design of titin aids active contraction of cardiac muscle in multiple ways. It will be worth studying how the connectivity between titin spring stiffness and cardiac contractility may change in failing hearts.

4. Titin PTMs

Cardiac filling and output are optimized according to the actual demands of the body, and these adjustments also employ changes to titin stiffness. The question is how do you fine-tune stiffness in such a large protein potentially on almost a beat-to-beat basis? One of the quickest ways to adjust protein activity is through PTMs. For titin, phosphorylation, oxidation, and more recently acetylation have been shown to modulate its spring stiffness. Differences in these modifications have also been detected under pathophysiological conditions, giving rise to novel treatment strategies aiming at reversing pathological passive stiffness of patient hearts.

4.1 Acetylation–deacetylation

Lysine acetylation/deacetylation has traditionally been studied as a modification of histones and a regulator of transcription but it is increasingly becoming recognized as a modulator of cytoplasmic proteins, particularly relating to cellular metabolism.[68] Acetylation regulation has become of interest to the cardiac community, not least because of its association with obesity,[69] a worsening global burden of disease and a major comorbidity in HF. Acetylation has been abundantly detected along the length of titin (*Figure 3A*);[70] however, both the native acetylation state of titin and how it changes in CMs require additional studies, especially on human hearts. Proteomic analysis has revealed an increase in titin acetylation in obese mice[69] but no significant difference in acetylation levels in HFpEF models compared with the experimental controls.[70,71]

Proposed treatments targeting acetylation pathways have also uncovered changes to titin acetylation, which may play a role in the modulation of cardiac stiffness. The caloric restriction mimetic, nicotinamide (NAM), is a precursor of nicotinamide adenine dinucleotide (NAD^+), which is required for the activation of deacetylase enzymes, such as sirtuin-1

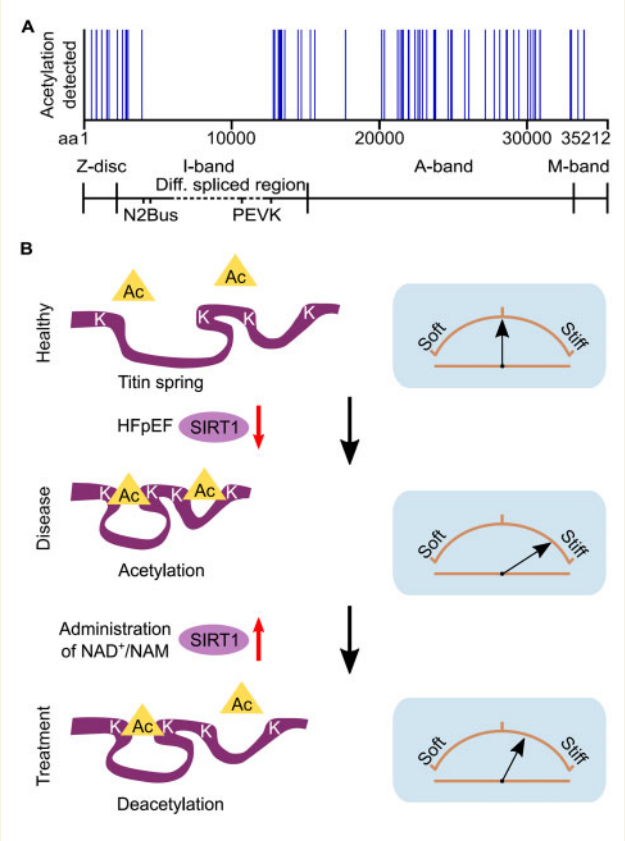


Figure 3 Locations and proposed mechanism of titin acetylation. (*A*) Titin acetylation sites detected in rat heart tissue by mass spectrometry (data obtained from Ref.[70]). (*B*) Locally increased acetylation of elastic titin in HFpEF may be due to reduced deacetylase activity of SIRT1; this increases titin-based stiffness. Treatment with NAD^+/NAM restores SIRT1 activity to reverse this process. Brown arches depict titin spring stiffness. aa, amino acid.

(SIRT1). NAD^+ has been suggested as a potential therapeutic for HFpEF.[70] Direct application of SIRT1 on skinned rat CMs was found to reduce their stiffness.[70] This was suggested to be a contributing factor of improved diastolic function in HFpEF after treatment with NAM.[70] However, conflicting results in similar animal models have shown that the use of histone deacetylase inhibitors can also ameliorate diastolic dysfunction and increase myofibril relaxation.[71,72] although changes in the acetylation state of titin after treatment were not assessed.

Another open question is how deacetylation causes a decrease in titin stiffness. The additional negative charge added to positively charged lysines during acetylation has been shown to stabilize enzymes and decrease the rate of unfolding.[68,73] Based on our findings,[70] we propose that the increased acetylation of the titin spring, e.g. in HFpEF due to reduced SIRT1 activity, promotes intramolecular interactions within elastic titin via addition of negative charges in a positively charged environment; as a consequence, titin becomes stiffer (*Figure 3B*). Conversely, the increased deacetylation of titin, e.g. by elevated SIRT1 activity following NAD^+/NAM treatment,[70] partially reverses this stiffening (*Figure 3B*). Additionally, titin acetylation may play a role in competitive PTM cross-talk with titin ubiquitination as both occur on lysine residues.[68] The exact role acetylation plays in titin regulation and its association with cardiometabolic disorders provides some exciting prospects for future research and therapeutics.

