**Abstract:** The giant sarcomeric protein titin has multiple important functions in striated muscle cells. Due to its gigantic size, its central position in the sarcomere and its elastic I-band domains, titin is a scaffold protein that is important for sarcomere assembly, and serves as a molecular spring that defines myofilament distensibility. This review focuses on the emerging role of titin in mechanosensing and hypertrophic signaling, and further highlights recent evidence that links titin to sarcomeric protein turnover.

**Keywords:** chaperones; connectin; heat shock proteins.

**Introduction**

The sarcomere is the smallest contractile unit of striated muscle cells and is mainly composed of three filament systems: the myosin-based thick filament; the actin-based thin filament, supplemented with the regulatory protein tropomyosin and the troponin complex; and the titin filament. The giant protein titin is encoded by a single titin gene and has a potential size of 4.2 MDa, allowing one titin molecule to span an entire half-sarcomere from the Z-disc to the M-line. Differential splicing of the titin gene is the basis for numerous species- and muscle-specific titin isoforms with molecular weights ranging from 3.0 MDa to 3.7 MDa. Most of the alternative splicing events occur in the I-band region of titin, but to a smaller degree they also affect the Z-disc and the M-band portion of the molecule (Krüger and Linke, 2011). The molecular mechanisms that control the complex splicing events of the titin mRNA are still not completely understood, but recent studies have identified the splicing factor RBM20 as an important player in this setting (Guo et al., 2012; Li et al., 2013).

The mammalian heart expresses two main isoform types of titin: the longer and more compliant N2BA isoforms (3.2–3.7 MDa) and the shorter and stiffer N2B isoform (3.0 MDa). During cardiac development the heart expresses a compliant fetal N2BA isoform that is replaced after birth by the smaller N2BA and the N2B isoform (Opitz et al., 2004). Depending on the species, this isoform shift is more or less pronounced (Krüger et al., 2006); in adult rat hearts the N2B isoform predominates, with approximately 90% of total titin; whereas in healthy human hearts the relative expression ratio is about 35% N2BA:65% N2B (Neagoe et al., 2002). Skeletal muscles express a third isoform type called N2A titin (3.3–3.7 MDa) with many muscle-specific splice variants (Freiburg et al., 2000; Neagoe et al., 2003; Prado et al., 2005).

**Structure and function of titin**

The structural composition of titin is mainly characterized by the sequential arrangement of immunoglobulin-like domains (Ig-domains), fibronectin-type-3 domains and several so-called unique sequences (Bang et al., 2001). The NH₂-terminal end of titin is anchored in the sarcomeric Z-disc via nebulin or the cardiac isoform nebulette (Witt et al., 2006), α-actinin 2 (Labeit et al., 2006) and telethonin (Granzier and Labeit, 2004; Miller et al., 2004; Lange et al., 2006). In the I-band part titin is composed of a series of proximal Ig-domains, the cardiac specific N2B-domain (including the N2-B unique sequence, N2-Bus), the middle Ig-domain region (not in the N2B isoform), the PEVK domain [predominant amino acids: proline (P), glutamic acid (E), valine (V) and lysine (K)] and the distal Ig-domains. This I-band part can be sequentially extended during sarcomere stretch and represents the main elastic segment of titin (Linke et al., 1996, 1999; Trombitas et al.,...
1998; Li et al., 2002). With a size of about 2 MDa, the A-band portion of titin is the largest part of the molecule (Bang et al., 2001) and it is tightly associated with myosin and myosin-binding protein C (Tskhovrebova and Trinick, 2004; Lange et al., 2006). The M-band portion of titin is characterized by several inserted sequences and the titin–kinase-domain in the M-band periphery (Figure 1) (Bang et al., 2001; Gautel, 2011).

Titin has long been recognized as a scaffolding protein that serves as a ‘blue print’ for sarcomerogenesis and assists in the process of myofibrillar assembly (Ehler and Gautel, 2008; Tskhovrebova and Trinick, 2010). The Ig-domains and fibronectin-type-3 domains in the A-band are arranged in seven- and 11-domain super repeats, which are repeated six and 11 times, respectively. A detailed overview of titin domain structure in the different parts of the sarcomere is provided in Krüger and Linke (2011).

