

RBM20, a potential target for treatment of cardiomyopathy via titin isoform switching

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Abstract Cardiomyopathy, also known as heart muscle disease, is an unfavorable condition leading to alterations in myocardial contraction and/or impaired ability of ventricular filling. The onset and development of cardiomyopathy have not currently been well defined. Titin is a giant multifunctional sarcomeric filament protein that provides passive stiffness to cardiomyocytes and has been implicated to play an important role in the origin and development of cardiomyopathy and heart failure. Titin-based passive stiffness can be mainly adjusted by isoform switching and post-translational modifications in the spring regions. Recently, genetic mutations of *TTN* have been identified that can also contribute to variable passive stiffness, though the detailed mechanisms remain unclear. In this review, we will discuss titin isoform switching as it relates to alternative splicing during development stages and differences between species and muscle types. We provide an update on the regulatory mechanisms of *TTN* splicing controlled by RBM20 and cover the roles of *TTN* splicing in adjusting the diastolic stiffness and systolic compliance of the healthy and the failing heart. Finally, this review attempts to provide future directions for RBM20 as a potential target for pharmacological intervention in cardiomyopathy and heart failure.

Keywords Titin isoform switching · Alternative splicing · Cardiomyopathy · RBM20

Introduction

Cardiomyopathy, or heart muscle disease, can result in either systolic dysfunction with decreased myocardial contraction or diastolic dysfunction with impaired ability of ventricular filling (Daughenbaugh 2007; Henry 2003; Peters 2016). A number of factors can contribute to the onset and development of cardiomyopathy, including abnormal energetic metabolism, intracellular calcium handling, and sarcomeric structure and protein changes (LeWinter 2005; Miyata et al. 2000). Among the sarcomeric proteins, titin protein is increasingly regarded as one of the molecular origins of cardiomyopathy and heart failure (Linke and Hamdani 2014; Yin et al. 2015). Titin protein is a giant multifunctional sarcomeric filament that plays a critical role in elastic recoil of the cardiac myocytes and contributes to diastolic function during the left ventricular filling phase (Granzier and Irving 1995; Granzier and Labeit 2004; Herman et al. 2012; LeWinter and Granzier 2010, 2014; Taylor et al. 2011). Titin can function as a “molecular blueprint”, “molecular spring”, and “molecular signaling mediator” (Granzier and Labeit 2004, 2005; Krüger and Linke 2011; Linke 2008). During the past decade, evidence has shown that titin elasticity is highly variable in the developing and the adult normal healthy heart, and that it can be pathologically modified in cardiomyopathy and heart failure. Pathological modifications can greatly compromise the extensibility and the diastolic passive stiffness of myocardium, and, presumably, the mechanosignaling transduction sensitivity (Makarenko et al. 2004; Opitz and Linke 2005). Variable titin elasticity is, in large part, attributed to titin isoform switching that results from alternative splicing (Granzier and Labeit

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2004; Linke 2008). However, mechanistic study of titin isoform switching had never been addressed until the *TTN* splicing regulator, RNA binding motif 20 (RBM20), was cloned and identified recently in 2012 (Guo et al. 2012). This finding opened a new avenue for the identification of novel potential targets for therapeutic intervention for titin-based pathological alterations in cardiomyopathy and heart failure. In our review, we begin with an introduction to titin, including its structure, function, and arrangement in the sarcomere, and provide an update on known alternatively used exons and *TTN* splicing patterns. We then cover some details of *TTN* splicing mechanism(s) regulated by RBM20 and discuss the potential therapeutic strategies and future directions.

Titin structure, function, and arrangement in the sarcomere

The sarcomere is the smallest contractile unit in myofibrils of striated muscle fibers and contains four major filament systems: actin-thin, myosin-thick, titin, and nebulin filaments (Trinick 1996; Tskhovrebova and Trinick 2003). Titin filament is the third one (after myosin and actin filaments), composed of a gigantic fibrous protein titin, which is the largest protein by far found in vertebrate animals (Gigli et al. 2016; Maruyama 1976; Wang et al. 1979; Yin et al. 2015). Titin spans half of a sarcomere and connects the Z-band to the M-band located in the center of the sarcomere. Its carboxy (C)-terminal cross-links with another titin's C-terminal in the M-band, and its amino (N)-terminal attaches another titin's N-terminal from adjacent sarcomere and anchors in the Z-band (Fürst et al. 1988; Labeit et al. 1990; Bang et al. 2001), which allows titin molecules to form a continuous system along the myofibril. Such structural arrangement of titin enables it to function as a molecular blueprint for the maintenance of sarcomere integrity and precise assembly of the regulatory, contractile, and structural proteins located in the sarcomere (Freiburg and Gautel 1996; Granzier and Labeit 2004; Labeit et al. 1992; Tskhovrebova and Trinick 2003) and transduce force generated during contraction at the Z-band (Gutierrez-Cruz et al. 2001; Labeit and Kolmerer 1995) (Fig. 1). Besides titin serving as a “molecular blueprint”, it also acts as a “molecular spring” and a “molecular signaling mediator” (Granzier and Labeit 2004, 2005, 2006; Kontogianni-Konstantopoulos et al. 2009; Krüger and Linke 2011; Linke 2009). Titin contains both highly repetitive and non-repetitive domains. Approximately 90% of the titin sequence is repeating immunoglobulin (Ig) and fibronectin-3 (Fn3) domains, each around 100 residues. Each molecule contains between 240 and 300 repeats, depending on different isoforms. The remaining approximately 10% of non-repetitive sequence consists of unique sequences (e.g., N2B and N2A), 28–30 residue PEVK [proline (P), glutamate (E),