4.2 Oxidation–reduction

Oxidative stress is a generalized term for an imbalance between reactive oxygen species (ROS) and antioxidants. While low levels of ROS are required for normal cellular function and regulation, higher levels are associated with the pathogenesis of many diseases including cardiovascular disorders.[74] Excess ROS leads to the modification of various cellular processes including modifications of amino acids containing thiol groups (commonly cysteines) in either a reversible or irreversible manner. Titin is a prime target of oxidation and I-band titin oxidation–reduction modulates titin-based stiffness.[75]

Mass spectrometric analyses of cardiac titin have detected oxidation sites along the entire molecule (*Figure 4A*).[76] In mouse hearts exposed to oxidative stress, the highest relative increase in titin oxidation was found within I-band 'hotspots', specifically, the N2Bus and Ig domains of the distal I-band region.[76] However, both the location of oxidation site and the type of oxidation are critical in determining the consequence on titin stiffness: titin domains can undergo either S-glutathionylation, which leads to an increase in titin compliance, or disulphide bonding, which causes increased titin stiffness (*Figure 4B*). These modifications are reversible, e.g. under the influence of reductants like dithiothreitol or glutathione.[76–78]

Mechanistically relevant is that titin oxidation within Ig domains may rarely occur spontaneously but may rather require cysteines buried within the Ig domains (cryptic cysteines) to be exposed through domain unfolding[77,78]—a process we have coined UnDOx (unfolded domain oxidation).[76] If UnDOx occurs, these domains cannot refold back to their native state, which affects titin-based stiffness. Importantly, the type of oxidation that occurs at the cryptic cysteines determines the effect on stiffness.[76] UnDOx, through S-glutathionylation, prevents the Ig domain from refolding, thereby maintaining a longer contour length and lowering titin stiffness (*Figure 4B*, left).[76–78] Additionally, this unfolded oxidized state allows for the controlled homotypic interactions and in-register aggregation of the distal I-band region (*Figure 4C*), which may aid stiffness regulation and force propagation.[76] Alternatively, UnDOx can involve disulphide bond formation (*Figure 4B*, top right). A disulphide bridge can arise between any two (of a diad, triad, or quadripartite group of) cysteines within an unfolded Ig domain and prevent the extension of the domain to its full contour length,[78] therefore increasing the passive stiffness of titin.[76] It has been suggested that the formation of disulphide bonds in titin may fine-tune muscle work production by providing additional power during Ig-domain refolding under a (low) stretch force.[65] The formation of disulphide bonds in Ig domains may also be aided by intermediatory S-sulfenylation,[79] but due to the highly volatile nature of S-sulphenylation, there is yet to be any evidence showing that this occurs *in vivo*. On the other hand, disulphide bond formation also occurs within the N2Bus region of I-band titin (*Figure 4B*, bottom right) and contributes to increased titin stiffness by producing additional scaffolding to this normally disordered I-band region and preventing it from full extension.[80,81]

Both S-glutathionylation and disulphide bonding are a natural consequence of exertion and increased metabolism. Therefore, low levels are detected in cardiac titin under healthy conditions, but they rise with an increase in cardiac preload or afterload (*Figure 4C*).[76] However, in the case of cardiac ischaemia or chronic HF due to metabolic syndrome (e.g. HFpEF), there is a loss in the balance of these oxidative modifications and what was once a normal short-term regulatory process becomes an extensive modification and marker of disease (*Figure 4C*).[76] Targeting titin