Another important function of titin is provided by the possibility of reversibly extending the elastic I-band domains upon mechanical stretching, thus allowing titin to act as a molecular spring and thereby defining the passive properties of the myofilaments. In cardiac myocytes the titin-based passive myofilament stiffness is mainly determined by the expression ratio of the N2BA and N2B titin isoforms (Krüger and Linke, 2011). In end-stage heart failure, the ratio of N2BA:N2B is often increased and results in a reduced passive stiffness (Neagoe et al., 2002; Makarenko et al., 2004; Nagueh et al., 2004). It has, however, also been shown that in some end-stage dilated and hypertrophic cardiomyopathy patients the titin isoform ratio remains unchanged and passive stiffness

Figure 1 Titin-mediated mechanosensing and hypertrophic signaling. Various titin–protein interactions at the Z-disc, in the I-band, and in the M-band link titin to hypertrophic signaling pathways. CaM, Ca2+-calmodulin; ERK 1/2, extracellular signal-regulated kinase; FHL1/2, four-and-a-half LIM domain protein 1/2; Fn-like domain, fibronectin-like domain; Ig-like domain, immunoglobulin-like domain; MEK 1/2, mitogen-activated protein kinase kinase 1; MLP, muscle LIM protein; MuRF 1/2, muscle ring finger protein 1/2; Nbr1, neighbor-of-BRCA1-gene-1; NFAT, nuclear factor of activated T-cell; p62, nucleoporin p62; Raf, rapidly accelerated fibrosarcoma protein; SQSTM1, sequestosome 1; telethonin, telethonin (titin-cap).
Posttranslational modification of titin modulates titin stiffness more dynamically, e.g., by phosphorylation of the elastic I-band regions N2-B and PEVK. To date, phosphorylation sites have been identified for cAMP-dependent protein kinase (Yamasaki et al., 2002; Krüger et al., 2009; Kötter et al., 2013), cGMP-dependent protein kinase (Krüger et al., 2009; Kötter et al., 2013), Ca\(^{2+}\)-dependent protein kinase C\(\alpha\) (Hidalgo et al., 2009), extracellular signal regulated kinase 1/2 (Erk1/2) (Raskin et al., 2012) and Ca\(^{2+}\)/calmodulin-dependent protein kinase II delta (CaMKII\(\delta\)) (Hamdani et al., 2013b; Hidalgo et al., 2013). Phosphorylation of the cardiac-specific N2-Bus by cAMP- and cGMP-dependent protein kinase and CaMKII\(\delta\) increases the persistence length of this region, and thereby decreases the passive stiffness (Krüger et al., 2009; Hamdani et al., 2013b). In contrast, phosphorylation of the PEVK domain by Ca\(^{2+}\)-dependent protein kinase C\(\alpha\) decreases the persistence length of the PEVK region and causes an increase in titin-based passive tension (Hidalgo et al., 2009). Why the addition of phosphates results in an increase of the persistence length in one titin region whereas it causes a decrease in another is not fully understood. One possible explanation is the different amino acid composition of the domains. The introduction of a negatively-charged phosphate group into an already negatively-charged environment, such as the N2-Bus, can have an opposite impact to introducing it into a positively-charged sequence, such as the PEVK region. This could lead to intramolecular electrostatic repulsion, thereby altering the persistence length of the domains (Kötter et al., 2013). Chronic changes in the phosphorylation status of titin without concomitant changes in titin isoform expression have been observed in different studies analyzing biopsies from heart failure patients and have resulted in severe alterations of titin-based myofilament stiffness (Kötter et al., 2013; Hamdani et al., 2013a). The role of titin phosphorylation in modulating diastolic function has recently been reviewed in more detail (Linke and Hamdani, 2014). Recent evidence from in vitro studies showed posttranslational modification of titin by S-glutathionylation, and a subsequent reduction in titin-based myofilament stiffness (Avner et al., 2012; Alegre-Cebollada et al., 2014). This modification raises the possibility that myofilament stiffness could be modulated by S-glutathionylation under acidic conditions, such as cardiac ischemia. The analysis of titin modification is an emerging field, however, and will doubtlessly result in the identification of many more modifications of titin and titin function in the near future.

