Gigantic Business
Titin Properties and Function Through Thick and Thin

Wolfgang A. Linke, Nazha Hamdani

Abstract: The giant protein titin forms a unique filament network in cardiomyocytes, which engages in both mechanical and signaling functions of the heart. TTN, which encodes titin, is also a major human disease gene. In this review, we cover the roles of cardiac titin in normal and failing hearts, with a special emphasis on the contribution of titin to diastolic stiffness. We provide an update on disease-associated titin mutations in cardiac and skeletal muscles and summarize what is known about the impact of protein–protein interactions on titin properties and functions. We discuss the importance of titin-isoform shifts and titin phosphorylation, as well as titin modifications related to oxidative stress, in adjusting the diastolic stiffness of the healthy and the failing heart. Along the way we distinguish among titin alterations in systolic and in diastolic heart failure and ponder the evidence for titin stiffness as a potential target for pharmacological intervention in heart disease. (Circ Res. 2014;114:1052-1068.)

Key Word: heart failure

With every heartbeat, our cardiomyocytes are exposed to variable degrees of stress and strain of both internal and external origin. The unique mechanical properties of these myocytes are determined by the sarcomeric cytoskeleton. The actin (thin) and myosin (thick) filaments of the sarcomere are mainly relevant for active force generation, whereas the titin filaments determine sarcomeric viscoelasticity. The giant protein titin, which is the focus of this review, has various roles beyond viscoelastic force generation: (1) it keeps the thick filaments centered in the sarcomere, allowing optimum active force development; (2) it is important for the assembly of the sarcomeres; (3) it participates in mechano-chemical signaling events via some of its binding partners; and (4) it may be crucial for the length-dependent activation of the contractile apparatus, which underlies the Frank–Starling law. During the past decade, we have learned that titin elasticity is highly variable in the developing and the adult healthy heart and that it can be pathologically altered in heart disease. These alterations greatly affect the extensibility and the diastolic passive stiffness of myocardium and presumably also the mechano-sensitivity. Moreover, TTN, which encodes the titin protein, has been recognized as a major human disease gene. In this context, the properties and functions of cardiac titin have become of interest in the continuing search for the molecular origins of human heart disease and the identification of novel potential targets for therapeutic intervention. In our review, we begin with a short clinical perspective establishing the link between diastolic stiffness and titin and briefly compare the importance of titin versus collagen for myocardial passive stiffness. We then cover some details of titin protein structure and elasticity, before summarizing how titin-binding partners affect titin properties and broaden the range of titin functions, with a special focus on the standing of titin in pathways of protein quality control. We continue with a current overview of titin mutations in human skeletal muscle and heart disease. The remainder of the review deals with the contribution of titin to diastolic stiffness and how it is modulated in normal and failing hearts by mechanisms such as titin-isoform switching, titin phosphorylation (including heart failure [HF]-related alterations of it), and oxidative modifications.

Clinical Context: Diastolic Function and Diastolic Abnormalities

Diastolic function has moved into the focus of HF research, because an increasing number of patients with HF (≥50%) present with diastolic rather than systolic dysfunction. Common abnormalities of diastolic function include impaired left ventricular (LV) relaxation, decreased LV distensibility, increased LV end-diastolic stiffness, and pericardial and right ventricular constraint. These abnormalities have been suggested to be contributing factors in diastolic HF or HF with a preserved ejection fraction (HFpEF). HFpEF is accompanied by high levels of mortality (5-year survival rate, 25%–35%) and morbidity (>50% of patients readmitted within a year) and is often associated with aging, hypertension, obesity, diabetes mellitus, and other metabolic risk factors affecting mostly elderly patients, particularly women. Next to abnormal diastolic function, HFpEF exhibits concentric hypertrophy and normal or reduced LV volume and thus differs from HF with a reduced ejection fraction. In this respect, the unique properties and functions of cardiac titin can be crucial for the understanding of diastolic heart failure and the identification of novel pathways of therapeutic intervention.

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fraction (HFrEF), which is characterized by abnormal LV systolic properties, eccentric hypertrophy, and progressive LV dilatation. Both phenotypes also differ in cardiomyocyte size and shape and in molecular composition.6,6 Contrasting with the numerous advances in HFrEF research, the pathophysiology of HFrEF remains poorly understood, despite its clinical importance. Improving our knowledge of the molecular origins of diastolic abnormalities is an important step toward understanding HFrEF pathophysiology and will eventually help the development of targeted treatments for HFrEF.

Titin as a Contributor to Diastolic Passive Stiffness

The elements contributing to myocardial diastolic stiffness include the titin filament network of the cardiomyocytes and the collagen fibers of the extracellular matrix, along with additional factors such as the diastolic Ca2+ levels (Figure 1A). Myocardial collagen is mainly composed of type I and III fibers, and their expression ratio affects diastolic ventricular compliance.7 Other mechanically relevant properties of collagen are the expression level, fiber geometry, glycation, and cross-linking. Any of these properties can potentially be altered in heart disease and modify diastolic stiffness. At the level of the cardiomyocytes, the stiffness of titin depends on the expression ratio of compliant versus stiff isoforms, the phosphorylation status of some titin spring elements, and certain oxidative modifications of elastic titin domains (Figure 1A), all of which are discussed in detail below. A general principle first proposed some 20 years ago9 is that titin/cardiomyocyte stiffness dominates total myocardial stiffness over most of the physiological sarcomere length (SL) range, whereas collagen/extracellular matrix stiffness becomes relatively more important beyond this range (Figure 1B). However, the stiffness contribution from titin, measured in absolute values, also continues to grow beyond physiological SLs and cannot be neglected under disease conditions. The respective contributions from the extracellular matrix and from titin to total diastolic stiffness presumably vary among different hearts, species, and types of heart disease, and a dynamic adaptation of both components takes place under different physiological and pathophysiological conditions.