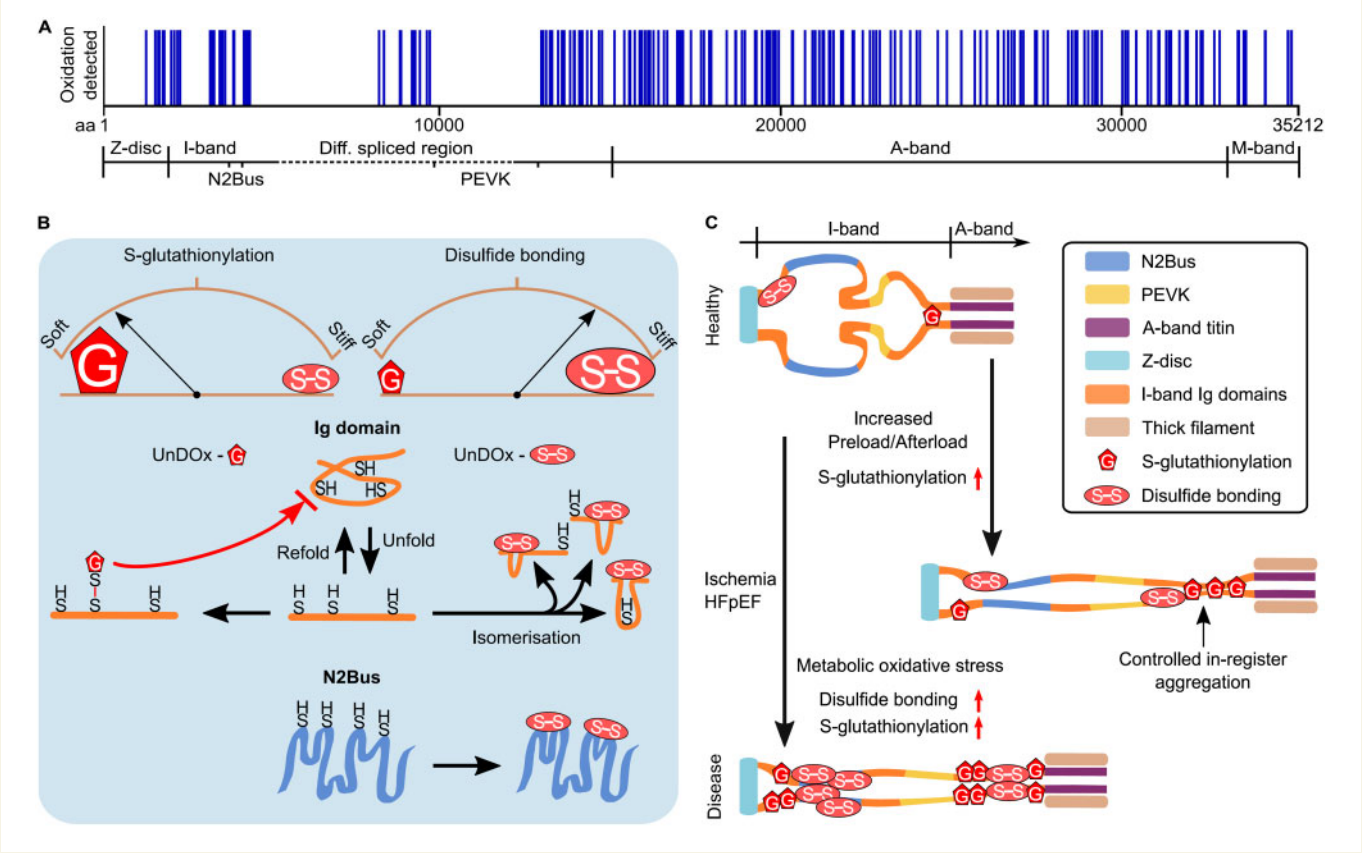


Figure 4 Titin oxidation sites and mechanisms of how S-glutathionylation and disulphide bonding alter titin stiffness in health and disease. (*A*) Titin oxidation sites detected in mouse heart tissue by mass spectrometry (data obtained from Ref.[76]). (*B*) Changes in titin stiffness are dependent on whether more S-glutathionylation or more disulphide bonding occurs in unfolded I-band titin domains. Increased compliance is mediated via the S-glutathionylation mechanism involving Ig domains and increased stiffness via the disulphide bonding mechanism involving Ig domains or the N2Bus. Brown arches depict titin spring stiffness. (*C*) Observed changes in I-band titin oxidation under cardiac stress (increased preload/afterload) or in diseased states (ischaemia, HFpEF) modulate titin stiffness in a complex manner. S-glutathionylation under cardiac stress can cause controlled in-register aggregation of distal I-band titin, whereas disease states can be associated with a general increase in titin oxidation. aa, amino acid; HFpEF, heart failure with preserved ejection fraction; Ig, immunoglobulin-like; UnDOx, unfolded domain oxidation.

oxidation therapeutically could be useful in the treatment for these cardiac conditions to modulate myocardial stiffness.

4.3 Phosphorylation–dephosphorylation

Phosphorylation is a well-established PTM in many cellular processes. In the heart, it regulates cardiac output on multiple levels. Phosphorylation is also the best-studied titin PTM, modulating the molecule's stiffness.[82] Over 300 titin phosphorylation sites have been detected using proteomic (mass spectrometric) techniques, with some phosphosites consistently being identified in multiple studies (*Figure 5A*).[83] At this stage, it appears that the titin phosphorylation-dependent changes in CM stiffness are related to where the titin spring becomes phosphorylated and the net protein charge present in that titin region (*Figure 5B*). Caution needs to be taken with this assumption, however, due to the bias in where along the titin molecule phosphorylation has been assessed historically.

The gigantic size of titin currently makes it impossible to recombinantly express the whole protein. Therefore, small regions of titin in specific locations, such as the Z-disc or M-band, or with unique mechanical properties, such as the PEVK and N2Bus regions, have been the focus of most low-throughput approaches (e.g. antibody-specific targeting or mutagenesis of phosphosites). Initial studies determined that phosphorylation within the Z-disc region could be attained by cyclin-dependent kinase (cdc2) and extracellular signal-regulated kinase (ERK)2[84] and similarly, cdc2 could also phosphorylate the M-band titin region.[85] Additionally, early studies showed that the titin kinase domain (TK) also required phosphorylation of tyrosine residue 32341, as well as the binding of Ca^{2+}/calmodulin, to enable activation (*Figure 5B*, right);[86] however, TK currently is considered an inactive pseudo-kinase.[87] Since then, two main regions, the N2Bus and PEVK segments, have been the focus of most titin phosphorylation studies. There is much compelling evidence to suggest that phosphorylation at these regions plays a crucial role in titin-stiffness regulation.

Interestingly, the phosphorylation of the N2Bus and PEVK regions has opposing effects (*Figure 5B*). Increased phosphorylation of the N2Bus causes increased titin compliance through an increase in persistence length, as determined by single molecule atomic force microscopy stretch experiments.[88,89] Conversely, phosphorylation of the PEVK region leads to increased titin stiffness.[90,91] The mechanisms behind this change in stiffness are still not completely clear; however, we have