Apart from its importance for sarcomeric stiffness, titin plays an important role as a mediator of myocyte signal transduction. The following section will place special emphasis on titin’s putative involvement in hypertrophic signaling and its role in the complex regulatory mechanisms of protein quality control.

### A mediator of mechanosensing and hypertrophic signaling

Sensing of mechanical stress and regulation of specific stretch responses is an important and highly conserved function in both cardiac and skeletal muscle. The adaptation of muscle function to increasing mechanical demands involves the initiation of a hypertrophic gene program to increase the number of sarcomeres, and often requires re-activation of fetal gene expression (Hunter and Chien, 1999). The existence of a specific signaling pathway that couples mechanical stress and muscle gene expression seems very likely; however, the molecular mechanism of this process is still unresolved.

Due to its large size and the longitudinal position within the sarcomere, titin is a very potent candidate sensor of mechanical load. Titin–protein interaction has been reported for more than 20 different proteins and is localized to three major sites of the molecule: the Z-disc region, the elastic I-band domains N2-B and N2-A, and the M-band proximity including the titin kinase domain (for a more detailed review of titin–protein interactions, see Linke and Krüger, 2010). Some of the identified binding partners of titin have been linked to hypertrophic signaling.

The Z-disc part of titin is mainly composed of the so-called Z-repeats and the Ig-domains Z1 and Z2, which form the very NH\(_2\) -terminal end of titin that protrudes from the Z-disc region. Telethonin connects the NH\(_2\)-terminal parts of two titin molecules from one sarcomere in a palindromic manner (Zou et al., 2006), an interaction that has been reported to be essential for proper sarcomere integrity (Gregorio et al., 1998). Cardiac telethonin is a substrate for atypical members of the Ca\(^{2+}\)/calmodulin-dependent kinase family, including titin kinase, CaMKII, and protein kinase D. Recent evidence suggests that constitutive bis-phosphorylation is critical for normal telethonin function that further involves maintenance of transverse tubule organization and intracellular Ca\(^{2+}\) transients (Candasamy et al., 2014). Missense mutations in telethonin have been associated with limb girdle muscular dystrophy 2G in skeletal muscle (Moreira et al., 2000) and with hypertrophic...
and dilated cardiomyopathies (Hayashi et al., 2004). Interestingly, some of the reported missense mutations of telethonin may interfere with the phosphorylation of telethonin and thereby disturb its proper function (Candasamy et al., 2014). An important binding partner of telethonin is the muscle LIM protein MLP, a zinc-finger protein of the LIM-only protein family, which has been shown in MLP-deficient mice to play a crucial role in myogenic differentiation (Arber et al., 1997). In conjunction with the Z-disc component actinin, it has been proposed that MLP, telethonin and MLP form a mechanical stretch sensing complex (Knöll et al., 2002, 2011) (Figure 1). MLP contains a nuclear localization signal, allowing cytoplasmic as well as nuclear localization of the protein. Increased nucleo-cytoplasmic shuttling of MLP has been observed in response to biomechanical stress and has been associated with the initiation of hypertrophic remodeling in stressed myocytes (Boateng et al., 2009). As MLP lacks a DNA binding site, however, the precise function of nuclear MLP remains to be elucidated (for more details on MLP, see Buyandelger et al., 2011). The importance of MLP has been stressed by the identification of several missense mutations associated with dilated cardiomyopathy, possibly caused by loss or disturbance of the MLP/telethonin/titin sensor complex (Knöll et al., 2010).

The MLP/telethonin/titin sensor complex also provides a direct link to hypertrophic signaling, which involves activation of the calcineurin/nuclear factor of activated T-cells (NFAT) signaling cascade. Telethonin has been shown to interact with calsarcin, a protein that tethers calcineurin to the Z-disc (Figure 1) (Frey et al., 2000). Calcineurin is a calcium/calmodulin-dependent serine/threonine phosphatase, which plays an important role in striated muscle signal transduction. The main function of calcineurin is the dephosphorylation of NFAT, thereby inducing NFAT translocation to the nucleus and activation of target genes (for a review, see Olson and Williams, 2000). Constitutive activation of calcineurin in the hearts of transgenic mice induces severe hypertrophy and leads to heart failure and sudden death. Evidence suggests that MLP plays a crucial role in the activation of calcineurin when associated with the sarcomeric Z-disc (Heineke et al., 2005).