valine (V), and lysine (K)] motifs, and a C-terminal Ser/Thr kinase domain interspersed between the Ig and Fn3 repeats (Guo et al. 2010; Kontogianni-Konstantopoulos et al. 2009; Meyer and Wright 2013; Puchner et al. 2008; Tskhovrebova and Trinick 2004). The repeating Ig domains located in the I-band are not extensible, while the non-repetitive unique sequences N2B and the PEVK motifs are. These elastic segments in titin determine that titin is a spring-like protein and serves as a molecular spring (Granzier and Labeit 2002; Kellermayer et al. 1997; Linke et al. 1996, 1999; Trombitás et al. 1998; Watanabe et al. 2002) (Fig. 1). Titin's modular structure with repetitive and non-repetitive domains provides diverse binding sites for a variety of proteins, which include myofibrillar proteins, membrane components, enzymes, and signaling molecules. Over 20 proteins have been found bound to titin, linking it to multiple potential mechanical signaling pathways (Krüger and Linke 2011; Linke 2008). Titin runs through distinct sarcomere regions: the Z-band, the I-band, and the A-band regions, each of which may transduce stress signals (Fig. 1). For example, at the Z-band, protein complexes with titin, telethonin/T-cap, MLP, and calsarcin-2 can activate the calcineurin/nuclear factor of activated T-cells (NFAT) signaling to induce hypertrophy, leading to heart failure (Fig. 1) (Hoshijima 2006; Lange et al. 2006; Linke 2008); at the I-band, the protein complexes of N2B, FHL1, FHL2, and MAPK may activate MAPK signaling cascade (raf/Mek1/2/Erk2) to induce hypertrophic signaling or repress calcineurin/NFAT signaling for pathological cardiac growth (Fig. 1) (Hojayev et al. 2012; Kötter et al. 2014; Lange et al. 2002; Scholl et al. 2000; Sheikh et al. 2008); at the M-band, titin can also mediate hypertrophic signaling through its kinase (TK) domain. Activated TK may interact with protein complexes of Nbr1, p62/SQSTM1, and MuRF1/2/3 to induce hypertrophic signaling (Fig. 1) (Kötter et al. 2014; Lange et al. 2005). Thus, this giant protein is believed to serve as a molecular signaling mediator, a central player for hypertrophic signaling.

TTN splicing

The human *TTN* gene is located on chromosome 2q31 and consists of 294-kilo base pairs (kb). This single mammalian gene contains 364 exons, with 363 coding ones (the first one is a non-coding exon) that can be transcribed to a more than 100-kb-long mRNA. Deduced from the mRNA sequence, a total of 38,138 amino acid residues with a molecular weight (MW) of 4.2 MDa could be produced (Bang et al. 2001). Interestingly, translation of full-length titin with 4.2 MDa has never been reported. Reported titin sizes range from approximately 3.0 to 3.9 MDa, resulting from alternatively used exons (Guo et al. 2010; Warren et al. 2004). Nearly all exons in the Z-band, A-band, and M-band regions are constitutively

