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Review

Sense and stretchability: The role of titin and titin-associated proteins in myocardial stress-sensing and mechanical dysfunction†

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Mechanical stress signals transmitted through the heart walls during hemodynamic loading are sensed by the myocytes, which respond with changes in contractile performance and gene expression. External forces play an important role in physiological heart development and hypertrophy, but disruption of the well-balanced stress-sensing machinery causes mechanical dysregulation, cardiac remodelling, and heart failure. Nodal points of mechanosensing in the cardiomyocytes may reside in the Z-disk, I-band, and M-band regions of the sarcomeres. Longitudinal linkage of these regions is provided by the titin filament, and several 'hot spots' along this giant protein, in complex with some of its >20 ligands, may be pivotal to the myofibrillar stress or stretch response. This review outlines the known interaction partners of titin, highlights the putative stress/stretch-sensor complexes at titin's NH_2 and COOH termini and their role in myopathies, and summarizes the known disease-associated mutations in those titin regions. Another focus is the elastic I-band titin section, which interacts with a diverse number of proteins and whose main function is as a determinant of diastolic distensibility and passive stiffness. The discussion centers on recent insights into the plasticity, mechanical role, and regulation of the elastic titin springs during cardiac development and in human heart disease. Titin and titin-based protein complexes are now recognized as integral parts of the mechanosensitive protein network and as critical components in cardiomyocyte stress/stretch signalling.

1. Introduction

Mechanical stresses play a central role in the regulation of physiological processes, and the heart is no exception. Physical forces promote cardiac development and hypertrophy, but dysregulation of mechanical signalling can lead to chronic heart diseases, such as hypertrophic (HCM) or dilated cardiomyopathy (DCM). Research on mechanotransduction in normal and diseased heart is aimed at elucidating the molecular mechanisms by which myocardial structures sense physical loads and transduce them into biochemical signals to alter gene expression and modify cellular structure and function.[1] The propagation and sensing of mechanical forces in myocardium involves many different components, including (but not restricted to) the extracellular matrix (ECM), the costameric protein network at focal adhesions,[2–4] the adherens-junction at intercalated disks,[5] and protein complexes associated with the sarcomeres.[6–8] In the mechanotransduction network of the heart, external force signals (such as those imposed during hemodynamic load) are transmitted from the ECM to the cardiomyocyte cytoskeleton, while at the same time the sarcomeres themselves generate forces, which propagate in the opposite direction. This bidirectional force transduction is mediated by highly specialized nodal points of mechanosignalling: Z-disks and M-bands.[7–11]

Like a myofibrillar backbone, >1-μm-long filaments of titin (also called connectin) run from the Z-disk to the M-band, or center of the sarcomere (*Figure 1A*), and it is conceivable that the titin strands are part of the stress-responsive machinery. During diastolic distension, titin filaments behave as passive-force generators in parallel with the contractile apparatus. Moreover, titin is attached to the thin filaments at the Z-disk and runs along the thick filaments, bound to myosin in the A-band/M-band regions (*Figure 1A*). These sections of the titin molecule, along with some of their interaction partners (*Figure 1B*), could sense the forces generated in the sarcomere during both diastolic stretch and systolic contraction. In addition, the multiple linkages involving those titin regions (*Figure 2*) may provide a means for sensing stresses from various directions. This review deals with the functions of titin, the

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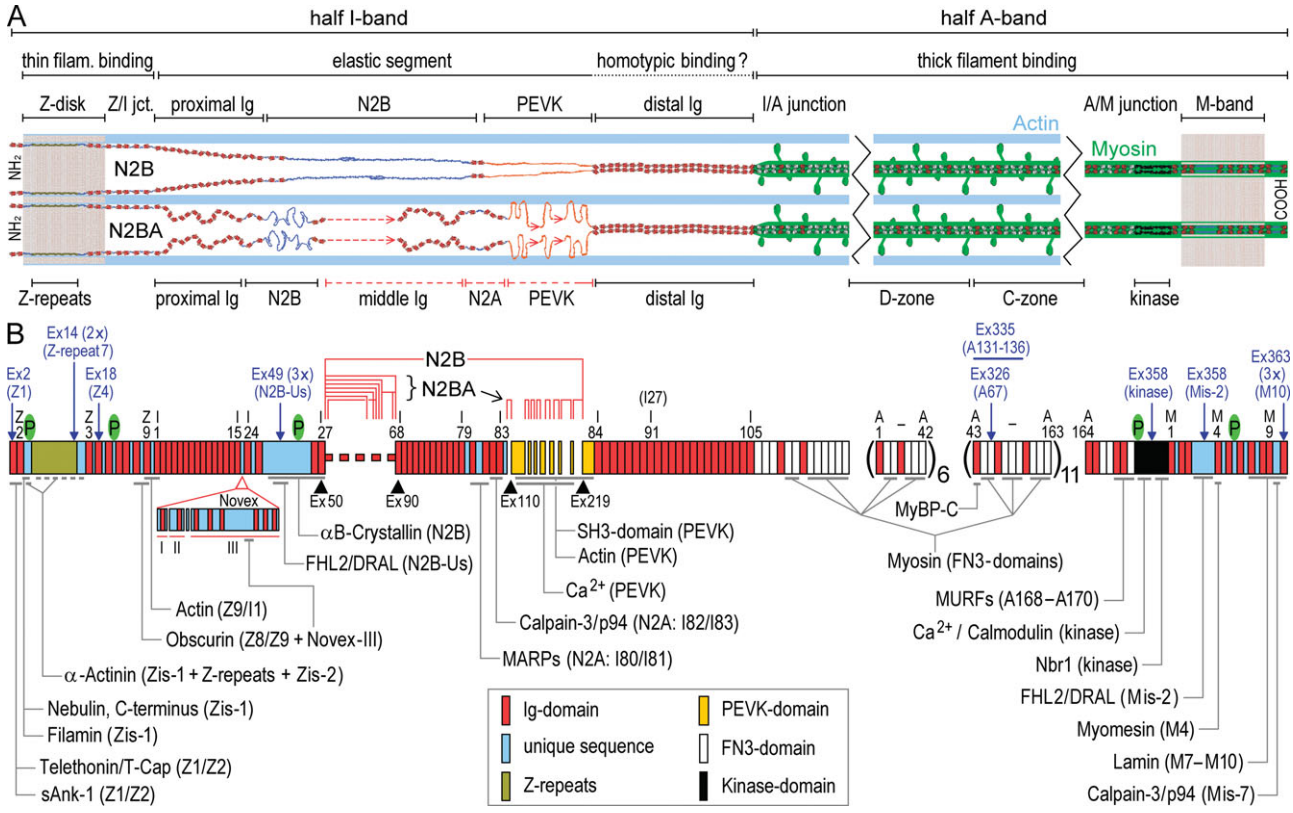

Figure 1 Titin architecture and binding partners. (*A*) Layout of cardiac titin-isoforms, N2B and N2BA, in the half-sarcomere. Dashed red lines indicate differentially spliced segments in I-band titin. (*B*) Domain structure of human cardiac titin and binding sites of known titin-ligands (listed in parentheses behind the ligand name). For full names of titin-ligands, see legend to *Figure 2*. Red lines illustrate splice-pathways for N2B and N2BA–titins between exons (Ex) 50 and 219 and highlight the Novex-domains. Blue arrows/text indicate mutations found in human titin. Domain names (nomenclature from[12]) are shown for selected Ig/FN3 modules above the titin sequence. Ig-domain I91 is also called I27 (old nomenclature from[20]). P, titin-phosphorylation site.

known titin–ligands, and putative 'hot spots' of stress-sensing along the titin molecule. Disease-causing mutations in human titin are highlighted and the plasticity, mechanical role, and regulation of the titin springs in heart development and disease are discussed. I apologize to anyone whose contribution relevant to the topic is not cited here; space limits precluded a full coverage.