The main site of protein–protein interaction in the I-band is the elastic cardiac-specific N2-B region, which consists of several Ig-domains and a so-called unique sequence (N2-Bus). The N2-Bus has been shown to bind the two isoforms of the four-and-a-half LIM domain protein FHL-1 (Sheikh et al., 2008) and FHL-2 (Lange et al., 2002). Both isoforms are highly expressed in response to biomechanical stress (Lim et al., 2001; Sheikh et al., 2008), can shuttle to the nucleus and act as transcriptional co-activators (Scholl et al., 2000). FHL-2 is expressed early during cardiogenesis and remains highly expressed in adult tissue; however, its function in the heart is not entirely understood. Recent data from a FHL-2 knockout mouse model and from cellular overexpression studies suggest that FHL-2 is an endogenous suppressor of calcineurin and thereby represses pathological cardiac growth (Hojayev et al., 2012). Apart from its I-band localization, FHL-2 has been shown to associate with the myofilament at the M-band and the Z-disc. In contrast, FHL1 association to titin may provide a link to the mitogen-activated protein kinase (MAPK) signaling cascade (Raf/Mek1/2/Erk2). Under non-stimulating conditions MEK1/2 acts as a scaffold protein for ERK1/2, and anchors ERK in the cytoplasm (Tanoue et al., 2000; Chuderland and Seger, 2005). Upon activation, MEK1/2 phosphorylates ERK1/2 and induces a conformational change that leads to its dissociation and translocation to the nucleus, where ERK2 is responsible for the activation of transcription factors such as c-Myc, c-Fos, and cAMP response element-binding (Mebratu and Tesfaigzi, 2009). ERK2 has further been shown to phosphorylate titin's N2-Bus sequence in vitro (Raskin et al., 2012) and possibly modulate myofilament stiffness. Interestingly, knockdown of FHL1 in mice resulted in increased myofibrillar compliance and reduced hypertrophic signaling (Sheikh et al., 2008). The N2-B/FHL-1/MAPK complex may therefore represent another potent biomechanical stress sensor in cardiomyocytes (Figure 1).

The third hotspot for titin-mediated hypertrophic signaling is the M-band region of the molecule, especially the titin kinase (TK) domain located in the M-band periphery. To date, the only substrate of the TK domain that has been identified is telethonin (Mayans et al., 1998; Weinert et al., 2006). Structurally, the titin kinase has some similarities with other kinases of the myosin light-chain kinase family, and shows a strong auto-inhibition (Gautel, 2011). Unlike other classical myosin light-chain kinases, however, the modulation of TK activity by Ca²⁺-calmodulin is only very weak (Mayans et al., 1998). Instead, it has been proposed from force-probe molecular dynamics simulations that activating changes to the conformational state of the TK could be induced biomechanically (Gräter et al., 2005). The TK therefore could be another biomechanical stress sensor and mediator of hypertrophic signaling.

Activated TK has been shown to interact with the ubiquitin-associated zinc-finger protein neighbor-of-BRCA1-gene-1 (Nbr1), which forms a signaling complex with p62/SQSTM1 and the muscle-specific ubiquitin E3 ligases MuRF1, MuRF2, and MuRF3 (Lange et al., 2005). It has been suggested that MuRFs have act as transcriptional
repressors in mechanically inactive cardiac and skeletal muscle cells (Gautel, 2011). In the absence of mechanical activity, MURFs shuttle to the nucleus, where MuRF2 has the ability to down-regulate the levels of nuclear serum response factor and thereby suppress serum response factor-dependent muscle gene expression (Lange et al., 2005). By interactions with several members of the MAPK signaling pathway, including p38, P62/SQSTM1 is likely involved in myocyte enhancer factor-2- and MyoD-controlled myogenic differentiation and hypertrophic growth (reviewed in Gautel, 2011). Interestingly, a human mutation in the titin protein kinase domain that disrupts the formation of this signaling complex has been shown to cause severe hereditary muscle disease (Lange et al., 2005).