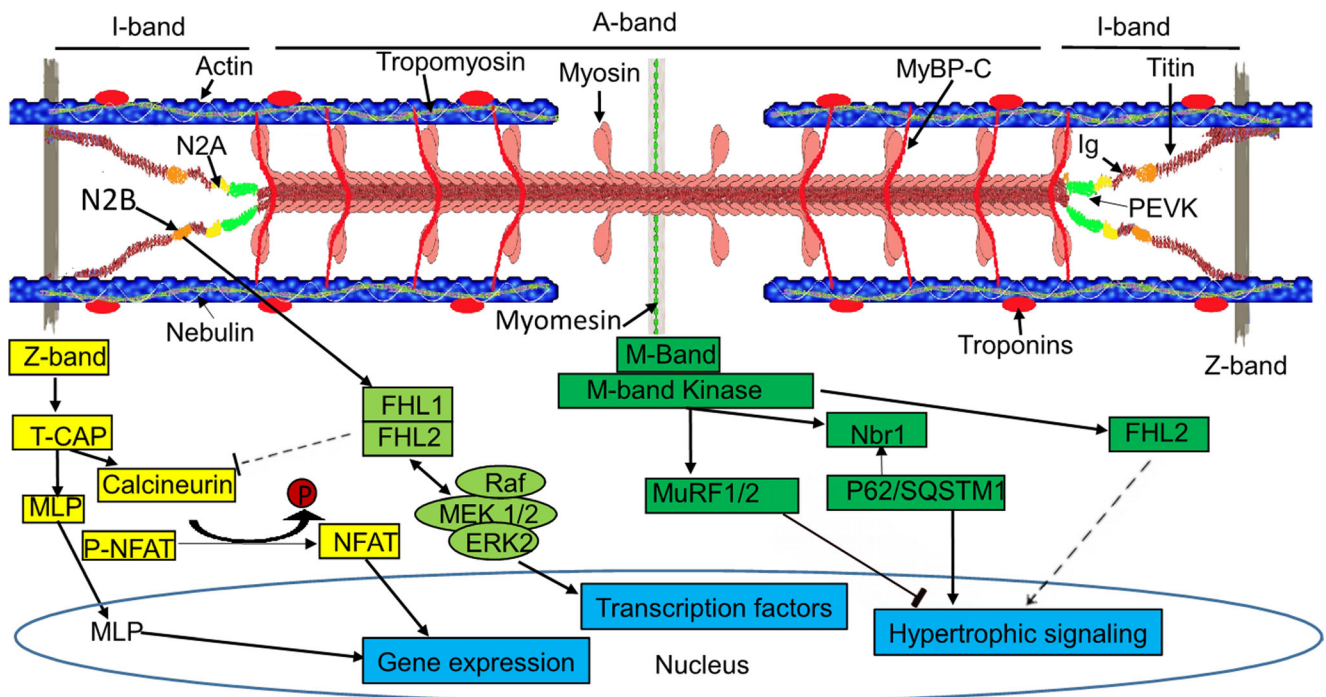


Fig. 1 The striated sarcomere structure and *TTN*-mediated signaling pathway. The upper schematic structure shows the layout of the sarcomere and the domains of *TTN*. The lower scheme shows the hypertrophic signaling pathways through *TTN* binding partners at the Z-band, I-band, and M-band. MLP, muscle LIM protein; NFAT, nuclear

factor of activated T-cells; FHL1/2, four-and-a-half LIM domain protein 1/2; Raf, rapidly accelerated fibrosarcoma protein; MEK 1/2, mitogen-activated protein kinase kinase 1; ERK 1/2, extracellular signal-regulated kinase; Nbr1, neighbor-of-*BRCA1*-gene-1; p62, nucleoporin p62; QSTM1, sequestosome 1

expressed in human striated muscle, while the I-band undergoes extensive alternative splicing with two splicing hot regions: the variable-length Ig region and the PEVK domain (Labeit and Kolmerer 1995; Linke and Kruger 2010). The splicing patterns of the PEVK domain are exceptionally complex. PCR results from a previous study indicated that at least 20 exons are absent in the PEVK region (Guo et al. 2010). If these 20 exons are independently spliced, the combinations of these splicing events could lead to millions of different splicing pathways or isoforms. However, using a well-recognized large protein electrophoresis gel detection system, vertical SDS-agarose gel electrophoresis, for titin isoform separation (Warren et al. 2003b), only six titin isoforms are detectable in rodent cardiac muscle, and one isoform is visible in most vertebrate skeletal muscles. The explanation for the few detectable isoforms could be that: (1) many isoforms have nearly equal sizes as the six isoforms and are overlapped at the same gel band as the six isoforms; (2) other potential isoforms are expressed in very low levels, which are undetectable; (3) both situations are combined. The six isoforms have been named as N2BA-N1 and -N2 (embryonic and neonatal forms) with apparent sizes of approximately 3.7 and 3.6 MDa, respectively; N2BA-A1 and -A2 (adult forms), with sizes of about 3.4 and 3.2 MDa, respectively; N2B (a cardiac-specific isoform), with a size of approximately 3.0 MDa (Warren et al. 2004); and N2BA-G (an unusual giant isoform), with a size of

approximately 3.9 MDa, caused by a gene mutation (Greaser et al. 2005, 2008). In addition, one isoform in skeletal muscle is known as N2A, with variable sizes from approximately 3.2 to 3.7 MDa (Cazorla et al. 2000; Freiburg et al. 2000; Labeit and Kolmerer 1995) (Fig. 2a). These isoforms are called N2B, N2BA, and N2A because they contain either N2B or N2A or both unique domains. Finally, each isoform represents a class of titin isoforms resulting from the combinations of alternatively spliced exons, mainly in the I-band Ig domain and the PEVK region. The N2B unique domain is solely found in cardiac muscle and the N2A unique domain can be found in both skeletal and cardiac muscle. If the isoforms contain both N2B and N2A unique domains, they are called N2BA, which are also exclusively found in cardiac muscle (Guo et al. 2010; Labeit and Kolmerer 1995). The giant N2BA isoform (N2BA-G) is assumed to result from nearly all the alternatively used exons being spliced in. N2BA-N1 is expected to contain all the exons for expression of the middle Ig domain and N2BA-N2 is proposed to lack the sequence from exons 72 to 79. Likewise N2BA-A1 and N2BA-A2 are suggested to skip exons 51–70 and 51–89, respectively (Greaser et al. 2005; Guo et al. 2013). N2B skips exons 51–218 (Cazorla et al. 2000; Freiburg et al. 2000; Greaser et al. 2005; Guo et al. 2013). Human soleus N2A is proposed to skip exon 49 expressing for N2B unique sequence and rabbit psoas N2A is lacking exon 49 and exons 53–69