### Layout in the Sarcomere and Domain Structure of Titin-Isoforms

Titin has a molecular mass of up to ≈3800 kDa and is encoded by a single gene containing 363 exons in humans.11 Throughout this review, we refer to the canonical amino-acid sequence of human titin according to UniProtKB entry Q8WZ42-1 (isoform 1; April 18, 2012; Version 4), which has 34,350 residues. The titin molecule extends from the Z-disk (NH2 terminus) to the M-band (COOH terminus) of the half sarcomere.12 Multiple alternative splicing events, particularly in the I-band region of titin (≈230 exons), result in 3 main isoform variants: N2B (≈3,000 kDa) and N2BA (≈3,200–3,800 kDa) in the heart and N2A in skeletal muscles (Figure 2).13 Rare isoforms of titin include the full-length variants Novex-1 and Novex-2, and the truncated Novex-3 (625 kDa).11 The canonical titin sequence has 9 immunoglobulin-like (Ig) domains in the Z-disk segment, interspersed with the Z-repeats and unique sequences. The elastic I-band portion contains 2 types of extensible segments: (1) tandem Ig-domain regions proximal-Ig (Ig10–Ig20), middle-Ig (Ig24–Ig76; alternatively spliced), and distal-Ig (Ig80–Ig95) and (2) intrinsically disordered structures, including a unique sequence within the cardiac-specific N2-B element (N2B unique sequence of titin [N2-Bus]) and the titin region rich in proline, glutamate, valine, and lysine (PEVK),13 which contains many 26- to 28-residue long motifs (PEVK1–PEVK31 in human titin). The PEVK repeats 27 to 31 are constitutively expressed in the full-length titin-isoforms. NH2-terminal to PEVK, the N2BA and N2A isoforms also contain the N2-A element. UniProtKB entry Q8WZ42-1 apparently misses several Ig domains in the proximal and distal-Ig segments, which probably have 15 and 22 domains, respectively, and it also underestimates the number of PEVK repeats.11,13 A-band titin comprises both Ig and fibronectin type-III (FNIII) domains and is characterized by the presence of 2 types of super-repeat: a 7-domain super-repeat occurring 6x (Ig97–Ig108/FNIII12–FNIII41) and an 11-domain super-repeat occurring 11x (Ig109–Ig139/FNIII42–FNIII1129; 2 Ig domains missing in UniProtKB entry Q8WZ42-1).14 Adjoining the M-band region, titin contains a kinase (titin kinase [TK]) domain.15 M-band titin has 10 Ig domains (Ig143–Ig152) interspersed with unique sequences. Altogether, the canonical titin sequence is predicted to have 132 FNIII and 152 Ig domains (Bang et al11: 132 FNIII/167 Ig domains).

### Molecular Mechanisms of Titin Extensibility and Elasticity

The molecular mechanism of titin extensibility and elasticity has been studied in detail.23,30 According to the generally accepted extension model for I-band titin, low stretch forces...
first straighten the short linkers between the Ig domains, causing the Ig segments to elongate; intermediate to high stretch forces then extend the PEVK segment, followed by (only in cardiomyocytes) the N2-Bus.16–18 Titin-Ig domain unfolding, which increases with the stretch force, contributes to sarcomeric viscoelasticity, although the number of Ig domains unfolded at any time is probably low at physiological SLs.19–21 If a molecular spring element is removed from titin by gene manipulation in mice, the cardiomyocytes become stiffer, confirming the mechanical function of these elements.22–24 Mouse hearts with a knockout of the N2-Bus show hypertrophy, and all of these hearts have diastolic dysfunction.

The force–extension relationship of I-band titin can be explained well by models of entropic elasticity, such as the wormlike-chain or freely jointed chain models.20,25 Descriptions of titin as an entropic spring consider that the molecule is a polymer undergoing thermal fluctuations, which cause it to adopt a coiled-up state of low energy but high entropy; to extend this molecule out of its high entropy state, a force is needed which must overcome the entropic recoiling force. However, titin elasticity also has nonentropic components, for instance, because of electrostatic and hydrophobic interactions within the PEVK domain.26–28 As will be discussed below, the elasticity of titin in the heart is highly variable in health and disease.

**Titin Functions Acquired Through Ligand Binding**

Titin binds to >25 other proteins (Figure 2B). This connectivity serves multiple roles, such as the build-up and maintenance of a stable sarcomeric structure, the optimization of myocyte contractility and viscoelasticity, the transcriptional repression or activation in the context of mechanosensing, and the myofilamentary protein quality control. Because many of these properties have been reviewed in detail elsewhere,1–3,10,15 we will cover them only briefly here. However, we will highlight recent findings revealing the involvement of titin in mechanisms of protein quality control.

For the titin NH2 terminus, a firm anchorage to the Z-disk is achieved presumably through the binding of the Z-repeats to α-actinin.29,30 The association of titin’s 2 NH2-terminal domains with small ankyrin-131 and with the titin-capping protein telethonin32,33 could be relevant for organizing the sarcoplasmic reticulum and the T-tubule, respectively, around the Z-disk.34,35 The well-documented strong binding of telethonin to the Ig1/Ig2 domains31 is not crucial for anchoring titin firmly to the Z-disk.36 Telethonin interacts, among others, with the E3 ubiquitin ligase mouse-double-minute-2-homolog,37 which links Z-disk titin to the ubiquitin-proteasomal degradation pathway (Figure 3). Interactions with nebulin/nebulette38 and filamin-C39 connect the first unique sequence at titin’s NH2 terminus to other components of the intra- and extrasarcomeric cytoskeleton (Figure 2B), providing additional structural stability. At the periphery of the Z-disk region, binding of titin-Ig8/Ig9 to obscurin40 and of Ig9/Ig10 to actin41 may stabilize this segment and support sarcomeric force transmission and mechanosensing at this site.

1-Band titin associates with putative mechanosensory protein complexes at the N2-B, N2-A, and PEVK elements (Figure 2B). The cardiac-specific N2-Bus interacts with the four-and-a-half-LIM-domain proteins (FHL1 and FHL2), which target protein kinases (PKs) to N2-Bus, such as those of the mitogen-activated protein-kinase–extracellular signal–regulated kinase-2 (ERK2) pathway,42 as well as metabolic enzymes.43 The N2-Bus–FHL1–MAPK complex could be central to a strain-sensing mechanism mediating the cardiac hypertrophic response to increased load.44 Mechanosensory activity has also been suggested for the N2-A element in complex with muscle-ankyrin repeat proteins, which include cardiac-ankyrin repeat protein, diabetes–related ankyrin repeat protein, and ankyrin repeat domain–containing protein-2.44 The PEVK segment associates with actin,45,46 which entails a viscous force in working sarcomeres.45,47 PEVK also binds Ca2+ ions48 and presumably various SH3 domain–containing proteins,49 which might again be relevant for myofilamentary mechanosensitivity. Parts of I-band titin bind tropomyosin in vitro,50 but the functional implications of this interaction are unknown. Moreover, elastic titin contains a binding site for calpain-1 within the proximal-Ig region (Ig13), thus affording a reservoir to the myofibrils, from which the peptidase can be released...
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according to cellular requirements (Figures 2B and 3). Titin also binds skeletal muscle–specific calpain-3 at the N2-A element,52,53 as well as matrix metalloproteinase-2 at the Z-/I-band region54; both proteinases cleave titin at various sites. Relatively high basal proteolytic activity toward I-band titin could be the reason why protein gel electrophoresis of heart tissue usually reveals, next to intact titin (T1, \( \geq 3000 \) kDa), \( \geq 1 \) proteolytic titin fragments (T2, 2000–2500 kDa) containing the molecule's A-band portion but lacking most of the I-band region.