A central part of sarcomeric protein quality control

Sarcomeres are highly dynamic structures that require permanent synthesis and turnover of their constituent parts (McKenna et al., 1985; Sanger et al., 1986; Mittal et al., 1987). There are three main turnover processes in the cell: the ubiquitin–proteasome system (UPS), autophagy and the calcains. The degradation of muscle proteins by the UPS plays an important role in the healthy heart and is often disturbed in patients with cardiac diseases, such as heart failure (Tsukamoto et al., 2006; Wang and Robbins, 2006; Predmore et al., 2010; Kohlschlaeger et al., 2010; Watkins et al., 2011), cardiomyopathies (Liu et al., 2006; Carrier et al., 2010, Predmore et al., 2010; Schlossarek and Carrier, 2011), hypertrophy (Howe et al., 2003; Depre et al., 2006; Meiners et al., 2008), atrophy (Attaix et al., 2005; Razeghi et al., 2006), ischemia-reperfusion (Powell and Divald, 2010; Li et al., 2011) and atherosclerosis (Herrmann et al., 2004). Despite this, autophagy is increasingly recognized as an important regulator of protein degradation and cellular signal transduction.

The following paragraphs will highlight the emerging evidence suggesting that the giant molecule titin represents an important signaling node in the processes of sarcomeric protein quality control, and at the same time could be its biggest challenge.

In the past several studies have directly or indirectly linked titin to important players of the protein quality control machinery and the ubiquitin proteasome system. In the Z-disc a potent connection to the proteasomal system is provided by the interaction of the ubiquitin–ligase Mice-double-minute 2 (Mdm2) with the titin-capping protein telethonin. The titin M-band domains A168–170 interact with MuRF-1 and MuRF-2, E3-ligases that are involved in the proteasomal degradation of several muscle proteins including troponins, telethonin and nebulin (Pizon et al., 2002; Centner et al., 2003; Gregorio et al., 2005; Witt et al., 2005) (Figure 2). Through MuRF-1, titin is also linked to the ubiquitin binding protein 9 (McElhinny et al., 2002).

Another important link between titin and protein turnover processes is mediated by the interaction of the titin-protein kinase domain with the Nbr1/p62/SQSTM1 complex and the subsequent recruitment of MuRF-2 to the sarcomere (Figure 2) (Lange et al., 2005). By targeting ligands to polyubiquitin chains, P62/SQSTM1 can induce the assembly of larger signalosomes and promote their proteasomal degradation (Seibenhener et al., 2007). Via interaction with the autophagosomal membrane anchor LC3, p62 and Nbr1 have been shown to target polyubiquitinated proteins to the autophagic protein turnover machinery (Pankiv et al., 2007; Waters et al., 2009). These data demonstrate that, apart from its role in hypertrophic signal transduction, p62/SQSTM1/Nbr1 is an important signaling complex in the crosstalk between the UPS and autophagosomal processes.

A direct contribution of titin to protein quality control mechanisms is provided by the association of the proteases calpain-1 to the proximal Ig-region of titin, and calpain-3 (skeletal muscle-specific isoform) to the N2A-domain and the M-band region of titin (Raynaud et al., 2005; Cowper et al., 2008, Hayashi et al., 2008). It has been suggested that the binding to titin keeps calpain-3 in an autoinhibited state and thereby regulates its proteolytic activity (Hayashi et al., 2008). Moreover, calpains, and particularly calpain-1, seem to be required for ubiquitin ligases to mark sarcomeric proteins for degradation by the proteasome (Fareed et al., 2006).

Much faster than expected from its giant size, titin can be rapidly exchanged in the sarcomere within approximately 14 h, which is probably enabled by a cytoplasmic pool of titin (da Silva-Lopes et al., 2011). How these processes allow an accurate integration of titin in the working sarcomere is still entirely unknown. It is also still unresolved whether titin degradation is mainly performed by the proteasome or the autophagosomal system or a combination of both. As titin is firmly integrated in the Z-disc, the A-band and M-line structure, it seems likely that the giant molecule is subjected to some kind of pre-digestion that allows subsequent proteasomal degradation. This has been demonstrated earlier in models of skeletal muscle cachexia, sepsis and muscle wasting. The pre-digestion has been performed by calpains (Williams et al., 1999; Hasselgren and Fischer, 2001). More recently, titin
Figure 2  Sarcomeric protein quality control and chaperone-mediated titin protection.