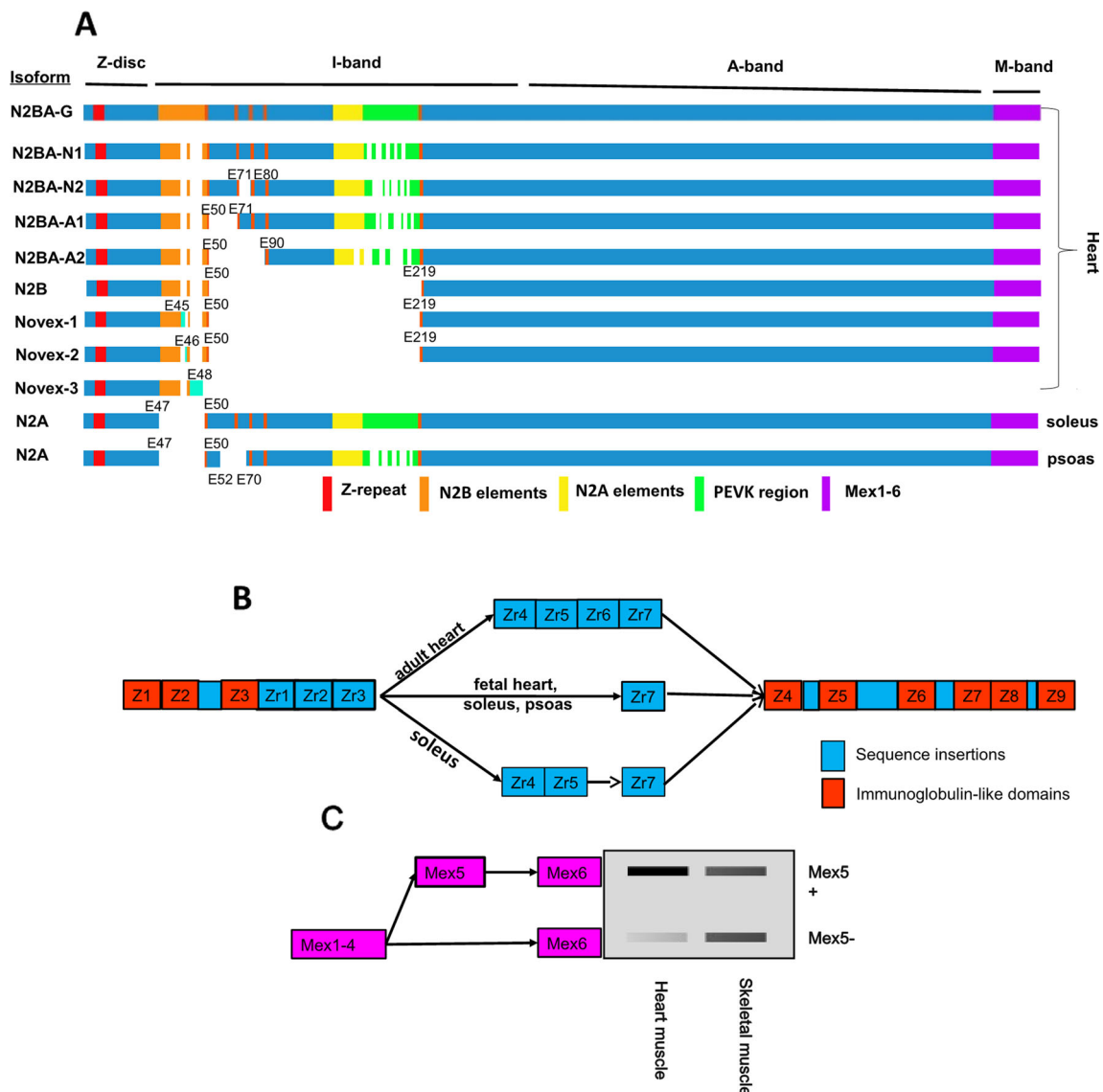


Fig. 2 Known *TTN* isoforms and *TTN* splicing patterns at the I-band, Z-band, and M-band. **a** Known *TTN* isoforms in striated muscles. **b** Nine Ig domains with insertions in the Z-band and overview of the Z-repeat isoforms expressed in different types of striated muscles. The repeats Zr1, 2, 3, and 7 express in all striated muscles, whereas Zr4, 5, and 6

are alternatively spliced, depending on the tissue type and the developmental stage. **c** Alternative splicing of the M-band. The right panel shows the approximate ratio of Mex5+:Mex5- in striated muscles. E, exon; Z, Ig domain repeats; Zr, Z-repeats between Z3 and Z4; Mex, M-band exon

(Greaser et al. 2005; Guo et al. 2010) (Fig. 2a). Besides the N2B, N2BA, and N2A isoforms, three other isoforms have also been reported, which are Novex1, Novex2, and Novex3. The Novex1 and Novex2 isoforms result from alternative splicing and have similar size and splice patterns as the N2B isoform. Novex1 expresses exon 45, and Novex2 exon 46, while these two exons are not present in the major isoforms (N2B, N2BA, and N2A) (Bang et al. 2001; Linke 2008). Novex3 expresses an approximately 650 kDa protein and is produced from an alternative terminal coding exon 48 instead of alternative splicing, and the rest of the exons in Novex3 have the same splice pattern as the major isoforms (Bang et al. 2001; Chauveau et al. 2014; Linke 2008) (Fig. 2a).