Molecular chaperones involved in complex formation at certain I-band titin domains have been shown to protect the spring region. One such mechanism targets the N2-A element, where a unique sequence encoded by titin-exon 104 binds lysine methyltransferase SET and MYND domain–containing protein-2 (Smyd2), and both proteins form a complex with heat shock protein-90 (HSP90; Figures 2B and 3).55 Smyd2, although not methylating the N2-A element, mono-methylates HSP90 at lysine K615 (dimerization domain). Importantly, only the methylated HSP90 can interact with N2-A (Figure 3).55 The Smyd2–methyl-HSP90 complex stabilizes N2-A and helps maintain the sarcomeric I-band structure. Knockdown of Smyd2 in zebrafish specifically disrupts the Z-/I-bands and impairs muscle and heart function.55,56 To elucidate the conformational changes in methyl-HSP90, which presumably trigger the binding to N2-A, an atomic structure of the complex with Smyd2 would be useful.

Another recently identified protective mechanism for I-band titin domains involves the small HSPs (sHSPs) HSP27 (HSPB1) and \( \alpha \)B-crystallin (HSPB5). These chaperones are abundantly expressed in skeletal and cardiac myocytes and are further induced by stress, such as that associated with cardiac ischemic injury or end-stage failure. HSP27 and \( \alpha \)B-crystallin are translocated under stress preferentially to the sarcomeres, in particular, to the I-band region. The 2 sHSPs bind to Ig domain–enriched segments of the titin spring, including the proximal-Ig region and the N2-A element, but not to the intrinsically disordered PEVK domain (Figure 2B).57,58 The sHSPs also bind to the N2-B element,57,58 where \( \alpha \)B-crystallin mechanically stabilizes the N2-Bus59 (Figure 3) and, together with HSP27, could help organize macromolecular complexes.58 In the presence of \( \alpha \)B-crystallin, titin-Ig domains show a reduced probability of unfolding in vitro.57,59 However, of particular physiological relevance is that the interaction of sHSPs with I-band titin is much enhanced by factors that increase titin-domain unfolding, such as stretch and expression of the stiff N2B isoform.58 Unfolded proteins typically expose cryptic hydrophobic sites and risk aggregation and loss of function. The unfolded Ig regions of titin in cardiomyocytes, however, are protected against aggregation by the sHSPs, and both the Ig-aggregation propensity and the sHSP-mediated protection against it are enhanced by acidic stress (Figure 3).58 In contrast, the disordered titin spring elements (N2-B, PEVK) are

Figure 2. Domain structure of titin-isoforms and binding sites of titin ligands. A, Layout of titin-isoforms in the cardiac half-sarcomere, showing stiff N2B coexpressed with compliant N2BA.
b, Domain structure of the canonical titin sequence (UniProtKB identifier Q8WZ42-1), main splicing pathways (green lines), and binding sites of known titin ligands. Domain numbers in blue refer to UniProtKB entry Q8WZ42-1, black numbers in parentheses to Ref. 11. Bin1 indicates bridging integrator-1; CMYA5, cardiomyopathy-associated protein-5; Ex, exon in genomic human titin sequence; FH1 and 2, four-and-a-half-LIM-domain protein-1 and -2; FNIII, fibronectin type III–like domain; HSP27, heat shock protein-27; HSP90, heat shock protein-90; Ig, immunoglobulin-like domain; MARPs, muscle ankyrin repeat proteins; MURF1/2, muscle-specific RING-finger protein-1/-2; MyBP-C, myosin-binding protein-C; Nbr1, neighbor of BRCA1 gene-1; P, titin phosphosite (kinase known); PEVK, titin region rich in proline, glutamate, valine, and lysine; sANK-1, small-ankyrin-1 isoform; Smyd2, SET and MYND domain–containing protein-2; and TK, titin kinase domain.
inherently protected against aggregation and stretched PEVK is not recognized by sHSPs as unfolded. Isolated human cardiomyocytes, in which titin-Ig unfolding is promoted, become stiffer under acidic conditions, but do not so in the presence of HSP27 or αB-crystallin.58 In failing human hearts, the 2 sHSPs colocalize with titin spring elements (except PEVK and distal-Ig domains), unlike in healthy hearts, where they are in the cytosol and at the Z-disk. These findings suggest that aggregation of unfolded titin-Ig domains under mechanical and acidic stress stiffens cardiomyocytes, but that sHSPs translocate to these domains to prevent this aggregation and protect against the stiffening, thus preserving diastolic function.

Consistent with this, mouse hearts deficient in αB-crystallin and HSPB2, when exposed to ischemic insults, have normal systolic function but increased passive stiffness and diastolic dysfunction.60 The obvious beneficial effects of sHSPs on titin-based passive stiffness may be worth exploring as a potential therapeutic option especially in patients with HFpEF.

Within A-band titin, interactions of the FNIII domains with myosin and of the first Ig-domain in each large super-repeat with myosin-binding protein-C14,61 provide a strong connection with the thick filaments (Figure 2). However, sarcomere stretch causing the titin springs to pull on the thick filaments could give rise to minute changes in the axial orientation of the myosin heads.62 This could in theory promote active force development and, together with changes in the transversal interfilament lattice, be a factor in the phenomenon of length-dependent activation, the molecular basis for the Frank–Starling relationship.10,62,63 Furthermore, the 11-domain super-repeat gives rise to a 43- to 44-nm periodicity, which is essentially the same as that provided by the myosin heads and myosin-binding protein-C. Therefore, A-band titin was suggested to be a molecular blueprint for A-band assembly.14 However, new data have challenged this concept.64

M-band titin, including the TK and several adjoining domains, is established as a hotspot for protein–protein interactions (Figure 2B). Deletion of this region in mouse models or in cardiac muscle cell culture disrupts the sarcomeric structure.65,66 A stretch of 3 titin domains just outside the M-band (Ig141/Ig142/FNIII132) interacts with muscle-RING-finger proteins 1 and 2 (MURF1/2).67,68 MURFs are E3 ubiquitin ligases whose members have a dual function in myocytes by marking proteins (perhaps also titin) via ubiquitination for proteasomal degradation and by regulating a cardiac hypertrophic program triggered by serum response factor.69,70 The TK domain of titin belongs to the family of Ca2+/calmodulin-dependent myosin light-chain kinases15,71 and is activated by mechanical force.72 Activated TK binds the zinc-finger protein neighbor of BRCA1 gene-1, which forms a signaling complex with Sequestosome-1.69 The latter associates with MURF2 and ubiquitin and links to the autophagy/lysosomal pathway (Figure 3).15 Additional binding partners of titin at the COOH terminus are FHL2 and calpain-3, suggesting some shared functionality between M-band and I-band titin elements. Important for the stability of the M-band probably is the interaction of titin domain Ig146 with myomesin, which in turn binds myosin.1,73,74 Furthermore, the association of a unique sequence between Ig149–Ig152 to lamin might occur in the nucleus (ie, be relevant only for nuclear titin), where this protein forms structural filaments.76 Two ligands of the last Ig-domain in titin (Ig152) are myospryn
and obscurin/obscurin-like 1, both of which have structural and signaling functions. Obscurin and myospryn have also been observed at the Z-disk, raising the possibility that these proteins shuttle between 2 sarcomeric hubs depending on developmental stage or disease condition.