2. The titin gene and tissue-specificity of titin expression

Members of the titin family are considered to be the largest known proteins. The human titin gene on chromosome 2q31 is 294 kilobases and encompasses 363 exons predicted to code for a total of 38,138 amino-acid residues or a polypeptide with a maximum molecular mass of 4200 kDa.[12] There is a single gene for titin in humans coding for many different isoforms in cardiac and skeletal muscles.[13] Interestingly, zebrafish has two orthologous titin genes, *ttna* and *ttnb*, located in tandem-array on chromosome 9.[14] In the zebrafish heart, *ttna* (but not *ttnb*) is required for sarcomere assembly and the establishment of cardiac contractility, and *ttna* was found to be an early marker for cardiomyocyte differentiation.

Titin-isoforms are also found in human smooth muscle tissues: aorta, bladder, carotid artery, and stomach each express 80–100 of the 363 titin–exons encoding parts of the Z-disk, I-band, and A-band regions of titin, resulting in proteins with a molecular mass of $\leq$1000 kDa.[15] The functional role(s) of the smooth–muscle titins still await exploration and the relationship between these titins and a previously described titin-like protein in smooth muscle, smitin,[16] also remains to be shown. Furthermore, human non-muscle cells, such as fibroblasts and platelets, express multiple isoforms of cellular titin (c-titin), products of the human titin gene that are associated with stress-fibers and apparently contain many of the titin domains found in striated muscles.[17] It will also be interesting to follow up on scattered reports suggesting that titin is a nuclear protein in non-muscle cells, potentially providing elasticity and structural flexibility to chromosomes.[18,19]

3. Titin-isoform diversity and functions in the heart

Approximately 90% of the mass of the titin molecule is made up of globular domains of the immunoglobulin (Ig) or fibronectin-type-III (FN3)-like folds; the remainder is composed of insertions of unique sequence (*Figure 1*).[20] Nearly all Z-disk and A-band/M-band titin domains are constitutively expressed in the human striated muscle titin-isoforms. Differential splicing occurs mainly in the I-band-titin segment ($\sim$800–1500 kDa) and gives rise to the presence of two distinct isoforms in mammalian heart,[12] N2B ($\sim$3 MDa) and N2BA (3.2–3.7 MDa) (*Figure 1*). Both isoforms have a 'proximal' (I1–I15) and a 'distal' (I84–I105) Ig-domain region, an "N2B"-domain (not expressed in skeletal muscles), and a "PEVK"-domain, so called for its high