In addition to the hot splicing region in the I-band, alternative splicing can also occur in the Z-band and M-band. The Z-band titin is encoded by the first 28 exons consisting of nine Ig domains (Z1–Z9) interspersed with large inter-domain insertions from Z2 to Z7 and between Z8 and Z9. The insertion between Z3 and Z4 contains alternatively spliced Z-repeats, termed Zr1 to Zr7. Alternative splicing leads to the differential expression of Z-repeats varying from 4 to 7. The repeats Zr1–3 and Zr7 are constitutively expressed in all striated muscles, while the repeats Zr4–6 are alternatively spliced depending on the developmental ages, the species, and the type of striated muscles. For example, in the adult heart, all seven repeats are included, whereas the fetal heart skips Zr4–6. In the slow

muscle soleus, two isoforms could be identified, with one excluding Zr4–6 and the other skipping Zr6. However, in the fast twitch rabbit psoas muscle, only the isoform skipping Zr4–6 could be found (Fig. 2b) (Chauveau et al. 2014; Gautel et al. 1996; Guo et al. 2010; Linke 2008; Sorimachi et al. 1997). Kolmerer et al. (1996) searched for alternatively expressed exons in the M-band region of titin, and they found that there were six exons in the M-band, termed Mex1 to Mex6. Intriguingly, only Mex5 was found to undergo alternative splicing. Mex5 is mainly included in heart muscle with a higher ratio of Mex5+:Mex5–, but it seems that skeletal muscle contains equal or lower ratios of Mex5+:Mex5–. Alternative splicing of Mex5 was not observed during mouse development (Fig. 2c) (Granzier and Labeit 2002; Guo et al. 2010; Kolmerer et al. 1996).

***TTN* splicing patterns with development stages, species, and muscle types**

The splicing patterns of individual alternative exons have not been well-characterized for different development stages, species, and muscle types (Lahmers et al. 2004; Ottenheijm et al. 2009; Opitz et al. 2004, Opitz and Linke 2005). In this section, we mainly focus on the discussion of the changes or switching of major classes of isoforms (N2B, N2BA, and N2A) resulting from alternative splicing. Patterns are primarily based on electrophoretic studies.

Titin plays a major role in determining passive tension in stretched muscle (Granzier and Labeit 2002; Linke and Fernandez 2002). Titin-based stiffness constitutes a major proportion of the total myocardial passive stiffness in the normal adult human heart and at approximately the time of birth. The heart modifies the mechanical properties of the titin spring to adjust to the global mechanical requirements (Opitz and Linke 2005). In cardiac muscle, N2B has a shorter spring-like domain than N2BA, and, thus, produces higher passive stiffness. Therefore, switching between these two isoforms provides variable titin-based passive stiffness in order to meet physiological requirements, depending on development stages and species. In the larger mammalian heart, including the cat, rabbit, pig, sheep, bovine, and human heart, all hearts express four major titin isoforms (N2BA-N1, N2BA-N2, N2BA-A1, and N2B). In the fetal stage, cardiac muscle expresses only the N2BA-N1 isoform (~3.7 MDa). During development, the N2BA-N1 isoform is gradually replaced by one neonatal isoform (N2BA-N2, ~3.6 MDa) and two or three adult isoforms, N2BA-A1/2 (~3.2–3.4 MDa) and N2B (~3.0 MDa). Nevertheless, the ratios of N2BA-A1 to N2B in the adult heart vary in distinct species. The estimated order is rabbit < sheep < human = pig < cat < bovine (Fig. 3a) (Freiburg et al. 2000; Fukuda et al. 2003; Neagoe et al. 2003; Opitz and Linke 2005; Opitz et al. 2004). By comparing