**Titin as a Major Human Disease Gene**

The titin gene locus 2q31 has been known for >15 years to be mutated in both human skeletal muscle and heart disease. However, only recently TTN has been established as a major human disease gene, made possible by novel high-throughput sequencing methodology. As of December 2013, to our knowledge, some 125 disease-associated mutations have been reported for human titin. They are listed in Online Table I (mutations at protein level, with annotations), and Figure 4 illustrates their positions along the canonical human titin sequence (UniProtKB entry Q8WZ42-1). TTN mutations have been found in patients with dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy, arrhythmic right ventricular cardiomyopathy, and various types of skeletal myopathies. Already the first reports on human titin mutations suggested that they can occur in all parts of the molecule and the 125 currently known mutations are indeed scattered all over the TTN gene, without a clear hotspot (Figure 4). A notable exception may be a rare muscle disease, hereditary myopathy with early respiratory failure, probably is a polymorphism. The 8 arrhythmogenic right ventricular cardiomyopathy–associated mutations identified to date are distributed all over the TTN sequence. Some TTN mutations disrupt the interaction of the protein with binding partners (Online Table I) and may compromise the functions of titin in myofibril assembly or mechanical signaling. Specific mutations in I-band Ig domains could weaken the structural integrity of these domains and thus be disease causing, as suggested for a proximal titin-Ig-domain mutated in arrhythmogenic right ventricular cardiomyopathy. For most of the mutations, however, the molecular pathomechanisms are not understood. Almost all affected patients carry a heterozygous TTN mutation and in many cases one cannot be sure whether the mutation is disease causing or predisposing the carrier to heart/muscle disease only in the presence of additional mutation(s) in another or in the same gene. A few TTN mutations associated with heart or skeletal muscle disease have also been identified in the general population represented by the 1000 Genomes Project. In fact, polymorphisms in the TTN gene are relatively abundant in the healthy population. It remains to be seen whether any truncated titin mutant is actually expressed at the protein level in affected patient hearts. In a DCM patient mimicking knockin mouse model of truncated A-band titin, an extremely low amount of truncated protein was detected in the hearts of animals heterozygous for the mutation. Whether the truncated titin variant is built into the sarcomere is another (difficult to resolve) issue. In summary, TTN has emerged as an important disease gene in human skeletal and cardiac myopathies. TTN mutations account for a large share of inherited cases of DCM. However, the pathophysiology of titinopathies is only beginning to be elucidated.

Figure 4. Titin mutations identified in human heart and skeletal muscle disease. Titin-domain arrangement and positions of mutations according to the canonical titin sequence (UniProtKB entry Q8WZ42-1). An annotated list of these mutations (current as of December 2013) is provided in Online Table I. ARVC indicates arrhythmic right ventricular cardiomyopathy; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; PEVK, titin region rich in proline, glutamate, valine, and lysine; RCM, restrictive cardiomyopathy; and TK, titin kinase domain.
Adjustment of Titin Stiffness by Isoform Switching in Nonfailing and Failing Hearts

For the remainder of this review, we focus on the elastic function of the I-band titin springs and how it is dynamically adjusted in health and disease. One type of stiffness adjustment lasting days to weeks relates to isoform transitions of cardiac titin (Figure 5). The expression ratio of the N2BA and N2B isoforms differs among mammalian species. The N2BA proportion in the heart is relatively low (5%–20%) in adult rodents, whereas it is much higher in larger adult mammals.103,104 Healthy adult human hearts express 30% to 40% N2BA and 60% to 70% N2B isoforms.105 Because N2BA is longer/more compliant than N2B (within the elastic I-band region), the ratio of coexpressed titin-isoforms affects the passive SL-tension curve of the cardiomyocytes, which therefore varies greatly among species and is generally steeper in small rodents than in larger mammals.103,104 Moreover, the right heart typically expresses a higher proportion of N2BA than the left heart and the atria have relatively more N2BA than the ventricles.104 In addition, the titin-isoform composition is developmentally regulated. The prenatal heart initially expresses a unique, compliant, fetal N2BA isoform (≈3.7 MDa).106 This isoform is replaced during perinatal development by smaller, less compliant, N2BA variants and the N2B isoform, causing a gradual increase in titin-based passive stiffness.106–108 Thus, adult cardiomyocytes are stiffer than fetal cardiomyocytes (Figure 5B).

Titin-isoform switching also accompanies various types of HF (Figure 5A). An increased proportion of the compliant N2BA variants was observed in end-stage failing human hearts, including those from ischemic cardiomyopathy,105 non-ischemic DCM,109,110 and other patients with HFrEF,111 compared with nonfailing donor hearts (45%–50% versus ≈30%). In patients with aortic stenosis, the N2BA:N2B expression ratio was somewhat higher than in healthy control hearts in one study,111 but unaltered112 or reduced113 in other studies. In the hearts of mice exposed to transverse aortic constriction, the N2BA:N2B ratio was significantly increased in comparison with healthy mouse hearts.114 Relatively more N2BA than in healthy hearts was also observed in rat hearts with chronic ischemic cardiomyopathy because of a ligation of the left anterior descending coronary artery (LAD model).105 Likewise, a hypothyroid rat model showed elevated cardiac N2BA:N2B expression ratios compared with normothyroid rat hearts.115 The direction of a titin-isoform switch, if any, is less well established in HFpEF. An early report demonstrated a reduced N2BA:N2B ratio in a limited number of HFpEF versus HFrEF patient hearts6 and a follow-up study showed an increased ratio in advanced HFpEF compared with nonfailing donor hearts.111 In contrast, a hypertensive dog model with diastolic dysfunction revealed a reduced cardiac N2BA:N2B ratio versus healthy dogs116,117 and slightly decreased N2BA proportions appeared in the spontaneously hypertensive