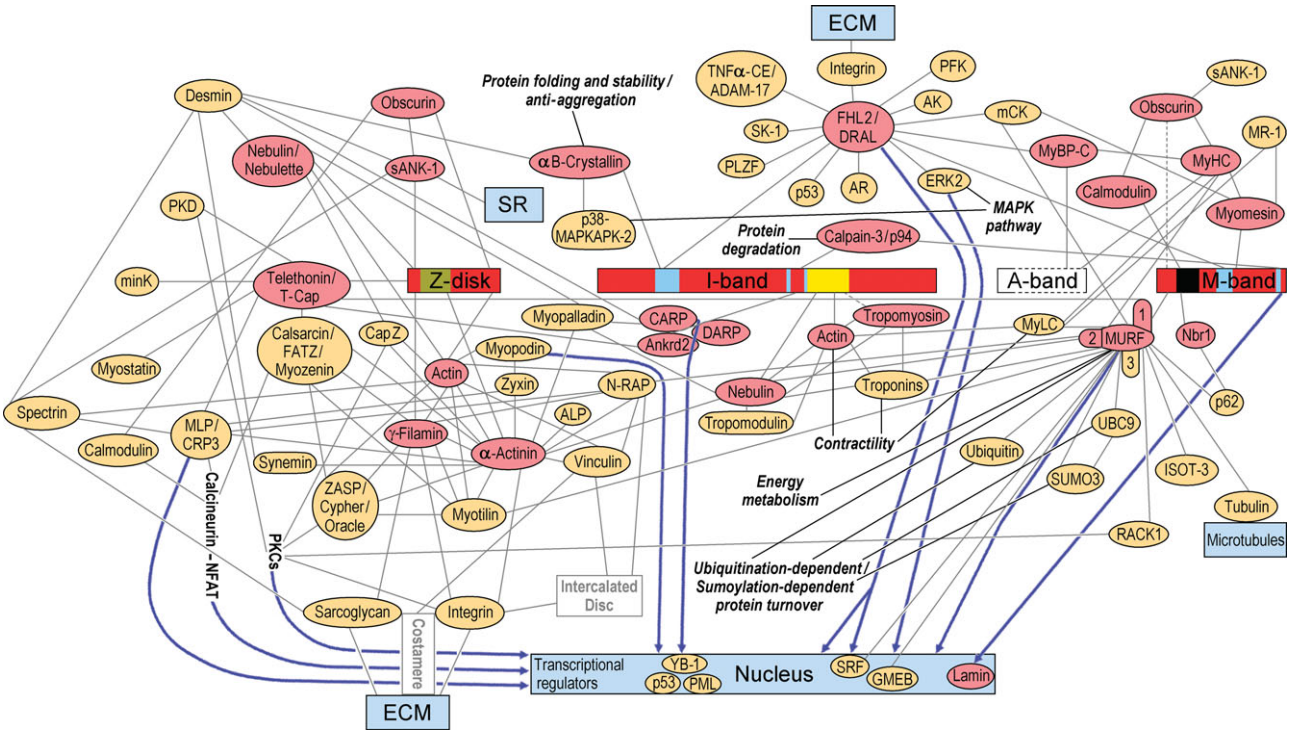

Figure 2 Current state of the titin-interactome. Summary of known connections between titin (Z/I/A/M-band region) and its direct (red ovals) and indirect (yellow ovals) ligands. Blue arrows indicate capacity for cytoplasmic-nuclear shuttling. SR, sarcoplasmic reticulum; ECM, extracellular matrix. AK, adenylate-kinase; ALP, actinin-associated-LIM-protein; Ankrd2, ankyrin-repeat-domain-protein-2; AR, androgen-receptor; CARP, cardiac-ankyrin-repeat-protein; DARP, diabetes-related-ankyrin-repeat-protein; ERK2, extracellular-signal-regulated-kinase-2; FATZ, filamin-/actinin-/telethonin-binding-protein-of-the-Z-disk; FHL2 (DRAL), four-and-a-half-LIM-domain-protein (down-regulated-in-rhabdomyosarcoma-LIM-domain-protein); GMEB, glucocorticoid-modulatory-element-binding-protein; ISOT-3, isopeptidase-T-3; MAPK, mitogen-activated-protein-kinase; mCK, muscle-creatine-kinase; minK, potassium-channel subunit; MLP (CRP3), muscle-LIM-protein (cysteine-rich-protein-3); MR-1, myofibrillogenesis-regulator-1; MURF, muscle-specific-RING-finger-protein; MyHC, myosin-heavy-chain; MyLC, myosin-light-chain; Nbr1, neighbor-of-BRCA1-gene-1; NFAT, nuclear-factor-of-activated-T-cells; N-RAP, nebulin-related-anchoring-protein; p38-MAPKAPK-2, p38-activated-MAPK-activated-protein-kinase-2; PFK, phosphofructokinase; PKCs, isoforms of protein-kinase-C; PKD, protein-kinase-D; PLZF, promyelocytic-leukemia-zinc-finger-protein; PML, promyelocytic-leukaemia-protein; RACK1, receptor-for-activated-C-kinase-1; sANK-1, small-ankyrin-1 isoform; SK-1, sphingosine-kinase-1; SRF, serum-response-factor; SUMO3, small-ubiquitin-related-modifier-3; TNFα-CE (ADAM-17), tumor-necrosis-factor-alpha-converting-enzyme (A-disintegrin-and-metalloprotease-17 protein); UBC9; ubiquitin-conjugating-enzyme-9; YB-1, Y-box-binding-protein-1; ZASP, Z-band-alternatively-spliced-PDZ-motif-protein.

content of proline (P), glutamic-acid (E), valine (V), and lysine (K) residues. In the N2B–titin-isoform, exon 50 (Ig-domain I27) is spliced directly to exon 219 in the PEVK-segment (*Figure 1*), skipping many exons coding for Ig-domains and unique sequences. N2BA–titin-isoforms contain in addition, a 'middle-Ig-domain' region (I28–I79), an 'N2A'-domain, and a PEVK-segment of variable length.[12] Extensive alternative splicing of exons occurs in the middle-Ig region and the PEVK-domain (*Figure 1B*), resulting in innumerable potential N2BA-isoforms, only some of which have been sequenced so far.

Titins are well known for their mechanical functions. The titin springs endow the sarcomeres with long-range elasticity and are main determinants of myocardial passive tension (PT), together with the ECM-based collagen fibers.[13,21] The elasticity of I-band titin may support elastic recoil in early diastole[22] and early systolic shortening.[23] Moreover, titin could be a factor in determining the length-dependence of Ca^{2+}-activated force development, the molecular basis for the Frank–Starling mechanism.[24] Apart from these mechanical roles, titin aligns structural, regulatory, and contractile proteins within the sarcomere (*Figure 1*) and may coordinate the precise assembly of myofibrils during muscle development and hypertrophy.[6,25] Direct and indirect links to many signalling molecules (*Figure 2*),[6,7] along with the presence of various phosphorylation sites in the Z-disk, I-band, and M-band segments (*Figure 1B*),[25] have implicated titin as a player in myocardial signalling. Further, there is a kinase domain near the COOH-terminus of titin,[26] the function of which is becoming increasingly clear.[27–32]

4. Interactions and properties of Z-disk titin

Z-disk titin is encoded by exons 1–28 and has nine Ig-domains (Z1–Z9) separated by unique sequences (*Figure 1B*). Up to Ig-domain Z4, this titin segment is an integral part of the Z-disk, whereas the remainder reaches beyond the Z-disk edge but is still tightly associated with the thin filament (*Figure 1A*).[33,34] Actin-binding was confirmed for the domains Z9-I1.[33]

4.1 Ig-domains Z1/Z2 and the Z-disk-based mechanosensor

Titin's most NH_2-terminal domains, Z1/Z2, interact with the sarcoplasmic reticulum membrane protein sAnk-1[35] (for full protein names, see legend to *Figure 2*), which has links to spectrin, desmin, and obscurin (*Figure 2*).[36,37] The ~800-kDa-protein obscurin is expressed in different

cardiac-isoforms, the composition of which can be altered in human DCM.[38] Obscurin also associates with the Ig-domains Z8/Z9, which are more distal in Z-disk titin,[39] suggesting connectivity between the SR, the Z-disk, and other cytoskeletal structures.

Additionally, the Ig-domains Z1/Z2 bind to telethonin (also called T-Cap),[6,7,13] which assembles two titin filaments entering the Z-disk from the same half-sarcomere into a tightly packed anti-parallel sandwich structure.[40] This Z1/Z2-telethonin complex is highly resistant to stretching forces, a property conferred by multiple hydrogen bonds that cross-link beta-strands of the two proteins.[41] Telethonin may have a key role in anchoring titin filaments in the Z-disk and in titin assembly. Other structural and signalling proteins are targeted via telethonin to the Z-disk. These proteins, covered in detail in recent reviews,[7,8,10] include (*Figure 2*): myostatin, potassium-channel subunit minK (I_{ks}), PKD, MURF1 and MURF2, Ankrd2, calsarcin (calsarcin-2 is also called FATZ or myozenin), and MLP (also known as CRP3). Mutations in telethonin that modulate the interaction with titin, MLP, or calsarcin cause either HCM or DCM in patients.[42,43] Together with titin's NH_2-terminus and MLP, telethonin is believed to be central to the Z-disk-based mechanosensor.[44]

MLP is highly expressed in myocardium where it interacts with numerous other proteins,[8,10] such as the cytoskeletal proteins, β-spectrin, α-actinin, zyxin, and N-RAP, as well as the Ca^{2+}-calmodulin-dependent phosphatase, calcineurin (*Figure 2*). The latter activates the transcription factor NFAT, triggering a cardiac hypertrophic response.[1] MLP itself can translocate from the Z-disk, cytosol, or intercalated disk to the nucleus, where it associates with the muscle transcriptional regulators, MyoD, MRF4, and myogenin.[8] Point mutations have been found in human MLP at sites involved in protein–protein interactions[8,10] and the affected patients develop either DCM or HCM. MLP is substantially down-regulated in end-stage failing human hearts.[45]