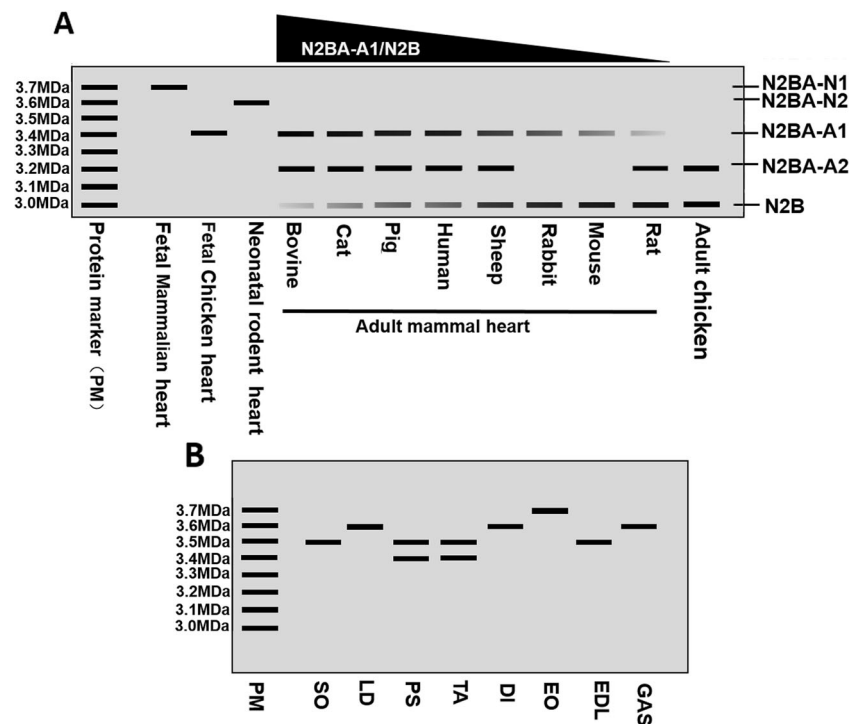
to large animals, small mammals such as mouse and rat also express the N2BA-N1 isoform during prenatal development. The larger isoform is later replaced by the neonatal form N2BA-N2 (~3.6 MDa) and adult forms N2BA-A1 (~3.4 MDa), N2BA-A2 (~3.2 MDa), and N2B (~3.0 MDa) in rat heart, and N2BA-A1 and N2B in mouse heart (Fig. 3a) (Cazorla et al. 2000; Greaser et al. 2005). The rat heart contains a lower ratio of N2BA:N2B than that of mouse heart (rat < mouse), but small mammals have lower ratios than larger mammals (Cazorla et al. 2000; Neagoe et al. 2003). Moreover, in chicken heart, the largest embryonic titin isoform is about 3.4 MDa, similar to N2BA-A1 in large and small mammals, and the adult forms are approximately 3 MDa and 3.2 MDa, similar to the N2B and N2BA-A2 isoforms (Fig. 3a) (Opitz and Linke 2005).

In addition to isoform switching in cardiac muscle, although skeletal muscles only express a single N2A titin isoform, the size of the N2A isoform vary from ~3.3 MDa to ~3.7 MDa (Granzier and Labeit 2002). Soleus (SO) and longissimus dorsi (LD) express a long single N2A isoform (~3.7 MDa), while psoas (PS) and tibialis anterior (TA) express doublet bands, with ~3.5 MDa and ~3.4 MDa in psoas and ~3.4 MDa and ~3.3 MDa in TA (Granzier and Labeit 2002; Guo et al. 2012; Li et al. 2012; Neagoe et al. 2003; Wang et al. 1991). Li et al. (2012) have estimated the titin sizes in several different rat muscles, including diaphragm (DI), extensor oblique (EO), SO, extensor digitorum longus (EDL), PS, LD, TA, and gastrocnemius (GAS), and they confirmed previous evidence and found that all titin sizes are in the range of ~3.3 MDa to ~3.7 MDa (Fig. 3b).

***TTN* splicing mechanisms**

The *TTN* gene has been known to undergo alternative splicing since the original gene sequence report (Labeit and Kolmerer 1995). However, the mechanism of how *TTN* splicing is regulated remained completely unknown until RNA binding motif 20 (RBM20), a splicing factor, was cloned and identified (Guo et al. 2012). As discussed above, titin expresses various sizes as a result of alternative splicing varying with development, species, and different muscle types. In the absence of RBM20 in both rat and mouse models, titin expresses a consistently larger isoform (~3.9 MDa) (Fig. 2a) under all conditions (developmental, species, and muscle types) (Greaser et al. 2008; Guo et al. 2012, 2013; Li et al. 2012; Methawasin et al. 2014). This suggested that RBM20 could function as a repressor of *TTN* splicing (Li et al. 2013). Our published data indicated that RBM20-mediated *TTN* splicing is dose-dependent. Cardiac muscle expresses mainly the smaller N2B isoform (N2BA:N2B = ~20:80) in wild-type rats and high levels of medium-sized N2BA (N1 and N2) (N2BA:N2B = ~60:40) in heterozygous RBM20 knockout

Fig. 3 Schematic diagram of *TTN* bands in the electrophoresis gel. **a** Cardiac *TTN* isoform migration and the ratio in the fetal and adult hearts in different species. **b** Skeletal muscle *TTN* migration in different skeletal muscle types. PM, protein marker; SO, soleus; LD, longissimus dorsi; PS, psoas; TA, tibialis anterior; DI, diaphragm; EO, extensor oblique; EDL, extensor digitorum longus; GAS, gastrocnemius



(KO) rats, whereas only the large N2BA-G isoform is expressed in homozygous RBM20 KO rats (Guo et al. 2012). In wild-type rats or those with RBM20 expression, *TTN* exons undergo dynamic changes with development, with fewer exons skipped in the prenatal development stage and more exons spliced out after birth to adult. This implied that the RBM20 level should increase along with more exon skipping according to a repressing regulatory mechanism of RBM20 (Guo et al. 2012; Li et al. 2013). Unexpectedly, the RBM20 level not only increases with development, but also decreases. One explanation for the contradictory phenomena is that other splicing-associated factors may be involved in *TTN* splicing regulation cooperatively with RBM20 (Ito et al. 2016; Li et al. 2013; Yin et al. 2015; Zhu et al. 2016). More evidence is needed to support this hypothesis.