Figure 5. Directions, causes, and consequences of titin-isoform switching in the heart. A, Directions of titin-isoform transitions reported for failing human or animal hearts, compared with healthy hearts. B, Consequences of titin-isoform switching for passive tension. C, Mechanisms altering the cardiac titin-isoform expression pattern. AngII indicates angiotensin II; AS, aortic stenosis; DCM, dilated cardiomyopathy; G, small G-protein; GPCR, G-protein–coupled receptor; HFpEF, heart failure with a preserved ejection fraction; HFrEF, heart failure with a reduced ejection fraction; ICM, ischemic cardiomyopathy; LAD, left anterior descending coronary artery ligation; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol-3-kinase; RBM20, RNA-binding motif protein-20; T3, thyroid hormone; TAC, transverse aortic constriction; and TR, thyroid hormone receptor.
A reduced N2BA:N2B expression ratio was also seen in tachypaced dog hearts.\textsuperscript{119,120} Taken together, eccentric remodeled hearts with systolic dysfunction (DCM/HFpEF/chronic ischemic cardiomyopathy) frequently seem to express higher N2BA proportions than healthy hearts (Figure 5A), thereby reducing myofibrillar passive stiffness (Figure 5B). An exception is the tachypaced dog HF model, which increases the proportion of stiff N2B titin.\textsuperscript{119,120} Patients with HFpEF also express increased N2BA:N2B ratios, which lower titin-based stiffness. In contrast, characteristic for concentric remodeled hearts (compensated hypertrophy) with diastolic dysfunction following from hypertensive heart disease may be a decreased N2BA:N2B ratio causing elevated myocyte stiffness (Figure 5A and 5B). Because the pattern of titin isoform expression correlates with parameters of diastolic function, such as the end-diastolic volume,\textsuperscript{110} it is likely that the titin isoform ratio does affect overall myocardial passive stiffness. Not all relevant studies, however, have reported altered titin isoforms in patients and HF models. No differences in titin isoform ratio, compared with healthy hearts, were seen in (1) a mixed cohort of human congenital cardiomyopathy hearts\textsuperscript{21}; (2) right ventricles from patients with pulmonary arterial hypertension\textsuperscript{122}; (3) human DCM and HCM hearts specially selected for their titin isoform pattern\textsuperscript{21}; (4) a small cohort of human HCM hearts\textsuperscript{124}; and (5) hearts of a metabolic risk–induced HFpEF model with hypertension and diabetes mellitus, the Zucker spontaneously hypertensive fatty l diabetic rat (lean or obese).\textsuperscript{125} In conclusion, titin isoform transitions add to the modification of total myocardial passive stiffness in heart development and presumably in a subset of patients with HF. The titin isoform shift can go in different directions in different types or stages of heart disease.

Recent efforts have provided mechanistic insight into principles that underlie the titin isoform switch in developing and failing hearts (Figure 5C). Thyroid hormone (T3) signaling was found to promote the titin isoform transition from fetal N2BA to stiff N2B in cultured embryonic rat cardiomyocytes.\textsuperscript{126} In turn, reducing the T3 level of adult rats increased the proportion of large N2BA isoforms in the heart.\textsuperscript{115} The N2B isoform could be boosted in cultured fetal rat cardiomyocytes by activating the phosphatidylinositol-3-kinase (PI3K)–Akt–mammalian target of rapamycin pathway (Figure 5C), through either T3\textsuperscript{132} or insulin,\textsuperscript{127} and somewhat also through angiotensin II.\textsuperscript{128} This developmental titin isoform shift was prevented by inhibiting Akt via blockade of PI3K or by blocking mammalian target of rapamycin with rapamycin.\textsuperscript{126,127} Reduced levels of T3, insulin, or other agonists of the PI3K–Akt–mammalian target of rapamycin pathway could well be responsible for the increased N2BA:N2B titin expression ratio frequently observed in end-stage human HF.

The splicing factor RNA-binding motif protein-20 (RBM20) was identified as a crucial player acting on titin as a splicing repressor (Figure 5C)\textsuperscript{128} while also regulating the splicing of 30 other cardiac proteins.\textsuperscript{129} Aberrant isoforms of these proteins were expressed in a rat model with a missense mutation in RBM20 and in a patient with severe DCM caused by mutation in the RNA binding domain of RBM20.\textsuperscript{129} In rat hearts homozygous for the mutation, an unusually large N2BA-titin isoform (3.83 MDa) was present, whereas the N2B isoform was missing. Rats and humans heterozygous for the mutation coexpressed large N2BA titins and low amounts of N2B. Thus, RBM20 is required for the expression of the adult N2B isoform (Figure 5C), and lack or dysfunction of RBM20 causes mis-splicing and the expression of aberrant large (not normal fetal!) N2BA isoforms.\textsuperscript{129,130} Future work may want to explore whether cross-connectivity exists between the (T3/insulin)–PI3K–Akt–mammalian target of rapamycin pathway and titin splice factor(s).

**Regulation of Titin Stiffness by Phosphorylation: Facts and Fancy**

The passive stiffness of the cardiomyocytes is modulated effectively on a short time scale by post-translational modifications of titin, including phosphorylation and certain oxidative modifications. Phosphorylation of sarcomeric proteins is well established as an important modifier of cardiac contractile function. Prime examples are troponin-I and myosin-binding protein-C, the phosphorylation of which alters the $\text{Ca}^{2+}$ sensitivity of the myofilaments. However, titin could well be the cardiomyocyte protein most affected by phosphorylation. Proteomic databases list hundreds of potential phosphosites in titin searchable by web-based resources, for example, http://gygi.med.harvard.edu/phosphomouse/index.php\textsuperscript{131} or http://cpr1.sund.ku.dk/cgi-bin/PTM.pl.\textsuperscript{132} The effects of site-specific phosphorylation on titin function and cardiomyocyte/myocardial performance are only beginning to be understood.

Efforts are in progress to identify the kinases that phosphorylate titin. Proline-directed kinases, such as ERK1/2 and cyclin-dependent PK-2, were shown earlier to phosphorylate specific motif repeats (XSPAR; KSP) within the Z-disk and M-band regions of titin (Figure 2B).\textsuperscript{133–135} The functional consequences of these modifications are still unknown, except that phosphorylation of a unique sequence between Ig147 and Ig148 regulates binding of M-band titin to bridging integrator-1.\textsuperscript{136} PKA was the first enzyme reported to phosphorylate the elastic I-band region of titin in vitro, specifically, the N2B element, and this modification was also inducible by $\beta$-adrenergic stimulation.\textsuperscript{136} This finding has since been confirmed for various mammalian species, including humans.\textsuperscript{123,137–140} Recent work on titin phosphorylation has focused mainly on the disordered spring elements, N2-Bus and PEVK, both of which are substrates of several major PKs activated by well-studied signaling pathways (Figure 6). N2-Bus is phosphorylated in vitro not only by PKA but also by cGMP-dependent PKG,\textsuperscript{123,140} ERK2,\textsuperscript{141} and the $\delta$-isoform of $\text{Ca}^{2+}$/calmodulin-dependent PK-II, $\text{Ca}^{2+}$/calmodulin-dependent protein kinase-Ib $\beta$ (CaMKIib)$\beta$,\textsuperscript{142,143} The PEVK region is also phosphorylated by CaMKIib$\beta$,\textsuperscript{142,143} and PKC$\alpha$.\textsuperscript{144}