An MLP-deficient mouse model has provided insights into the function of the putative Z-disk-mechanosensor. MLP-null mice develop DCM 2–4 weeks postnatally and have cardiomyocytes with widened and disorganized Z-disks.[44] In MLP-null hearts the PT of papillary muscles was reduced,[44] myocardial passive stiffness was decreased, and relaxation time was prolonged, but no alterations were found in most systolic characteristics.[46] Notably, stretching of cultured neonatal cardiomyocytes caused upregulation of the 'stress-marker' brain-natriuretic-peptide (BNP) in wildtype but not in MLP-null cells.[44] However, BNP could still be induced by pharmacological stimulation, consistent with a role for MLP specific to mechanosignalling. How could a stress-sensing mechanism via MLP and the titin-Z1/Z2-telethonin complex work? Extracellular or sarcomere-generated forces transmitted via the Z-disk may affect the fraction of Z-disk-bound versus nuclear MLP, and increased MLP in the nucleus may activate transcriptional (co)-factors. This could evoke a graded response in terms of variable changes in muscle–gene expression depending on the force level. Alternatively, mechanical forces may alter the interaction between the MLP–telethonin–titin ternary complex and a ligand, thus triggering a hypertrophy pathway, *e.g.*, via activation of calcineurin-NFAT or PKC; again, muscle–gene expression would be modified depending on the applied force level. Possibly, the force level is somehow fed back to the Z-disk sensor to alter its stress-responsive behavior.

4.2 Connectivity provided by unique-sequence insertions at titin's NH_2-terminus

Several interactions involve Zis-1, a sequence insertion adjacent to the titin-Z2 domain (*Figure 1B*). Zis-1 associates with an SH3-domain at the C-terminus of nebulin, which is also present in the cardiac-specific nebulette.[47] Nebulin (600–900 kDa) is expressed at very low levels in cardiomyocytes,[48,49] where it may regulate actin–filament dynamics, stabilize cytoskeletal linkages to the Z-disk by interacting with actin, desmin, CapZ, and myopalladin (*Figure 2*),[9,49] and possibly specify Z-disk width.[47]

Titin-Zis-1 also binds γ-filamin,[15] a striated muscle-specific filamin with multiple links in the myocardial stress-response pathway. Titin's NH_2-terminus is coupled via filamin to structural and signalling proteins (*Figure 2*), such as integrin and sarcoglycan at the costameres, α-actinin, actin, myotilin, ZASP (cypher/oracle) and calsarcin at the Z-disks, and N-RAP at the intercalated disks.[2,4] The filamin-mediated connection between titin and the focal–adhesion complex is particularly interesting, as external forces transmitted via the costameres are known to initiate a signalling cascade involving several stress-responsive proteins, including vinculin, melusin, talin, focal–adhesion kinase, integrin-linked kinase, Src-tyrosine kinase, zyxin, paxillin, PKC_ε, and members of the Rho-family GTPases.[2–4] Further downstream, the MAPK and AKT/PKB signalling pathways are activated, promoting gene expression and cardiomyocyte growth.[1]

A third ligand of titin-Zis-1 is α-actinin (*Figure 1B*).[15] This interaction exists in addition to the α-actinin-binding sites within the seven 45-amino-acid repeats (*Figure 1B*) known as 'titin Z-repeats' (exons 8–14).[6,7,13] The Z-repeats are alternatively spliced between exons 9 and 12, generating a variable number of titin-α-actinin links depending on muscle type. Titin's NH_2-terminus is cross-linked via α-actinin, not only to actin but to a vast network of Z-disk-associated proteins (*Figure 2*), providing additional mechanical stability. In conclusion, by interacting with multiple ligands, the titin region between Ig-domains Z2 and Z3 supports Z-disk structure, force transmission, and perhaps mechanical signalling.

5. I-band titin: interactions and multifaceted roles in normal and diseased heart

The mechanically active element of titin in the I-band (exons 28–251) begins approximately 100 nm away from the center of the Z-disk.[33,34] Mounting evidence suggests that the titin springs not only generate passive force but also associate with multiple ligands and might serve as a "tensiometer" in the sarcomere.

5.1 Extensible elements in I-band titin

Distinct subsegments in I-band titin contribute differentially to the extensibility and passive-force generation of the cardiac myocyte (*Figure 1A*). When the sarcomere is stretched from slack length, initially the proximal and distal Ig-domain regions (and the middle-Ig region in

N2BA–titin) extend by straightening out their inter-domain linkers, whereas unfolding of individual Ig-domains is a rare event.[50–53] Once the extensibility of the (folded) Ig-domain regions is largely exhausted, the PEVK-domain and then also a 572-amino-acid-residue unique sequence in the N2B-domain (N2B-Us) extend, while passive force now rises much more steeply than during Ig-segment extension.[13,52] Thus, a step-wise titin-extension model has emerged, in which straightening of I-band Ig-segments at low stretch-forces is followed by extension of the long unique-sequence insertions at higher forces (also see *Figure 3C*).

5.2 Novex-domains

Exons 45, 46, and 48 in the titin genomic sequence, just COOH-terminal to the proximal Ig region (*Figure 1*), are known as Novex-I, II, and III, respectively.[12] The Novex-domains are not expressed in the main cardiac-isoforms, N2B and N2BA. Novex-I and Novex-II code for sequence in the ~3000-kDa Novex-1/N2B and Novex-2/N2B titin-isoforms, respectively, which are both expressed at very low levels in heart. Novex-III codes for a large domain that binds obscurin and can function as an alternative COOH-terminus in cardiac and skeletal muscle, thus generating a (low-abundance) Novex-3 titin-isoform of ~650 kDa that integrates into the Z-disk lattice but is too short to reach the A-band.[12]

5.3 Cardiac-specific N2B-domain: molecular spring and ligand-binding site

The N2B-domain (encoded by titin-exon 49) encompasses the Ig-domains I24–I26 and the intervening, extensible, N2B-Us. The latter associates with FHL2 (also called DRAL) (*Figure 1B*), which in turn targets metabolic enzymes (*Figure 2*) to the sarcomere.[54] Another interaction site for FHL2 exists in the M-band titin region. FHL2 has >50 binding partners belonging to different functional classes, including receptors, structural proteins, signal-transducers, transcription factors and cofactors, splicing factors, and DNA replication and repair enzymes.[55] Some of them (detected in heart) are illustrated in *Figure 2*. FHL2 itself, as well as several of its ligands, *e.g.*, SRF and ERK2, can translocate to the nucleus where they act as modifiers of gene expression (*Figure 2*). FHL2 links I-band titin to MAPK-dependent stress signalling via ERK-binding. FHL2 also ties the N2B-region of titin to integrin and the integrin-related mechanotransduction pathway.

Titin's N2B-Us and the Ig-domains I26/I27 also associate with αB-crystallin (*Figure 1B*), a member of the small heat-shock-protein family.[56] In the heart, αB-crystallin moves to the myofibrils under conditions of stress, such as ischaemia, and may act as a protector of cytoskeletal proteins, including titin[57] and desmin.[58] N2B–titin domains might be protected from stretch-induced unfolding when αB-crystallin binds to intermediate folding states.[56] The stress-protective effect of αB-crystallin is phosphorylation-activated and mediated by p38-MAPKAPK-2,[58] again linking a titin-ligand to MAPK-signalling (*Figure 2*).