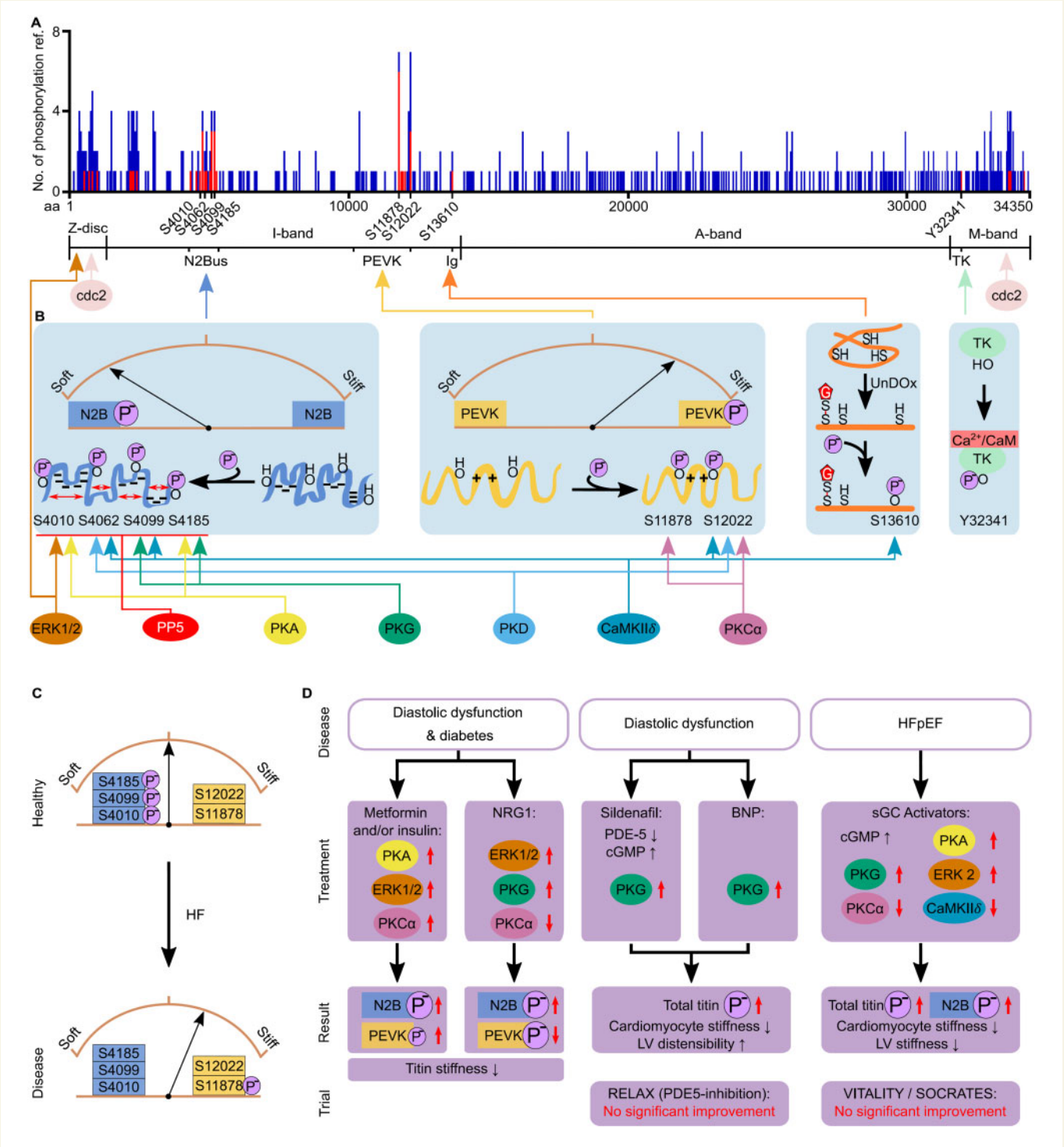


Figure 5 Where and how titin becomes phosphorylated, how this affects titin stiffness, and the role titin phosphorylation changes play in heart disease. (*A*) The number of times a titin phosphorylation site has been referenced (ref.) in the literature from either high-throughput (mass spectrometry; blue) or low-throughput, site-specific (antibody detection, mutagenesis; red) detection in human heart tissue. Data obtained from www.phosphosite.org.[83] Note that phosphosite S4062 is currently not detected in this database but has been added based on the publication by Ref.[95] (*B*) Specific phosphorylation sites detected in different regions of titin, known PKs/phosphatases involved, and differential effects of I-band titin phosphorylation on titin stiffness. (*C*) Hypo-phosphorylation of the N2Bus and hyper-phosphorylation of constitutively expressed PEVK titin are thought to increase titin stiffness in HF. (*D*) Proposed treatment strategies in pre-clinical tests aimed at correcting titin phosphorylation in animal models of heart disease, to reduce titin-based cardiac stiffness. 'Trial' highlights results of large clinical trials on human HF patients using the respective drugs. Brown arches depict titin spring stiffness. aa, amino acid; BNP, B-type natriuretic peptide; CaM, calmodulin; CaMKIIδ, Ca^{2+}/calmodulin-dependent protein kinase IIδ; cGMP, cyclic guanosine monophosphate; ERK, extracellular signal-regulated kinase; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; Ig, immunoglobulin-like; LV, left ventricular; NRG1, neuregulin 1; PDE-5, phosphodiesterase type 5; PKA, protein kinase A; PKCα, protein kinase Cα; PKD, protein kinase D; PKG, protein kinase G; PP5, serine/threonine protein phosphatase 5; sGC, soluble guanylyl cyclase; TK, titin kinase domain; UnDOx, unfolded domain oxidation.

previously suggested that the difference may be due to the N2Bus region and the PEVK having different localized net charges.[82] The N2Bus has a net negative charge and therefore, the introduction of additional negative phosphate groups could cause electrostatic repulsion within the N2Bus region, leading to improved extensibility and a decrease in titin-based force. Conversely, the positive net charge of the PEVK region (notably: only the constitutively expressed and not the differentially spliced segment, which has a net negative charge!) would see increased intramolecular interactions, reduced extensibility, and increased force (*Figure 5B*). Firm experimental proof for this 'charge theory' is still lacking.

To complicate matters further, there is an array of protein kinases (PKs) that can phosphorylate multiple sites in titin. At least four main phosphoserines have been identified in the human N2Bus region (*Figure 5B*): S4010, S4062, S4099, and S4185 (according to human titin consensus sequence, UniProKB #Q8WZ42-1). The first three are evolutionary conserved (mouse equivalents S3991, S4043, and S4080, respectively). These phosphosites have been identified using site-specific methods, such as mutagenesis of phosphorylated residues or antibody-specific targeting. The N2Bus sites have been determined to be phosphorylated by various PKs, such as PKA and PKG,[44,46,88,92] PKD,[93] ERK2,[89,94] and/or CaMKIIδ.[95,96] These phosphosites have also been shown to be dephosphorylated by serine/threonine protein phosphatase 5,[97] a highly regulated protein phosphatase with low basal activity.[98] Similarly, within titin's PEVK region, several sites have been detected, which are phosphorylated by PKCα, CaMKIIδ, and/or PKD, in particular conserved phosphoserines S11878 and S12022 (mouse equivalents S12742 and S12884).[44,46,90,91,93,95] In part, this would imply conflicting signalling as both PKD and CaMKIIδ phosphorylate N2Bus and PEVK titin—which in theory should have opposite effects on titin stiffness (*Figure 5B*). However, both kinases cause a decrease in titin-based force of isolated cardiac preparations,[89,93,95] suggesting the effect on N2Bus dominates. However, caution is required when interpreting these results, considering the number of phosphorylation sites that have been detected but not yet thoroughly investigated in titin (*Figure 5A*). Indeed, at least one of the above PKs, CaMKIIδ, can also phosphorylate Ig domains in I-band titin, but only after domain unfolding, and the process is further promoted by UnDOx through S-glutathionylation (*Figure 5B*).[76] This additional possibility for titin regulation by phosphorylation may aid in stabilizing the unfolded state of the domain and support mechano-chemical signalling events. In summary, titin-stiffness regulation by phosphorylation is complex and involves several signalling hotspots within I-band titin and various PKs/phosphatases; currently, it is the best-understood mode of titin regulation by PTMs backed by a large body of evidence.

4.3.1 Titin phosphorylation in heart disease

Irregular phosphorylation of titin is also highly implicated in HF. Frequently, HF is associated with pathologically increased myocardial and CM passive stiffness, at least in HFpEF patients.[40] Some of this stiffness increase can be explained by hypo-phosphorylation of phosphoserines within the N2Bus region of titin and hyper-phosphorylation of select sites within the PEVK region, e.g. S11878 (*Figure 5C*).[44,46,97] However, exceptions exist depending on the form of human HF and specific heart chamber investigated, or the animal model used.[44,82,95,99] A detailed review of this topic has recently been compiled.[82] Generally, it is more informative to determine site-specific titin phosphorylation rather than global titin phosphorylation, considering the huge number of potential phosphosites in the full-length titin protein.[82] However, given where phosphorylation occurs affects how titin stiffness is modulated, more site-specific information on titin phosphorylation is needed to better understand the diseased state. Altogether, reduced phosphorylation of titin's N2Bus region appears to be a frequent alteration in various HF types, and this alteration may be an important contributor to increased CM stiffness in disease.