All PKs phosphorylate >1 residue in N2-Bus and PEVK, respectively (Figure 6). The first phosphoserine identified within N2-Bus using a site-specific method (back-phosphorylation by PKA/PKG and autoradiography of recombinant wild-type and mutant N2-Bus) was S4185 of human titin, which is not conserved in other mammalian species.\textsuperscript{146} This serine was confirmed to be PKA/PKG dependently phosphorylated in a recent mass spectrometric analysis of recombinant N2-Bus.\textsuperscript{123} This analysis identified additional phosphosites within N2-Bus, including evolutionary conserved S4010.
(PKA-dependent) and S4099 (PKG-dependent), as well as nonconserved S4065 (PKA-dependent) and S4092 (PKG-dependent). ERK2 was also shown in vitro to phosphorylate S4010 within the N2-Bus, along with nonconserved S3918 and S3960.\textsuperscript{144} Interestingly, the ERK2 phosphosites on N2-Bus are shielded by the N2-Bus binding partner FHL1, suggesting an additional level of regulation important for the putative strain-sensing function of this titin domain.\textsuperscript{144} Several potential CaMKII phosphosites were identified by mass spectrometry of recombinant human N2-Bus, most of them not conserved across species.\textsuperscript{142,143} One evolutionary conserved serine within human N2-Bus, S4062, was confirmed using a site-directed method (antibodies against phospho-S4062 in immunoblots of recombinant N2-Bus or heart tissue).\textsuperscript{142} A first study of quantitative titin phosphoproteomics in vivo used heart tissue from the stable isotope labeling of amino acids in cell culture mouse\textsuperscript{145} mixed with that of wild-type or CaMKII-δ double-knockout mice.\textsuperscript{142} Results suggested that CaMKII phosphorylates ≥17 different sites along the titin molecule, among them 3 cross-species conserved sites within PEVK, T12007, S12009, and S12022. These 3 sites were confirmed via back-phosphorylation by CaMKIIδ and autoradiography of human wild-type and mutant PEVK.\textsuperscript{142} S12022 was identified as a CaMKIIδ-dependent site in another mass spectrometry–based in vitro study, together with conserved S11878 and nonconserved T11969, T11922, and T11932.\textsuperscript{142} However, S11878 was excluded as a CaMKIIδ-site in the study from our laboratory, based on the results of a web-based prediction tool and the reactivity of a phosphosite-specific antibody.\textsuperscript{146} PKCα specifically phosphorylated 2 conserved serines of the PEVK region, S11878 and S12022, in vitro and in heart tissue, with S11878 being suggested as the preferred PKCα site.\textsuperscript{146} Future work will undoubtedly detect additional phosphosites in titin, reveal the responsible kinases, and aim to assess the relevance of these modifications for cardiomycocyte structure and function.

PKA, PKG, and CaMKIIδ all act to decrease titin-based passive tension, as evidenced in mechanical experiments on demembranated isolated cardiomycocytes or myocardial strips incubated with the respective PK (Figure 7A, 7B, and 7D). ERK2 presumably reduces the passive tension as well,\textsuperscript{144} but this still needs to be shown directly. The PKA effect on passive tension was reported for rat,\textsuperscript{156,157} cow,\textsuperscript{158} mouse,\textsuperscript{146} dog,\textsuperscript{152} and human\textsuperscript{131,132,139} cardiomycocytes (Figure 7A), the PKG effect for human,\textsuperscript{111,140} dog,\textsuperscript{145} and rat\textsuperscript{125} cardiomycocytes (Figure 7B). In contrast, PKCα-mediated phosphorylation increased the passive tension of skinned myocytes from mouse and pig heart, an effect amplified by pretreatment with protein phosphatase-1,\textsuperscript{144} whereas it did not alter the passive tension of dog cardiomycocytes\textsuperscript{177} (Figure 7C). The CaMKIIδ effect on passive tension was seen with skinned mouse cardiomycocytes,\textsuperscript{142} and additional evidence for this effect came from mechanical measurements using genetically modified mouse hearts: cardiomycocytes deficient in the 2 CaMKII isoforms, CaMKIIδ and CaMKIIγ (CaMKII-KO), had increased passive tension, whereas those of CaMKIIδ-overexpressing transgenic mice (CaMKII-TG) had reduced passive tension, compared with those of wild-type mice (Figure 7D).\textsuperscript{142} In summary, most kinases shown to phosphorylate the titin springs lower cardiomycyte passive tension, whereas PKCα has the opposite effect, albeit not in all species.

The mechanism behind the effects of phosphorylation on titin-based passive stiffness was addressed in single molecule mechanical studies using the atomic force microscope. The force–extension behavior of recombinant N2-B or PEVK constructs was investigated to parameterize their elasticity in the absence or presence of cGMP-PKG and PKCα, respectively.\textsuperscript{140,144} An important parameter describing the entropic elasticity of biopolymers such as titin is the persistence length, which resembles a (statistical) segment length and is a measure of the polymer’s bending rigidity.\textsuperscript{147} Simply speaking, the higher the persistence length, the lower the force needed.
CaMKIIδ phosphorylates both N2-Bus and PEVK, the net mechanical effect of this kinase on titin stiffness could have been zero. However, because CaMKIIδ decreased cardiomyocyte passive tension in the experiments (Figure 7D),142 the mechanical effect on N2-Bus seemed to prevail over that on PEVK. A possible explanation is that CaMKIIδ is enriched at the Z-disk region,149 such that the N2-Bus element experiences higher CaMKIIδ concentrations than the PEVK domain, as it is closer to the Z-disk.150 Another possibility is that CaMKIIδ has a different affinity for the N2-Bus and PEVK substrates. Alternatively, CaMKIIδ could of course alter the stiffness of N2-Bus and PEVK in the same direction by increasing the persistence length of both regions. In conclusion, altered entropic elasticity of the titin spring elements on phosphorylation can explain the kinase-mediated effects on cardiomyocyte passive stiffness, but some questions remain.

**Altered Titin Phosphorylation in HF and Implications for Diastolic Function**

Direct evidence linking altered titin phosphorylation with human HF was initially provided for a small number of end-stage failing DCM hearts, which showed a deficit for PKG-dependent titin phosphorylation compared with nonfailing donor hearts.140 Other studies have since demonstrated hypo-phosphorylation of total-titin in human HCM123 and in animal models of HFpEF.117,125 A few studies have reported an increased ratio of phospho-N2BA:phospho-N2B in human HF, including HFrEF and HFrEF.113,112 However, as the N2BA:N2B isoform-expression ratio was also increased in these patient hearts, the proportion of phospho-titin to all-titin (per isoform) may have been similar in the nonfailing and failing hearts. Unaltered total-titin phosphorylation was recently found in end-stage failing DCM patients, compared with nonfailing donor hearts.121 Although
the quantification of total-titin phosphorylation has provided some useful hints, in particular when back-phosphorylation assays with a specific kinase were performed, interpretation of these data is not straightforward, because the huge titin protein contains hundreds of phosphosites, which may be altered differentially in HF.