Knock-down of titin-exon 49 in mouse hearts leads to cardiac atrophy and diastolic dysfunction due to increased diastolic wall stress, and cardiomyocytes deficient in N2B-domain generate higher-than-normal PT and have reduced slack sarcomere length (SL).[59] In these knockout mice, FHL2, but not αB-crystallin, is down-regulated suggesting the FHL2-N2B-Us connection is important for the cardiac hypertrophic response. One can speculate that, if the affinity between FHL2 and the springy N2B-Us were dependent on the stretch state, this interaction could represent a *bona fide* stretch-sensor.

5.4 N2A-domain: a stress-sensing element?

The N2A-region (exons 102–109) encompasses the Ig-domains I80–I83 interspersed with a few unique sequences (*Figure 1*). I80/I81 interact with the three homologous muscle-ankyrin-repeat proteins (MARPs) (*Figure 1B*), CARP, DARP, and Ankrd2 (also known as Arpp).[60,61] MARPs may participate in muscle stress-activated pathways and are upregulated in both cardiac and skeletal muscles after mechanical or metabolic challenge. Cyclic stretching of cultured cardiomyocytes induced expression of CARP and DARP both in the nucleus and in the sarcomeric I-bands,[60] and end-stage failing human DCM hearts showed increased expression levels of MARPs.[62] CARP binds to myopalladin and desmin, likely via a potential coiled-coil dimerization motif that also mediates homo-dimer formation of other MARPs.[60,63] Ankrd2 additionally associates with telethonin and with the three transcription factors, YB-1, PML, and p53 (*Figure 2*),[64] which hints at the potential of MARPs to act as nuclear regulators of transcription. Thus, MARPs may provide a link between myofibrillar stress-response and muscle–gene expression, implicating the N2A-domain in a mechano-chemical signalling pathway.

Another ligand of the N2A-domain is the Ca^{2+}-dependent muscle protease, calpain-3/p94, which interacts with the Ig-domains I82/I83, but also has a second binding site in titin's M-band region (*Figure 1B*).[65] Binding of calpain-3 to the N2A-domain inhibits autolytic activation and disassembly of the protease.[65,66] Calpain-3 is not expressed in adult heart but is important in skeletal muscle, as loss-of-function mutations in the calpain-3 gene cause limb-girdle muscular dystrophy type-2A (LGMD2A) in humans.[66] A mouse model with a deletion mutation in the calpain-3-binding site of the N2A-region develops muscular dystrophy with myositis (MDM).[67] A notable feature in the MDM mouse is that CARP and Ankrd2 are strongly upregulated,[61] suggesting feedback between titin-N2A, calpain-3, and MARPs in a signalling complex associated with the central I-band region.

5.5 PEVK-domain: signalling and mechanical functions

Almost one third of the exons (110–225) in the human titin gene code for the PEVK-domain, a segment made up of conserved alternating motifs of 26–28 amino-acid repeats (PPAKs) separated by regions rich in glutamic-acid residues (polyE-motifs).[68] The PEVK-segment interacts with nebulin SH3-domains and may bind to SH3-domains of other proteins as well (*Figure 2*),[69] implying an as yet unappreciated role for this region in signalling processes during sarcomere assembly or in mechanosensing. Within the PEVK-domain three conformational states have been identified, polyproline-II helix, beta-turn, and random coil,[70] which may be important for the mechanical properties of this domain. PEVK-titin elasticity is thought to be largely based upon an entropic-spring mechanism[53,71] and *in-situ*