4.3.2 Titin phosphorylation as a therapeutic target

Disruption in phosphorylation regulation is a common feature in cardiac diseases not only affecting titin but also many other cardiac proteins. This makes phosphorylation regulation an attractive target for therapeutic treatment. Pathologically increased myocardial passive stiffness is a sign of diastolic dysfunction often seen in HF patients when associated with comorbidities, including diabetes mellitus.[43,100] Interestingly, conventional therapeutics for diabetes, including metformin and insulin, have been found to improve diastolic function in these patients. Specifically, metformin and insulin increased the activity of titin-targeting PKs (ERK1/2; PKCα; PKA), improving phosphorylation of the N2Bus (while marginally increasing PEVK phosphorylation) and causing a reduction in titin-based stiffness (*Figure 5D*).[100,101] Similarly, chronic admission of the cardiac growth factor neuregulin-1 was shown to activate ERK1/2 and PKG while suppressing PKCα, resulting in hyper-phosphorylation of N2Bus and hypo-phosphorylation of PEVK titin, both of which are conducive in reducing titin stiffness (*Figure 5D*).[100]

In this context, a main focus has been on the cyclic guanosine monophosphate (cGMP)-PKG pathway, because dysregulation of this pathway is strongly associated with cardiac remodelling and HF. There are now multiple substances available which stimulate this pathway (see Ref.[102] for a recent, comprehensive review on the role cGMP plays in the heart). Phosphodiesterase-5A inhibitors (such as sildenafil) and B-type natriuretic peptide (BNP), which boost cGMP levels, showed promise as a potential treatment for diastolic dysfunction in pre-clinical tests (*Figure 5D*). In regards to titin, both sildenafil and BNP increased (total) titin phosphorylation, reduced CM stiffness and increased left ventricular (LV) distensibility in a dog model of diastolic dysfunction.[46,103] However, the use of PDE5 inhibitors in the RELAX trial failed to show significant improvement of diastolic function in HFpEF patients that received the treatment (*Figure 5D*).[104] Similar trials are underway with PDE9A inhibitors, which also aim to increase the cGMP concentration in the heart and benefit cardiac function.[105] In mouse models of diastolic function, PDE9A inhibition reduced LV diastolic stiffness through reduction of CM stiffness; however, titin phosphorylation was not measured.[106] Furthermore, soluble guanylyl cyclase (sGC) activators/stimulators are considered a promising treatment for HFpEF, having been found to raise cGMP levels leading to increased PKG (and also PKA and ERK2) activity, while reducing PKCα and CaMKIIδ activity.[107,108] This again resulted in increased N2Bus and total titin phosphorylation, reduced CM stiffness and reduced LV stiffness (*Figure 5D*). However, these results were not replicated in either the VITALITY or the SOCRATES clinical trials where no improvement in diastolic function was seen in HFpEF patients treated with sGC-stimulator.[109,110]

Although these treatments showed promise in the laboratory setting, discrepancies between dosages and metabolic differences in animals vs. humans may in part be the cause of clinical trial failures.[102] This highlights one of the many barriers in finding effective new treatments for HF. Additionally, the complexity of pathways, such as cGMP-PKG, means that the regulation of intermediate steps in the pathway can also be unintentionally changed and counteract the desired effect of the

treatment.[102] Therefore, increasing local cGMP levels within titin microdomains by exploiting compartmentalization might prove more useful, but such microdomains are yet to be determined. Regardless, these treatments may still be successful for a subset of patients with only modestly impaired cGMP-PKG signalling.

5. Titin-PQC

Maintaining a protein as large as titin over a lifetime requires a sophisticated PQC system. Titin is thought to be turned over at least every ~3 days in cell culture,[111] but the protein's half-life in adult mice is 2–3 weeks.[19,112] This enables sarcomere maintenance and CM remodelling during development and repair of stress-related protein damage in adulthood.[113] However, with advancing age, the cellular PQC machinery, including the titin-directed PQC systems, may slow down.[34] Consequently, defective titin protein could accumulate intracellularly and form cytoplasmic aggregates that are cytotoxic. As shown for other proteins, this may be one of the contributing factors to the development of cardiac disease in the elderly.[113]

An initial step in PQC is to stabilize or repair (refold) damaged (unfolded) proteins under stress with the aid of chaperones.[114] This appears to be particularly important for the elastic I-band titin as it contains many regions that unfold during a stretch. Small heat shock proteins (sHSPs) are chaperones that protect unfolded proteins from permanent damage but do not necessarily mediate the refolding (which requires an ATP-dependent chaperone).[115] The sHSPs alpha-B-crystallin (HSPB5) and HSP27 (HSPB1) are abundant in the cytosol of CMs and are also present at the Z-disc under physiological conditions.[115,116] Upon physiological stretch, as well as in failing hearts (e.g. ischaemia; cardiomyopathy), these chaperones are up-regulated and play a protective role by translocating to the N2Bus, N2A, and proximal/middle I-band Ig-domain regions of titin (*Figure 6A and B*).[117–119] There, they stabilize unfolded segments and prevent aggregation.[117,118,120] Moreover, the ATP-dependent HSP90 associates with titin's N2B region[97] and also with the N2A element if first methylated by the co-chaperone and methyltransferase Smyd2, a direct N2A ligand (*Figure 6A*).[121,122] This interaction protects I-band integrity.[121–124] The protective roles of HSPs on titin may include the prevention of pathological stiffening;[117] however, their stabilizing function could also impede the elastic properties of titin and contribute to increased stiffness.[123]

If chaperones fail to protect the protein, it then becomes marked for degradation and turnover.[125] For titin turnover to occur, this large protein likely needs to be (partially) released from its binding partners within the sarcomere.[34] Proteases, such as calpain-1 and matrix-metalloproteinase (MMP)-2 can bind to the proximal I-band and Z-disc or M-band regions of titin (*Figure 6A*) and aid in the pre-digestion of titin.[126–128] Under ischaemic conditions or in the presence of anthracyclines used in cancer treatment (e.g. doxorubicin), there is an increase in the expression and activity of these proteases leading to increased titin breakdown and cardiac remodelling (*Figure 6B*).[129–131] Protection from titin degradation by these proteases would be possible by using protease inhibitors, as is known from *in vitro* work.