Differential changes in the phosphorylation level of N2-Bus and PEVK have already been detected in human and experimental HF using phosphosite-specific antibodies. In both DCM and HCM patient hearts, the PKA/PKG-dependent N2-Bus phosphosites S4010, S4099, and S4185 were hypo-phosphorylated compared with donor hearts, whereas the PKCα-dependent PEVK phosphosite S11878 was hyper-phosphorylated.123 Two CaMKIIδ-dependent phosphosites, S4062 within N2-Bus and S12022 within PEVK, were hyper-phosphorylated in human failing versus donor hearts.142 In a dog model of early HFpEF, phosphorylation of N2-Bus sites S4010 (PKA) and S4099 (PKG) was reduced in comparison with healthy dog hearts, whereas phosphorylation of PEVK site S11878 (PKCα) was increased.117 Likewise, in a metabolic risk–induced animal model of HFpEF, the obese Zucker spontaneously hypertensive fatty 1 rat, phosphorylation of S4010 (S3991 in rat) was lower, but that of S12022 (S12884 in rat) was higher, compared with healthy rat hearts, whereas phosphorylation of S11878 (S12742 in rat) was unaltered.123 In experimental mouse hearts exposed to transverse aortic constriction surgery, PEVK site S11878 (PKCα) was hyper-phosphorylated, but PEVK site S12022 (CaMKIIδ/ PKCα) was hypo-phosphorylated.114 These findings suggest that differential phosphorylation of elastic titin elements is common to failing hearts. Because phosphorylation of N2-Bus and PEVK can have opposite effects on cardiomyocyte passive tension, it is important to determine site-specific rather than total phosphorylation of titin in HF. To better characterize altered titin phosphorylation in failing hearts in the future, the combined use of in vivo (quantitative) phosphoproteomics and phosphosite-specific antibodies for quantification by immunoblotting seems most promising.

Among the kinases phosphorylating the titin spring elements, PKA and PKG are usually downregulated in expression/activity in failing hearts, whereas PKCα and CaMKIIδ are usually upregulated. Thus, the hypo-phosphorylation of several N2-Bus sites observed in human and animal HF is probably because of impaired PKA or PKG signaling (Figure 7E), the hyper-phosphorylation of PEVK sites because of increased PKCα or CaMKIIδ expression/activity. Because PKCα increases cardiomyocyte passive stiffness,144 whereas CaMKIIδ reduces it,142 the 2 effects could neutralize one another in HF if both kinases were increased. In contrast, the deficit for PKA- and PKG-dependent phosphorylation at the N2-Bus element may contribute substantially to the increased diastolic stiffness observed in failing hearts (Figure 7F). Support for this view comes from the observation that PKA or PKG (administered ex vivo) corrected the pathologically high passive stiffness of isolated cardiomyocytes in human HFpEF, HFrEF, and aortic stenosis with or without diabetes mellitus,5,111,112,149 as well as in animal models of HFpEF.117,125 (Figure 7F). Patients with HFpEF had higher cardiomyocyte passive tension than patients with HFrEF, and recombinant PKA or PKG tended to reduce this parameter more strongly in HFpEF than in HFrEF or aortic stenosis (Figure 7F),111,149 which could be because of a larger phosphorylation deficit at the N2-Bus in HFpEF than in HFrEF/aortic stenosis. Indeed, myocardial cGMP concentration/PKG activity was much reduced in patients with HFpEF versus HFrEF120 and also in dog HFrEF versus healthy dog hearts.117 Moreover, treatment of skinned cardiomyocytes with cGMP-activated PKG was sometimes more effective than treatment with PKA in reducing the pathologically high passive tension in HFpEF (Figure 7F).117 These findings suggest that, to normalize the titin phosphorylation deficit and the high titin-based passive tension in HFpEF cardiomyocytes, it may be particularly useful to boost the cGMP-PKG pathway. Feasibility of this concept was demonstrated when young healthy or old hypertensive HFpEF dogs were exposed to acute cGMP-enhancing treatment with phosphodiesterase-5A inhibitor sildenafil, followed by B-type natriuretic peptide.150 Sildenafil rapidly increased cardiac titin phosphorylation and lowered cardiomyocyte passive tension, and this was maintained with B-type natriuretic peptide, thus explaining the better diastolic distensibility observed in living dog hearts after the treatment.150 In summary, reducing titin stiffness through cGMP-enhancing therapy seems to be a reasonable strategy to correct the pathologically high diastolic stiffness characteristic of patients with HFpEF.

The effects of kinases other than PKA and PKG on titin phosphorylation and stiffness are only beginning to be explored in human HF. The PKCδ-dependent PEVK site S11878 was hyper-phosphorylated in human failing versus nonfailing hearts in 2 studies.123,142 Inhibition of the increased PKCδ expression/activity typically seen in HF could thus be useful to reduce myocardial passive stiffness by lowering titin stiffness. Furthermore, the hyper-phosphorylation of CaMKIIδ-dependent sites S12022 (PEVK) and S4062 (N2-Bus) in failing human hearts correlated with an increased expression and activity of CaMKIIδ in these hearts.142 If CaMKIIδ-mediated titin phosphorylation reduced cardiomyocyte passive stiffness in human hearts as it did in mouse hearts,142 one could envision both a beneficial and a detrimental side of this effect. The reduction in myocardial diastolic stiffness will clearly be beneficial, as it promotes ventricular filling. In addition, CaMKIIδ has a function in speeding up relaxation in a heart rate–dependent way.146 Efficient ventricular filling is ensured even at high beating frequencies through CaMKII-dependent phosphorylation of phospholamban, thus accelerating the Ca2+ reuptake into the sarcoplasmic reticulum by the SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase) pump. If myoplasmic Ca2+ removal is slow and relaxation is delayed in failing hearts, CaMKII δ signaling will be activated, which will also increase titin phosphorylation. The resulting improvement in cardiomyocyte extensibility will facilitate slow filling and correct for the deleterious effect of slow relaxation on the fast early filling phase. Apart from these positive effects, one needs to consider that titin-based passive stiffness is probably fine-tuned for the benefit of mechanosensory function. Increased CaMKII δ in HF causing reduced titin stiffness could well have adverse effects on stress-sensitive
cardiomyocyte signaling pathways. Along this line, the use of CaMKII inhibitors as a potential therapeutic strategy in HF could help improve some, although not all, of titin’s mechanical functions. Last but not least, one needs to take into account that cross-talk between kinase signaling pathways (such as CaMKII–ERK2–PKCα or CaMKII–PKA) might affect the phosphorylation of some titin sites and therefore, cardiomyocyte passive stiffness, in a hardly predictable manner. Despite these limitations, the stiffness of titin is now recognized as a highly variable and dynamic parameter depending on the activation or depression of kinase signaling pathways. Titin phosphorylation as an important modifier of myocardial stiffness is altered in HF, but is promising as a potential target for pharmacological intervention aimed at improving ventricular filling in patients with HF.