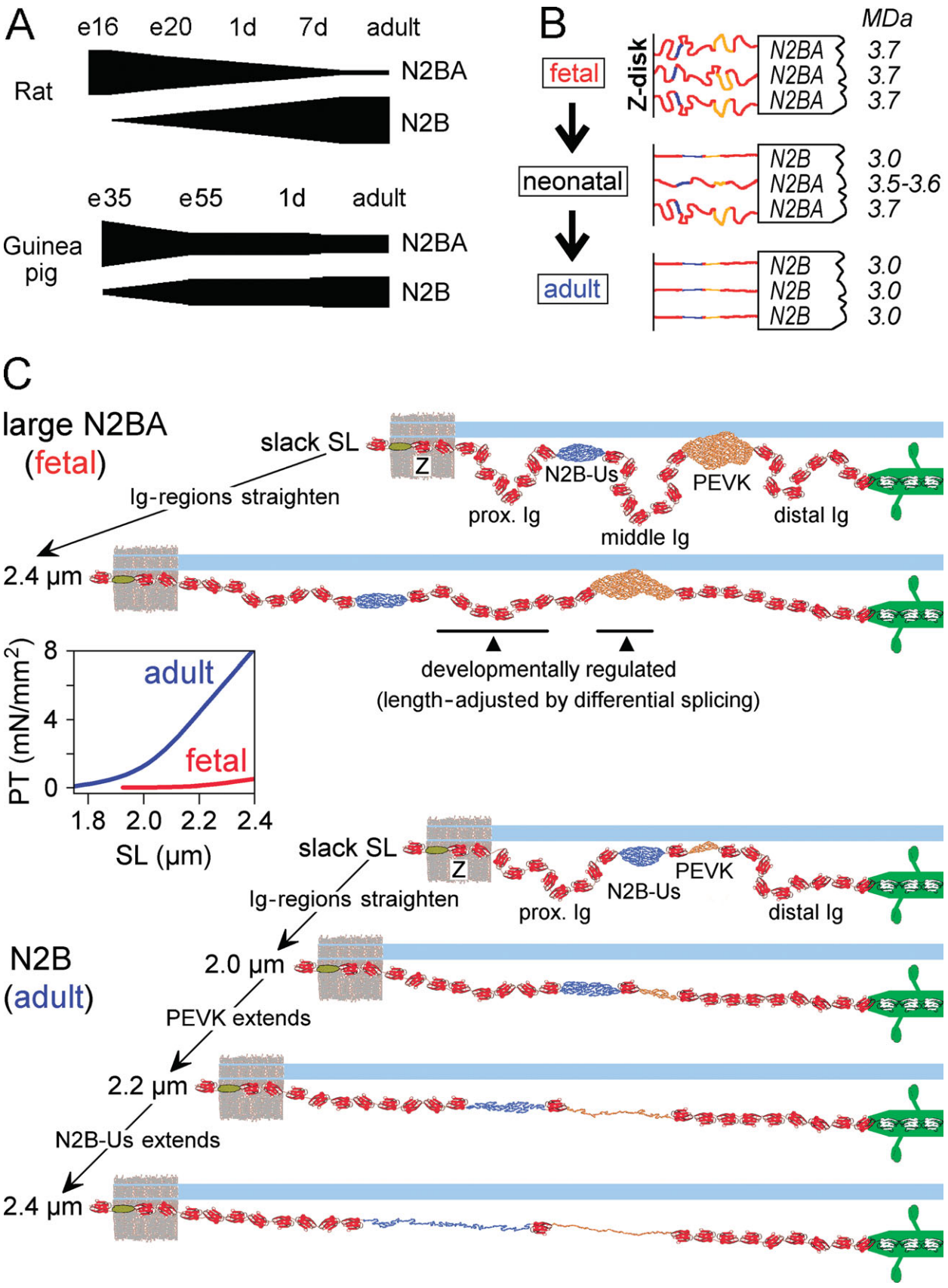

Figure 3 Developmental isoform transitions in cardiac titin and consequences for passive tension (PT). (*A*) Shifts observed in N2BA *versus* N2B titin-isoform composition during fetal/perinatal development of rat and guinea-pig hearts. e, embryonic day; d, postnatal day. (*B*) Illustration of changes in titin-isoform composition in a half-sarcomere during rat-heart development. (*C*) Model explaining the extension of elastic I-band segments in long N2BA–titin (top) and short N2B–titin (bottom), in the physiological sarcomere-length (SL) range. For clarity, schemes do not show the real number of titin-Ig-domains (see *Figure 1*). Inset: PT–SL relationships of isolated cardiac myofibrils from fetal (e16) and adult rat heart.

extension of this segment is associated with a significant rise in sarcomeric PT.[50]

The passive mechanical properties of the sarcomere are modified by interaction of the PEVK-domain with actin filaments. This interaction was suggested in one study to be regulated by Ca^{2+}/S100A1,[72] but found to be independent of Ca^{2+}/S100 and Ca^{2+}/calmodulin in another.[73] Actin-binding propensity is characteristic of both the constitutively expressed PEVK-titin (exons 219–225) and the differentially spliced PEVK-titin in the N2BA (and skeletal N2A) isoforms.[71,74] The actin–PEVK interaction is rather weak and might be further alleviated by physiological levels of Ca^{2+}[71] and the presence of tropomyosin, which itself binds to I- band titin.[75] Still, the interaction imposes a viscoelastic load on the passively stretched[73] or contracting[23] myocardium. Further, the stiffness of PEVK-titin is increased by Ca^{2+}-binding,[76] an effect mediated by the differentially spliced, but not the constitutively expressed, PEVK-titin.[77] In summary, the PEVK-domain is an intriguing titin region with a major function as a molecular spring that is tunable by ligand-binding.

5.6 Plasticity of titin in cardiac development

Differential splicing of titin's I-band segment not only generates great diversity of titin-isoforms in adult skeletal muscles[78] and in different compartments of the adult heart,[79,80] but also leads to dramatic size changes of the titin springs during fetal and perinatal heart development (*Figure 3*). At mid-gestational stages, mammalian hearts express a unique fetal N2BA-isoform of ~3.7 MDa but no N2B–titin.[81–83] The large N2BA-isoform is gradually replaced later during development by smaller N2BA–titins co-expressed with the N2B-isoform in the same half-sarcomere (*Figure 3A and B*).[81,82,84] The length differences between these developmentally regulated N2BA-isoforms result from differential splicing of the middle-Ig-region and PEVK-domain. N2B is the predominant titin-isoform in the adult left ventricles (LVs) of smaller mammalian species and also humans, whereas the N2BA–titins prevail over N2B in the adult hearts of larger mammals.[79,80] The developmental titin-isoform switching is particularly fast in mice or rats, where it occurs perinatally within 1–2 weeks (*Figure 3A*, top). In other species, *e.g.*, guinea-pig which has a comparatively long gestation period, the switching takes longer but is nearly complete before birth (*Figure 3A*, bottom).[85] These fetal/perinatal transitions from high to low N2BA:N2B ratios (*Figure 3A*) cause the myofibrillar PT to be much higher in adult than in fetal myocardium (*Figure 3C*, inset).

The molecular basis behind this phenomenon is illustrated in *Figure 3C*. Stretching a long N2BA-isoform in the physiological SL range of ~1.8–2.4 μm straightens out the Ig-domain regions, but does not significantly extend the PEVK and N2B-Us (*Figure 3C*, top). Titin-based passive force therefore remains low. In contrast, when the short N2B-isoform is stretched to the same SLs, the strain on the I-band segment is much higher and also the PEVK-domain and N2B-Us elongate (*Figure 3C*, bottom), causing passive force to rise steeply.[52] The overall passive stiffness then depends on the proportion of compliant N2BA *versus* stiff N2B springs expressed in the sarcomere.[86] The dramatic alterations in N2BA:N2B ratio during heart development serve to adjust the passive stiffness of myocardium to the changing hemodynamic situation during cardiac growth. Although nothing is known yet about the triggers for the developmental titin-isoform switching, it is conceivable that growth hormones and/or mechanical stress play a role, as is the case for other myofibrillar proteins.

5.7 Mechanical function of titin in human heart disease

Considering that titin is a major contributor to diastolic wall stiffness, what role do the titin springs play in the passive stiffening of the cardiac walls in chronic human heart disease? Earlier electron microscopical and immunohistological studies of end-stage failing human DCM hearts showed altered distribution and loss of titin.[87] When the N2BA:N2B titin-isoform ratio was analyzed in chronically ischaemic LVs of coronary-artery-disease (CAD) patients with congestive heart failure (HF), the mean percentage of N2BA-isoform was found to be elevated to nearly 50% of the total titin, compared to ~30% in the LVs of control donor patients (*Figure 4A*).[88] These titin-isoform changes were associated with a substantial decrease in myofibrillar PT (*Figure 4B*). The CAD hearts showed increased fibrosis and collagen accumulation and it was proposed that the shift towards more compliant N2BA-isoforms occurs in response to elevated ECM-based stiffness.[88]

Subsequent analyses of explanted non-ischaemic human DCM hearts (mean ejection fraction (EF), ~20%) again demonstrated increased proportions of N2BA-isoforms (*Figure 4A*),[38,62] and particularly the upregulation of N2BA-isoforms larger than 3.3 MDa.[38] As in the CAD hearts, the titin-isoform switching lowered passive myocyte