Protease pre-digestion of titin is thought to further expose binding sites for targeted degradation. Two interlinked pathways, the ubiquitin-proteasome (UPS) and autophagy-lysosomal systems, regulate the subsequent degradation steps.[132] Through the UPS, damaged or aged titin molecules can become ubiquitinated by ubiquitin E3 ligase(s) and thus marked for proteasomal degradation. The E3 ligase mouse-double-minute 2 homolog interacts with the titin-capping protein telethonin,[133] but it is unknown whether it ubiquitinates titin. Other E3 ligases, the muscle ring-finger proteins (MuRF)-1 and -2, bind in the titin A-/M-band transition zone and to the TK (*Figure 6A*).[87,134–138] They preferentially ubiquitinate A-band proteins, including the TK.[137,138] In diseased states, protective effects on sarcomere proteins (including titin?) and contractile improvements have been observed with MuRF1-interfering small molecules as a potential therapeutic approach.[139] It is likely that one or more other, not yet identified, E3 ligase ubiquitinate titin. The TK is a well-established hub for protein–protein interactions as it binds the Nbr1/SQSTM1(p62) complex (*Figure 6A*),[140] which acts as an autophagy receptor for ubiquitinated proteins.[141] Autophagosome activity reduces with age; however, the role of autophagy in heart disease is incompletely understood:[142] both increased and reduced activities have been reported (*Figure 6B*). A more common finding is that the activity of the UPS is reduced in HF and cardiomyopathy, resulting in the accumulation of ubiquitinated but not degraded proteins.[143] These alterations also include titin (*Figure 6B*).[4,144,145] In end-stage failing human DCM hearts due to a *TTN*-truncating variant (TTNtv), increased ubiquitination of wt-titin was found, whereas truncated (tr-)titin proteins were barely ubiquitinated and stably expressed (*Figure 6B*).[4] Truncated titin accumulated in cytoplasmic aggregates.[4] Thus, titin-degradation pathways appeared to be deregulated in TTNtv hearts. The changes observed in TTNtv-DCM patient hearts also included reduced MuRF1 expression, whereas autophagy was not impaired or even activated.[4] Taken together, despite recent advances, many details of the titin turnover and degradation processes are still poorly understood.

5.1 PQC as a therapeutic point of influence

Several approaches have been made to target the PQC machinery therapeutically in cardiac diseases, which may also work for disorders related to titin dysfunction. Chaperone induction in mice has been shown to improve cardiomyopathy associated with muscular dystrophy[146] and perhaps can also be employed to enhance titin protection against ischaemia or DCM (*Figure 6B*).[117] Moreover, autophagy activators, such as spermidine, have been shown to be protective against age-related cardiovascular disease, including animal models of diastolic dysfunction.[147] Further, the inhibition of MMP-2 or calpain proteases might be advantageous as a prophylactic therapy during cancer treatment, e.g. to reduce doxorubicin-induced cardiotoxicity (*Figure 6B*). Given the dysfunction of the UPS in several forms of heart disease, modulators of the UPS may show promise as a potential treatment, as demonstrated for UPS inhibitors.[148] However, the toxicity of proteasome inhibitors may limit their therapeutic value.[143] Interestingly, in hiPSC-CMs with a TTNtv, UPS-inhibition raised wt-titin-protein expression (as well as tr-titin-protein content) and boosted contractility.[4] Increased wt-titin levels may be beneficial as they promote the formation of sarcomeres.[4,19] At this stage, caution needs to be taken with any speculations on the therapeutic benefit of protease or UPS inhibitors, due to their unspecific nature and our limited understanding of their complex cellular interactions.

5.2 PQC and pathomechanisms of TTN-truncation cardiomyopathy

Importantly, deregulated PQC of titin is central to the pathomechanisms of *TTN*-truncation cardiomyopathy.[4] Earlier, groundbreaking work established a titin truncation as the most frequent genetic cause of human DCM: 15–25% of most DCM patient cohorts studied carry a heterozygous TTNtv—by far the largest share among all cardiomyopathy gene variants known.[3,149,150] Similarly, a TTNtv is the most common genetic