**Post-Translational Modifications of Titin Related to Oxidative Stress and Consequences for Titin-Based Myocardial Stiffness**

Oxidative stress, such as that seen in myocardial ischemia–reperfusion damage, obesity, or diabetes mellitus, impairs LV diastolic function. Interestingly, the stiffness of titin is strongly affected by oxidative stress, through several mechanisms (Figure 8). First, oxidizing conditions promote the formation of disulfide bridges within the disordered N2-Bus element of cardiac titin (Figure 8A, far left). The human N2-Bus contains 6 cysteines, which can form ≤3 disulfide bonds. Because

![Figure 8. Effects of oxidative stress on titin-based stiffness. A, Oxidative stress–induced pathway changes affecting post-translational modifications of titin, such as oxidation of cysteines in N2B–unique sequence of titin (N2-Bus) causing disulfide bonding (far left), S-glutathionylation of cysteines in unfolded Ig domains inhibiting domain refolding (left-middle), and reduced cGMP-dependent protein kinase-G (PKG)–dependent N2-Bus phosphorylation, because of oxidation of the heme moiety in soluble guanylyl cyclase (sGC) and the ensuing blockade of cGMP production (right). Graphs in B to D show oxidative stress–related effects on titin-based passive tension caused by S–S bonding within N2-Bus (B), S-glutathionylation of unfolded titin-Ig domains (C), or depressed cGMP-PKG pathway activation (D). S’GMP indicates guanosine-5’-monophosphate; cGMP, cyclic guanosine monophosphate; G, glutathione; GSSG, glutathione-disulfide; NO, nitric oxide; P, titin phosphorylation; PDE5, phosphodiesterase-5; pGC, particulate guanylyl cyclase; PKG, cGMP-dependent protein kinase-G; and sGC, soluble guanylyl cyclase.](image-url)
S–S bridges are covalent bonds, the internally cross-linked N2-Bus looses much of its extensibility, resulting in elevated cardiomyocyte passive tension (Figure 8B).152

A second, recently elucidated, oxidative stress–related mechanism targets the Ig domains, which make up the majority of elastic titin. If I-band Ig domains become unfolded, for example, through increased strain on the sarcomeres, they expose cysteic cysteines, which now become accessible to disulfide bonding or S-glutathionylation in the presence of oxidizing conditions (Figure 8A, left-middle).153 Importantly, the unfolded titin-Ig domains form almost exclusively mixed disulfides with glutathione, which weakens the mechanical stability of these domains and prevents their refolding. S-Glutathionylation substantially reduces the passive tension of stretched human cardiomyocytes incubated with oxidized glutathione (Figure 8C), and the effect is reversible with the incubation of reduced glutathione.153 A fascinating implication of this novel mechanism regulating titin elasticity is that it opens the possibility that titin-Ig domains represent mechanosensors responding to myocyte stretch coupled with oxidative stress with reversible mechanical softening, which could well be the origin of altered mechano-chemical signaling in stressed cardiomyocytes.

A third type of oxidative modification of titin stiffness relates to the alterations in cGMP-PKG signaling seen under oxidative stress. Normally, cGMP is augmented by nitric oxide (NO), which activates soluble guanylyl cyclase in a process involving the binding of NO to the heme moiety of soluble guanylyl cyclase (Figure 8A, right). Cyclic GMP modulates the activity of various phosphodiesterases and activates PKG.154 In addition, stimulation of natriuretic peptide receptors activates particulate guanylyl cyclase and subsequently promotes cGMP and PKG activation.155 The effect of oxidative stress on these pathways is to lower NO bioavailability, block soluble guanylyl cyclase activity, increase cGMP-specific phosphodiesterase-5A, and downregulate cGMP-PKG signaling altogether (Figure 8A, right).156 Reduced PKG activity resulting from oxidative stress will then lead, among others, to hypo-phosphorylation of titin, raising cardiomyocyte passive tension (Figure 8D). Consistent with this, the HFpEF patient hearts, which exhibited reduced cGMP concentration and PKG activity, hypo-phosphorylated titin, and high cardiomyocyte passive stiffness, also showed increased nitrotyrosine levels indicative of nitrosative/oxidative stress.149 These findings point to oxidative/nitrosative stress as an indirect modifier of titin phosphorylation and stiffness, which could lead to diastolic dysfunction. If so, many patients with HFpEF might develop diastolic dysfunction not least because they typically have various comorbidities, including old age, renal insufficiency, obesity, diabetes mellitus, or hypertension, all of which are likely to increase the level of oxidative/nitrosative stress.3 Future HFpEF treatment strategies may thus aim to treat the comorbidities through the use of NO donors, phosphodiesterase-5 inhibitors, and antioxidants, the beneficial effects of which would also include a correction of titin-based myocardial stiffness.

Conclusions and Outlook

Earlier studies provided evidence that the giant protein titin is crucial for muscle elasticity and myofibrillar assembly. The interactions detected with an increasing number of binding partners have reinforced a structural role for titin in myocytes and have now established the protein as a signaling hub linking to pathways of cardiac hypertrophy, mechanosensation, and protein quality control. Of particular interest has been the discovery that TTN is a major human disease gene, and one can expect that many more titin mutations will be detected in human heart or muscle disease in the near future. Ongoing investigations need to better establish the pathogenicity of TTN mutations and elucidate the pathomechanisms. Another exciting feature emerging during the past decade is that titin-based elastic force is highly variable in normal and diseased hearts. Titin-isofoms of greatly different elasticity are generated under the control of the splicing factor RBM20. Future work should aim to identify the regulators of titin splicing responsible for a reversion from the adult to a more fetal isoform pattern, which is observed in patients with HF. The dynamic modulation of titin stiffness occurs via post-translational modifications, with phosphorylation of titin spring elements being established as a major player mostly decreasing the stiffness. Altered titin phosphorylation in failing hearts seems to be a potential target for pharmacological intervention aimed at reducing diastolic stiffness in patients with HFpEF. Finally, oxidative stress–related post-translational modifications of the titin spring are emerging as important regulators of cardiomyocyte stiffness. Whereas S–S bonding within the N2-Bus increases titin stiffness, S-glutathionylation of mechanically unfolded titin-Ig domains prevents their refolding, thus decreasing titin stiffness. It will be interesting to examine to what extent these modifications occur in normal and diseased hearts and whether they contribute to cardiac dysfunction. Work on titin remains a big job for some time to come.

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Disclosures

None.

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Gigantic Business: Titin Properties and Function Through Thick and Thin
Wolfgang A. Linke and Nazha Hamdani

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