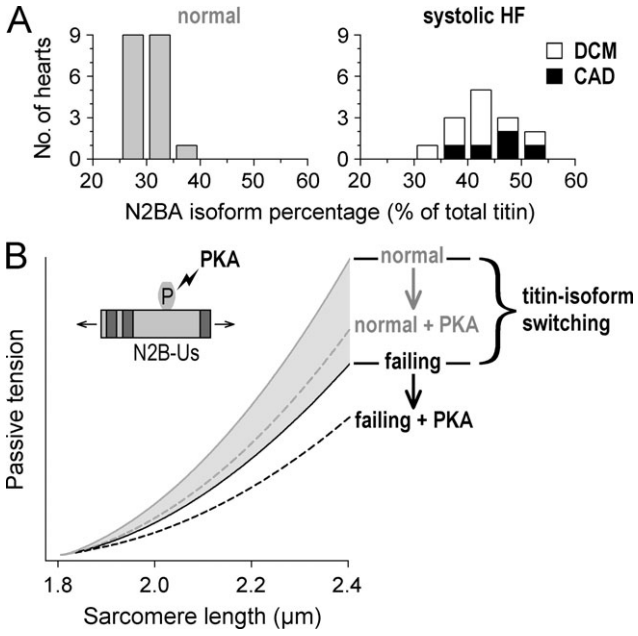

Figure 4 Regulation of titin spring force in normal and failing human hearts by titin-isoform switching and titin-phosphorylation. (*A*) Proportion of N2BA-isoform expressed in hearts from normal donors and from systolic heart failure (HF) patients with dilated cardiomyopathy (DCM) or coronary-artery-disease (CAD). (compiled from[38,88]) (*B*) Graph demonstrating changes in myofibrillar passive tension caused either by titin-isoform switching or by PKA-mediated titin-phosphorylation in normal and end-stage failing human hearts with systolic HF. Inset: PKA-phosphorylation site on titin's N2B-domain.

stiffness in comparison with normal donor LV (*Figure 4B*), and these changes affected diastolic filling. The findings suggest that the hearts of systolic HF patients express increased proportions of compliant N2BA-isoforms, which helps reduce wall stiffness, thus benefiting diastolic function. A drawback, however, may be that a reduced titin spring force in heart failure could compromise systolic function through impairment of the Frank–Starling mechanism and myocyte stretch-sensing. Interestingly, a recent analysis of left-ventricular biopsies from patients with diastolic HF (mean EF, 62%) found N2BA:N2B ratios that were much lower than those reported for systolic HF patients, while myocyte passive stiffness was high.[89] Possibly, a reduced N2BA:N2B ratio causing high titin-based stiffness is a general feature of human HF with preserved EF. In summary, the increased N2BA:N2B titin ratios and correspondingly reduced sarcomeric stiffness in human systolic HF may occur in response to global fibrosis, altered loading conditions, or altered humoral status.[90] Future work will need to define the triggers for the titin-isoform switch better and establish possible differences in the direction of the switching in systolic *versus* diastolic HF.

5.8 Regulation of titin stiffness by phosphorylation

The stiffness of the cardiac titin springs can also be dynamically regulated on a faster time-scale by protein-kinase-A (PKA)-mediated phosphorylation (*Figure 4B*).[91,92] Titin phosphorylation via the β-adrenergic pathway was first recognized to lower titin-based stiffness in rat and cow heart.[91,93] PKA was also found to decrease PT in skinned human cardiomyocytes and interestingly, this effect was much more pronounced in cells from diastolic HF patients than in those from systolic HF patients or control donors.[89,94] Thus, an abnormal titin-phosphorylation state may contribute to altered diastolic stiffness in diastolic HF.

In skinned muscle strips from normal donor hearts, PKA reduced the PT by ~20–40% (*Figure 4B*) and the mechanical changes correlated with phosphorylation of both the N2B and the N2BA titin-isoforms.[92] The PKA-dependent PT-drop in human-heart preparations was substantially larger when titin was first de-phosphorylated, suggesting that inherent phosphorylation of titin is important for the basal myocardial PT level.[92] PKA targets Ser/Thr phosphorylation sites in the N2B-Us (*Figure 4B*, inset),[91,95] but the molecular basis behind the PKA-effect on titin-spring force is still unresolved. The effect may be interesting from a therapeutic point of view, because raising myocardial PKA activity by beta-adrenoceptor stimulation could improve the left-ventricular diastolic function in patients with diastolic HF.[89] To conclude, modifications in titin-based passive stiffness triggered by titin phosphorylation represent a novel mechanosensitive signalling event in heart muscle, which is worth exploring in follow-up studies.

6. The scaffolding role of A-band titin

The segment of titin at the I-band/A-band junction and in the A-band is composed of Ig and FN3 modules that are mainly arranged in a super-repeat pattern with either seven (6×) or eleven (11×) domains (*Figure 1*).[20] The ~2-MDa A-band titin is functionally inextensible, since it is tightly bound via FN3-domains to myosin[25] presumably at a stoichiometry of six titin molecules per half-thick filament.[96] Moreover, within the 11-domain super-repeats the first Ig-domain interacts with MyBP-C.[7] Because A-band titin provides regularly spaced binding sites for other thick filament proteins, it is viewed as a molecular blueprint which controls the precise assembly and exact length of the myosin filaments.[6,25]

7. Structural and signalling complexes of M-band titin

The ~200-kDa COOH-terminal end of titin (exons 355–363) is at the A-band/M-band junction and the M-band (*Figure 1*). The involvement of this titin segment in thick-filament assembly, M-band formation and even maturation of other parts of the sarcomere was demonstrated by targeted homozygous deletion of the entire M-band-titin region in cardiomyocytes, which prevented sarcomere formation.[31]

7.1 The titin-kinase region: a putative stretch-sensor complex

The domains A168–170 just NH_2-terminal to the titin-kinase domain provide a binding site for MURF-1 (*Figure 1B*),[97,98] an E3 ubiquitin ligase that binds various other muscle proteins, including troponins, myosin-light-chain, myotilin, telethonin, N-RAP, and nebulin (*Figure 2*),[99] presumably to control their proteasome-dependent degradation. MURF-1 in turn associates with MURF-2,[97] which also interacts with the above muscle proteins and titin-A168,[99] unlike the microtubule-associated MURF-3.[97,99] MURF-1 interacts with many enzymes involved in ATP-production[99] and with various proteins that have nuclear functions (*Figure 2*).[97] Recent work on MURF-1-deficient mice suggested that MURF-1 is dispensable for normal cardiac development but has an inhibitory role in cardiac hypertrophy, likely by its direct association with the transcriptional co-factor SRF and perhaps also via inhibition of PKCε-activity through interaction with RACK1 (*Figure 2*).[100] Thus, the titin-MURF-1 linkage could be at the heart of a stress-dependent signalling pathway acting via the sarcomeric M-band.

The titin-kinase domain (encoded by titin-exon 358) was initially shown to be activated by phosphorylation of a tyrosine and subsequent binding of Ca^{2+}/calmodulin to the regulatory tail.[26] The titin-kinase phosphorylates telethonin but whether this is important for myofibrillogenesis, as suggested earlier,[26] remains controversial.[30] Deletion of the titin-exons 358 and 359 in a conditional knockout mouse caused sarcomere-disassembly in both skeletal and cardiac muscle and early death.[27] In a conventional knockout-mouse model containing the same deletion, the initial assembly of sarcomeres was unaffected, but the mice died in late-embryonic development owing to impaired cardiac hypertrophy.[30] A recent tamoxifen-inducible deletion of the titin-kinase region in adult mouse hearts produced severe cardiac hypertrophy and congestive heart failure, associated with an attenuated response to adrenergic stimulation and extracellular Ca^{2+}.[32] Surprisingly, despite the deletion of the MURF-1-binding site in this mouse model, MURF-1 was upregulated and PKCε and troponin-I were unchanged, which raises new questions about the role of the titin-MURF-1 signalling pathway in cardiac hypertrophy.