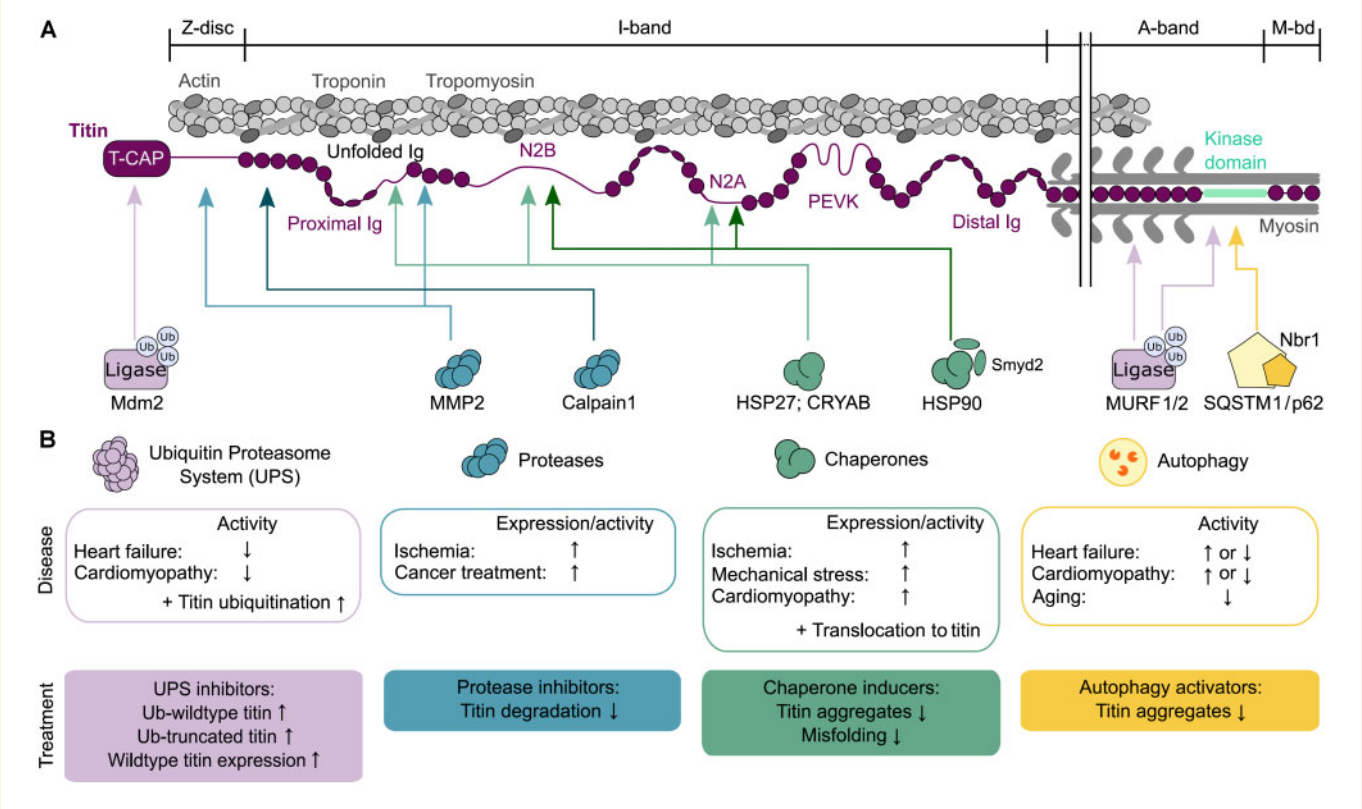


Figure 6 Titin-PQC pathways. (*A*) Interaction of different regions in cardiac titin (N2BA isoform) with components of the PQC machinery: UPS, ubiquitin-proteasome system (purple), proteases (blue), chaperones (green), and autophagy-lysosomal pathways (yellow). (*B*) Heart disease-related changes in PQC pathways, effects on titin, and suggestions for therapeutic approaches that could be taken to improve titin properties but are still speculative. CRYAB, alpha-B-crystallin; HSP27, heat shock protein 27; HSP90, heat shock protein 90; Ig, immunoglobulin-like domain; Mdm2, mouse-double-minute 2 homolog; MMP2, matrix metalloproteinase-2; MuRF1/2, muscle RING-finger protein-1/2; Nbr1, neighbor-of-BRCA1-gene-1; PEVK, segment rich in proline (P), glutamic acid (E), valine (V), and lysine (K) residues; Smyd2, SET and MYND domain containing protein 2; SQSTM1/p62, sequestosome 1; T-CAP, telethonin; Ub, ubiquitin.

predisposition in other types of inherited cardiac disorders, including restrictive, non-compaction, and peripartum cardiomyopathy, but also in acquired cardiomyopathies, such as those induced by alcohol abuse or cancer treatment.[151] Heterozygous TTNtv can occur anywhere along titin; however, the prevalence of having a *TTN* truncation and the odds of getting DCM from a TTNtv vary depending on the location of the pathogenic variant (*Figure 7A and B*). The odds ratio is highest if the truncation is in the A-band segment (prevalence in DCM 10.74% vs. control 0.24%, odds ratio 49.8), followed by truncations in constitutive exons of I-band *TTN* (*Figure 7B*).[3,149,150] In contrast, the odds ratio is lowest for central I-band titin (prevalence in DCM 0.24% vs. control 0.17%, odds ratio 1.5).[150] This is a consequence of the extensive alternative splicing of I-band *TTN*, where exon usage is low for all regions not expressed in the N2B titin isoform (*Figure 7A*). Thus, while TTNtv are found in 0.5–3% of the healthy (control) population, many of these variants occur in I-band titin exons with low percentage spliced-in (PSI) and do not cause DCM (*Figure 7B*).[3,149,150]

Previous suggestions about the possible pathomechanisms of TTNtv-DCM included sarcomere insufficiency,[152] modest nonsense-mediated decay of TTNtv-mRNA,[150] or translational deregulation,[153] whereas a poison-peptide (dominant-negative) mechanism was considered unlikely because truncated titin proteins were detected only in hiPSC-CMs[152] but not in adult heart tissue.[149–151] However, recent studies have unequivocally shown that tr-titin proteins are stably expressed in human end-stage failing, adult TTNtv-DCM hearts.[4,154] Their concentration reaches up to 50% of the total titin-protein pool but is highly variable. Strikingly, the higher the tr-titin-protein content of a TTNtv-heart, the younger the (adult) patients at the time of transplantation, suggesting that these proteins are disease-relevant.[4] The tr-titin proteins (unlike the wt-titin proteins) are not built into the sarcomeres at meaningful amounts but are sequestered in aggregates.[4] Thus, a poison-peptide mechanism is likely part of the pathomechanisms of TTNtv-DCM (*Figure 7C*).

Apart from a dominant-negative mechanism, TTNtv-DCM patient hearts contain less wt-titin protein than DCM hearts without TTNtv or non-failing (donor) hearts, which demonstrates titin haploinsufficiency.[4,154] However, nonsense-mediated decay of TTNtv-mRNA is not a prominent feature of TTNtv-DCM patient hearts (*Figure 7C*).[4,150,153] With wt-titin being lost, the CMs of TTNtv-DCM hearts also have fewer sarcomeres per unit area than non-TTNtv-DCM hearts,[4] confirming sarcomere insufficiency[152] and explaining chronic contractile deficiency.[4,152]

Recent findings[4,154] underscored that TTNtv-DCM hearts have a problem with intracellular PQC (*Figure 7C*; cf. section above). With increasing patient age, the UPS may be overwhelmed by the large amounts of tr-titin protein produced and partially shut down, whereas E3-ubiquitin ligases, such as MuRF1 become down-regulated.[4] Conversely, the intracellular aggregate formation may promote autophagy. Interestingly, disease modelling in hiPSC-CMs with a TTNtv