Titin has been demonstrated to interact with several members of the protein quality control machinery. Interaction with E3 ubiquitin ligases provides a potent link to the ubiquitin proteasome system, whereas the titin-associated Nbr1/p62/SQSTM1 complex may promote the autophagosomal degradation of ubiquitinated proteins. Inset: chaperone-mediated protection of I-band titin.

HSP27, heat shock protein 27; HSP90, heat shock protein 90; LC3, microtubule-associated protein 1 light chain 3; Mdm2, Mice-double-minute 2; Nbr1, neighbor-of-BRCA1-gene-1; p62, nucleoporin p62; Smyd2, SET and MYND domain-containing protein 2; SQSTM1, sequestosome 1; telethonin, telethonin (titin-cap).

has been shown to be degraded by the matrix-metalloprotease (MMP) MMP-2 in vitro and in vivo, e.g., after cardiac ischemia/reperfusion injury (Ali et al., 2010). Although usually located in the extracellular matrix, the intracellular and particularly the sarcomeric localization of MMPs have previously been convincingly demonstrated. Under conditions of oxidative stress, MMP-2 is responsible for the degradation of several sarcomeric proteins like troponin, myosin-light chain and α-actinin (Wang et al., 2002; Gao et al., 2003; Sawicki et al., 2005; Sung et al., 2007).

Chaperone-mediated protection of titin

Protein quality control not only involves the degradation and recycling of proteins but also their stabilization and protection from damage. Titin has been shown to associate with important components of the protective protein quality control machinery: the heat shock proteins (HSPs). HSPs play a major role in protein homeostasis by protecting unfolded proteins from aggregation and keeping them in a state that allows refolding or degradation (Vos et al., 2008). In the following section we will highlight some recent findings demonstrating the association of titin with HSP90 and small HSPs (sHSPs).

In skeletal muscle the elastic N2A-domain in the I-band region of titin associates with the methyltransferase SET and MYND domain containing protein 2 (Smyd2), which in turn recruits HSP90 to this region and monomethylates it on lysine residue K616 (Donlin et al., 2012). This Smyd2-mediated monomethylation is crucial for the formation of a complex consisting of titin-N2A, Smyd2 and HSP90, which protects I-band titin against degradation. Deletion of Smyd2 in a zebrafish model resulted in severe I-band damage and loss of muscle function in skeletal as well as cardiac muscle (Donlin et al., 2012, Voelkel et al., 2013). In the mammalian system, however, knockdown of Smyd2 did not display any visible phenotype in skeletal or heart
prevented the unfolding and aggregation of titin and may therefore represent an important mechanism to protect myocytes from abnormal passive stiffness under ischemic conditions (Kötter et al., 2014).

**Conclusion**

This review has briefly highlighted the recent data implicating a multifaceted role of titin in mediating hypertrophic signaling processes and sarcomeric protein turnover. Nevertheless, details of titin-associated downstream and upstream signaling pathways that maintain and modify sarcomeric structure and function under stress conditions are only just beginning to be elucidated. Future studies will certainly provide more mechanistic insight into the role of titin as a sarcomeric signaling node and will also shed light on the precise mechanisms that control and mediate integration, degradation, and possibly recycling of the titin molecule itself.

**Acknowledgments:** Some of the work reviewed here was supported by grant KR 3409/5-1 from the German Research Foundation.

**References**


After finishing his Biology studies at Münster University in 2007, Sebastian Kötter joined the Titin lab of Wolfgang Linke and obtained his PhD on the sarcomeric organization and phosphorylation of the titin filament, and its association to small heat shock proteins. In 2011 he joined the lab of Martina Krüger as a post doc and continued his work on titin modification and regulation of titin turnover.

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After graduating as a biologist in 1999, Martina Krüger obtained her PhD in 2004 at the Physiology Department of the University of Cologne. In 2005 she joined the Titin lab of Wolfgang Linke as a postdoc to study titin-based myofilament function in health and disease. In 2011 she became a full professor at Düsseldorf University. Her current research focuses on posttranslational modification of titin and the hormonal regulation of titin-based myofilament properties.