RBM20 consists of 14 exons and encodes an RNA-binding motif protein 20 with a prototypical RNA-recognition motif 1 (RRM-1), followed by an arginine/serine-rich (RS-rich) domain. These structural features are characteristic of a family of RNA-binding SR proteins that assemble in the spliceosome, a huge multiprotein complex that orchestrates constitutive and alternative splicing of pre-mRNA (Fig. 4a) (Brauch et al. 2009; Zhu et al. 2016). Usually, SR and SR-related proteins are frequently phosphorylated and function as splicing activators, recognizing exonic splicing enhancers (Lim et al. 2011; Manley and Tacke 1996). However, sporadic examples of SR proteins act as splicing repressors upon binding at intronic positions (Ibrahim et al. 2005; Kanopka et al. 1996; Shen and Mattox 2012; Wang et al. 2013). A recent study showed that RBM20 binds predominantly to introns

near the 3' and 5' splice sites of repressed exons and in close proximity to the binding sites of U1 and U2 snRNPs. The binding sites of RBM20 to RNA may contain the UCUU core sequence (Figure 4b) (Maatz et al. 2014). In addition, RBM20 could regulate *TTN* splicing via cooperation with *trans*-acting factors and binding with *cis*-acting elements. Recent studies also indicated that *TTN* splicing can be regulated by external stimuli, such as hormones (thyroid hormone and insulin). With increased thyroid or insulin hormone, the ratio of N2BA to N2B is decreased (Krüger et al. 2008, 2010). However, the role RBM20 plays in hormone-modulated *TTN* splicing remains unclear. Currently, our studies demonstrated that RBM20 is the essential mediator linking hormone stimulation to *TTN* splicing via the PI3K/Akt/mTOR signaling pathway. Activated Akt increases the expression level of RBM20, and, thus, regulates titin isoform switching (Fig. 4c) (Zhu et al. 2015). Akt is one of four known kinase systems to regulate gene alternative splicing through the phosphorylation of SR proteins (Lynch 2007); therefore, whether phosphorylation of RBM20 plays a critical role in *TTN* splicing via the PI3K/Akt signaling pathway needs further study.

TTN splicing and cardiomyopathies

Titin is emerging as a promising therapeutic molecular target by restoring compliance to the sarcomere and, thereby, improving diastolic function, or by increasing passive stiffness to the ventricular wall, and, thus, improving systolic function. Improving titin-based passive stiffness of the heart can take

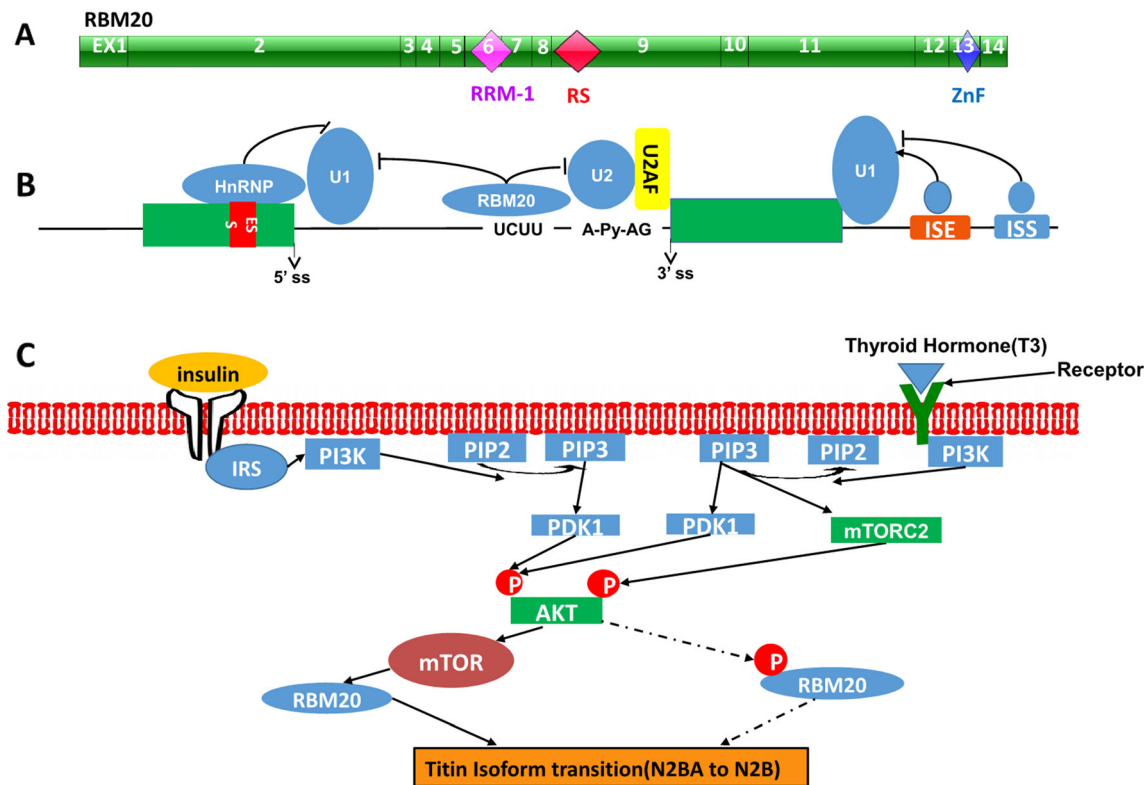


Fig. 4 RBM20 structure and potential *TTN* splicing mechanism(s) regulated by RBM20. **a** Schematic diagram of RBM20 structure. **b** RBM20 regulatory mechanism through binding to *cis*-regulatory elements in *TTN*. **c** *TTN* splicing mechanism via the PI3K/Akt/mTOR