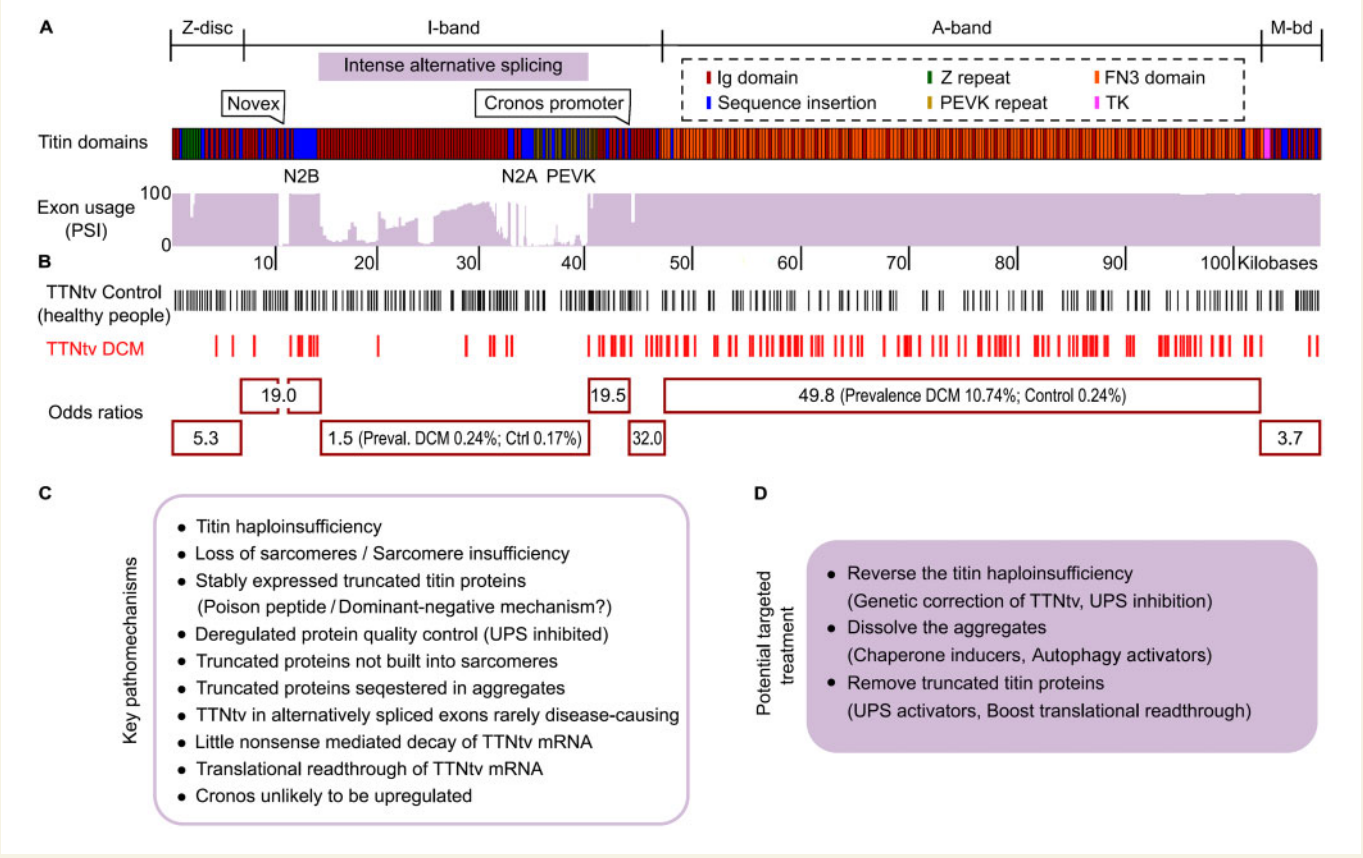


Figure 7 Pathomechanisms observed in human cardiomyopathy due to a *TTN*tv. (*A*) Titin domain architecture (according to human titin meta-transcript) and *TTN* exon usage in adult human hearts.[150] PSI above 90% indicates (nearly) constitutive expression, lower PSI values are due to differential splicing. (*B*) Location of heterozygous TTNtv detected by next-generation sequencing in the general population and in DCM patients, and odds ratio indicating the chance to get DCM if the TTNtv is in a specific titin region (marked by red boxes).[150] (*C*) Recently identified pathomechanisms of human TTNtv-DCM.[4] (*D*) Specific treatment strategies suggested.[4]

demonstrated that UPS-inhibition, but not autophagy-modulation, increased both tr- and wt-titin-protein content, with larger effects on tr-titin.[4] Reversal of the titin haploinsufficiency by UPS-inhibition improved TTNtv-hiPSC-CMs contractility, despite the raised tr-titin-protein content. If the TTNtv was repaired by CRISPR/Cas9 gene-editing, the titin haploinsufficiency was corrected, tr-titin proteins were absent, and contractility was fully recovered.[4] These findings can be exploited for new therapies of TTNtv-related cardiomyopathies (*Figure 7D*).

In summary, several key pathomechanisms come together in TTNtv-patient hearts, providing a rationale for phenotypic diversity. Disease mechanisms include titin haploinsufficiency as a life-long condition and truncated titin-protein enrichment with aggregate formation, as well as aberrant PQC, presumably both as additional late-onset pathomechanisms. Having a TTNtv may represent a major risk factor to get DCM later in life, especially when other stressors hit.

6. Conclusion and outlook

New technologies emerging over the last decade (e.g. next-generation sequencing, 2D and 3D culture of hiPSC-CMs, gene-editing, or 'omics') have allowed amazing progress to made also in the titin field. This has greatly improved our understanding of the role titin plays in HF and the disease mechanisms of *TTN*-truncation cardiomyopathy. However, much work still lies ahead of us. For example, *TTN* pathogenic variants include not only truncations but also missense variants whose pathophysiological relevance is only slowly evolving.[155] Missense and truncation variants can occur together in the same patient, typically amplifying the pathophenotype.[155] Moreover, the role of titin–isoform switch and titin PTMs in HF (notably HFpEF) remains fuzzy, as it is not yet clear whether these changes are a cause or consequence of the disease, and whether targeting these titin properties specifically (if possible) could improve the syndrome. The ageing population is also highlighting the importance of proteostasis regulated by PQC, and we are only just starting to appreciate how deregulated PQC in ageing and disease may affect titin. Similarly, the discovery that circular RNA of titin can also play a regulatory role in cellular function potentially opens the door to a whole new relevance of titin. Finally, this giant protein may still carry more molecular mysteries awaiting discovery. Perhaps we will know soon, how titin processes a stretch signal to boost active myocardial contraction and support increased cardiac output.

Acknowledgements

We thank the past and present members of the Linke lab for their research contributions to the titin topic.

Funding

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB1002 TPA08) and Interdisziplinären Zentrum für Klinische Forschung Münster (Li1/029/20) to W.A.L.

Data availability

This review article does not contain new original data.

Conflict of interest: none declared.

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