The sarcomeric M-band region may respond to mechanical stress by force-induced conformational changes in the titin-kinase.[29] Activation of the kinase by stretch allows interaction with the proteins Nbr1 and p62, the latter binding to MURF-2 (*Figure 2*).[28] MURF-2 then interacts with SRF to inhibit its nuclear localization and transcriptional activity, hence suppressing hypertrophic responses elicited by mechanical forces. Although this model is attractive, a recent study on MURF-2-deficient mice showed no changes in the hypertrophic response of the hearts to experimentally induced pressure-overload, compared to wildtype hearts.[100] These results suggest that the titin-MURF-2 signalling axis may be dispensable for normal cardiac response to mechanical stress. In conclusion, it is likely that the titin-kinase region is centrally involved in myocyte stress-sensing, but the molecular mechanism remains to be demonstrated unambiguously.

7.2 Interactions and functions of COOH-terminal titin domains

Titin-exons 358/359 code also for the Ig-domains M1–M7 and several intervening unique sequences, the largest of which is Mis-2, which contains a second binding site for FHL2/DRAL (*Figure 1B*).[54] Further, titin-M4 interacts with myomesin, a myosin-binding protein that cross-links the thick filaments and titin's COOH-terminus in an elastic manner.[7,11] Elasticity of myomesin may be important to rectify force imbalances between parallel thick filaments during active muscle contraction. Myomesin associates with creatine-kinase and MR-1 (*Figure 2*) and is regulated in its affinity to titin by phosphorylation.[11] The myomesin–titin–myosin complex is most likely the critical structure that maintains the stability of the M-band.

At the extreme COOH-terminus of titin, the domains M7–M10 bind to A- and B-type lamins (*Figure 1B*), proteins that form structural filaments in the nucleus.[19] This interaction likely involves nuclear forms of titin found in non-muscle cells,[18] which could contribute via their lamin-binding properties to nuclear organization during interphase. Finally, the last unique sequence in titin (Mis-7) encoded by exon 362 (Mex5) offers a second binding site for the protease calpain-3/p94.[65,66] In summary, association of titin's COOH-terminus with structural and signalling molecules implicates this titin region in thick filament assembly/turnover and would confer both mechanical stability and flexibility to the M-band.

8. Human titin as a candidate gene for hereditary myopathies

The titin gene locus on chromosome 2q31 has long been recognized as a strong candidate for familial DCM.[101] Currently, all segments in human titin (Z-disk, I-band, A-band, M-band) are known to be affected by mutations causing various forms of hereditary myopathies. Among them are DCM as well as HCM, but also skeletal-muscle diseases, such as tibial muscular dystrophy (TMD) (limb-girdle muscular dystrophy type-2J, LGMD2J), and hereditary myopathy with early respiratory failure (HMERF). The respective locations of the currently known mutations in human titin are highlighted in *Figure 1B* and *Table I*. Although relatively few mutations in titin (8 DCM; 2 HCM; 4 muscular dystrophies) have been reported so far, the huge size of this molecule and the prevalence of the mutations already found suggest that titin mutations may be a more common cause of human myopathies.[101,102] Many titin mutations are predicted to alter the interaction with a ligand (*Table I*), suggesting they could affect the putative stress-sensing function of those titin regions.

9. Conclusions and perspectives

Increasing evidence suggests that the myocardial stress-response machinery extends to the sarcomeres where distinct regions, including 'hot spots' along the giant titin molecules, participate in a mechano-chemical coupling. Titin's functional roles were once thought to be restricted to molecular scaffolding and providing myofibrillar elasticity, but

Table I Disease-associated mutations in human titin (also see *Figure 1B*)

Phenotype	Location on titin	Mutation	Remarks	References
DCM	Z1 (exon 2)	Val54Met point mutation	Decreased binding to telethonin	103
DCM	Z-repeat 7 (exon 14)	Ala743Val point mutation	Decreased binding to α-actinin	103
DCM	Z4 (exon 18)	Trp930Arg missense mutation	Predicted to disrupt IgZ4-fold	104
DCM	N2B-Us (exon 49)	Gln4053ter nonsense mutation	Predicted to generate truncated titin	103
DCM	N2B-Us (exon 49)	Ser4465Asn missense mutation	Mutation in FHL2-binding site	103
DCM	A67 (exon 326)	2-basepair insertion, frameshift mutation	Predicted to generate truncated A-band titin	104
DCM	A131-A136 (exon 335)	62890delG1 1-basepair deletion, frameshift mutation	Predicted to generate truncated A-band titin	105
DCM	Mis-2 (exon 358)	Arg25618Gln point mutation	Mutation in FHL2-binding site	106
HCM	Z-repeat 7 (exon 14)	Ala740Leu point mutation	Increased binding to α-actinin	107
HCM	N2B-Us (exon 49)	Ser3799Tyr point mutation	Increased binding to FHL2	103,106
HMERF	Titin-kinase (exon 358)	Arg279Trp in exon 358, point mutation	Mutation in Nbr1-binding site	28
TMD/LGMD2J	M10 (exon 363)	complex 11-bp deletion-insertion	Mutation near calpain-3-binding site; found in Finnish population	102,108
TMD/LGMD2J	M10 (exon 363)	Iso293329Asp point mutation	Found in Belgian family	109
TMD/LGMD2J	M10 (exon 363)	Leu293357Pro point mutation	Found in French family	108

this protein may have additional important duties as a stress-sensor. Titin together with some of its direct and indirect ligands in the Z-disk and M-band regions, and the N2B, N2A, and PEVK-domains in the I-band region, could act as a 'tensiometer' that when stretched, triggers downstream signalling events (*e.g.*, activation of transcriptional (co)-factors) leading to changes in muscle–gene expression and cardiac hypertrophy. Conversely, a compromised stress-response function of the titin-signalosome, for instance caused by mutations in protein–protein interaction sites, can result in mechanical dysregulation and congestive heart failure.

Future work may aim at detecting novel titin-ligands that participate in the mechano-chemical coupling and uncovering their stress-dependent interaction to the atomic detail. It will be useful to explore the involvement of titin-based 'hot spots' in the stress-sensing network of the cardiomyocyte by gene knock-down and functional tests. Additional studies should also identify the triggers that cause the large changes in titin-isoform composition during heart development and disease, which greatly affect myocardial passive stiffness and possibly, stress-dependent signalling. An intriguing property that warrants further research is the dynamic regulation of titin-based passive stiffness and titin's putative tensiometer function by phosphorylation of the N2B-domain. Some of the mysteries of the sensitive molecular giant titin have now been revealed, but we are likely to see many more of its secrets uncovered in the years to come.

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