signaling pathway through regulation of RBM20. EX, exon; RRM, RNA recognition motif-1; RS, arginine/serine-rich domain; ZnF, zinc finger domain; ESS, exon splicing silencer; ISE, intron splicing enhancer; ISS, intron splicing silencer; ss, splice site; Py, polypyrimidine tract

place in two ways. One is to switch the N2BA to N2B isoform ratio; the other can be tuned through post-translational modifications (PTMs) of these primary isoforms, particularly through the phosphorylation of titin's spring domains. Switching titin isoform ratios could contribute to ventricular wall stiffness in large part (Guo et al. 2012; Hidalgo and Granzier 2013; Methawasin et al. 2014; Trombitás et al. 2000). Recently, variable titin isoform switching resulting from *TTN* splicing in animal models and human patients has led to heart stiffness changes which have been associated with cardiomyopathies. In animal models, a dog tachycardia-induced model with dilated cardiomyopathy (DCM) showed an elevated ratio of N2BA:N2B under two-week mechanical challenge (Bell et al. 2000), while the ratio was decreased after four weeks of pacing (Wu et al. 2002). In addition, a hypertensive dog model with diastolic dysfunction demonstrated a reduced ratio of N2BA:N2B (Hamdani et al. 2013; Shapiro et al. 2007), and also a spontaneously hypertensive rat model (SHR) had slightly decreased ratio of N2BA:N2B in response to pressure overload (Warren et al. 2003a). In the mouse transverse aortic constriction (TAC) model, a commonly used experimental model for pressure overload-induced cardiac hypertrophy and heart failure, an elevated ratio of N2BA:N2B was observed (Hudson et al. 2011). In contrast, in a ligation of the left anterior descending

coronary artery (LAD) rat model with ischemic cardiomyopathy, the rat heart expressed a reduced ratio of N2BA:N2B (Neagoe et al. 2002). However, in human patients, altered titin isoform ratios have also been detected. By comparing to nonfailing donor heart, the larger and compliant N2BA levels were increased in ischemic cardiomyopathy (Neagoe et al. 2002), non-ischemic DCM (Makarenko et al. 2004; Nagueh et al. 2004), and patients with heart failure with a reduced ejection fraction (HFrEF) (Borbély et al. 2009). Overall, increased compliant N2BA isoforms can be commonly found in eccentric remodeled hearts with systolic dysfunction, such as DCM, HFrEF, and chronic ischemic cardiomyopathy, while reduced N2BA isoforms can frequently be observed in concentric remodeled hearts (compensated hypertrophy) with diastolic dysfunction developed from hypertensive heart disease. Therefore, the manipulation of titin isoform ratios could be a potential therapeutic strategy for variable titin isoform ratios caused by cardiomyopathies and heart failure. A couple of very recent reports with mouse models of RBM20 manipulation showed the feasibility of such an approach. Reducing RBM20 levels in N2B knockout-induced diastolic dysfunction could improve diastolic stiffness (Hinze et al. 2016) and the inhibition of RBM20 in cardiac muscle can reduce ventricular wall stiffness induced in the transverse aortic constriction (TAC) mouse model, and, thus, improve diastolic

function (Methawasin et al. 2016). Since RBM20 is a master regulator of titin isoform switching, it could be a promising therapeutic target for drug development to adjust the titin isoform ratio.

Future directions

Titin is increasingly recognized as a major human disease gene. Titin-associated heart disease is mainly based on the alteration of elastic force, which is highly variable between normal and diseased hearts. Titin-based passive stiffness in the heart can be affected by two main ways: titin isoform switching resulting from alternative splicing and post-translational modifications in *TTN* spring domains with particular phosphorylation. These two paths offer the therapeutic targets in titin to improve diastolic compliance or systolic stiffness and relieving symptoms. Titin isoform switching largely contributes to variable ventricular wall stiffness. As discussed earlier, such an approach could have the potential to adjust titin elasticity by the manipulation of *TTN* splicing under the control of the splicing factor RBM20. However, therapeutic modalities targeting RBM20 are currently largely theoretical due to the still less-defined mechanism(s) of how RBM20 regulates *TTN* splicing. Therefore, future work should aim to identify cooperative regulators, *TTN* pre-mRNA binding sites of RBM20, and the signaling pathways that control the expression and/or post-translational modifications of RBM20.

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Compliance with ethical standards

Conflict of interest Wei Guo declares that he has no conflict of interest. Mingming Sun declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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