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Tuning the molecular giant titin through phosphorylation-role in health and disease

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Abstract

Titin is a giant multi-functional filament that spans the half sarcomere. Titin's extensible I-band region functions as a molecular spring that provides passive stiffness to cardiac myocytes. Elevated diastolic stiffness is found in a large fraction of heart failure patients and thus understanding the normal mechanisms and pathophysiology of passive stiffness modulation is clinically important. Here we provide first a brief general background on titin including what is known about titin isoforms and then focus on recently discovered post-translational modifications of titin that alter passive stiffness. We discuss the various kinases that have been shown to phosphorylate titin and address the possible roles of titin phosphorylation in cardiac disease, including heart failure with preserved ejection fraction (HFpEF).

Titin isoforms in the adult heart

Titin is a giant multi-functional sarcomeric filament that spans from Z-disk to M-band (Fig. 1A) and that is responsible for passive stiffness generation of the sarcomere. This stiffness is important for maintaining the structural integrity of the contracting sarcomere (Horowits and Podolsky, 1987) and together with the extracellular matrix for defining diastolic stiffness(Granzier and Irving, 1995). The extensible I-band region of titin is comprised of three distinct elements: 1) tandem Ig segments that consist of serially-linked immunoglobulin(Ig)-like domains, 2) the spring-like PEVK (containing a high percentage of proline, glutamic acid, valine, and lysine resi dues), and 3) the spring-like N2B element (Fig. 1B)(Labeit and Kolmerer, 1995; Bang et al., 2001). Titin is encoded by a single gene and variable mRNA splice pathways result in distinct titin isoform classes that contain spring element length variants(Bang et al., 2001). In adult cardiac muscle two classes of titin isoforms are present: N2BA and N2B titin (Fig. 1B)(Bang et al., 2001; Lahmers et al., 2004; Greaser et al., 2005). These two titin isoform classes differ in their I-band region; the rest of the molecule (Z-disk, A-band, M-band localized regions) is largely identical(Bang et al., 2001). The tandem Ig segments can be visualized as "beads on a string" with folded Ig domains with a diameter of 4–5 nm separated by short peptide linkers(Tskhovrebova and Trinick, 2001). All isoforms contain a proximal tandem Ig segment (Ig1-15) and a distal tandem Ig segment (Ig84-105)(Labeit and Kolmerer, 1995; Bang et al., 2001). The N2BA

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isoform also contains a middle tandem Ig segment that is comprised of a variable number of Ig domains (Fig. 1B)(Lahmers et al., 2004). The N2B element is cardiac-specific and is found in all cardiac titin isoforms; it consists of 3 Ig domains and a large 550 residue unique sequence (referred to as N2B-Us) without a well-defined structure. In addition to behaving as a large molecular spring, the N2B element is a substrate for various kinases that affect its mechanical properties (see below). While the N2B element is found in both adult cardiac titin isoforms, the N2A element is only found in the N2BA isoform (hence its name)(Labeit and Kolmerer, 1995; Bang et al., 2001). Similar to the N2B element, N2A contains Ig domains and unique sequence. The third spring element is the PEVK region of titin. The N2B and PEVK spring elements dominate titin's extension towards the upper limit of the physiological sarcomere length range(Trombitas et al., 1999). The PEVK sequence is relative short in the N2B titin isoform (183 residues) while the PEVK region of N2BA titin contains a much larger PEVK (~up to 800 residues)(Greaser et al., 2002). The additional Ig domains and PEVK sequence and the inclusion of the N2A element make the N2BA titin isoform larger than the N2B isoform (~3.3 MDa vs. 2.97 MDa)(Bang et al., 2001).

The force required to stretch a titin molecule depends non-linearly on its fractional extension(Kellermayer et al., 1998). Because the N2B titin isoform has a shorter contour length (due to fewer Ig domains, a shorter PEVK segment, and absence of the N2A element), a given sarcomere stretch requires a larger fractional extension of the N2B isoform(Trombitas et al., 2000). Therefore more force is needed to stretch the N2B titin isoform—it is stiffer because it is shorter. For this reason, sarcomeres that express different titin isoforms develop levels of passive force that greatly differ (Fig. 1C). Adult cardiac muscles co-express N2B and N2BA cardiac titin at the level of the half sarcomere(Trombitas et al., 2001). The number of titin molecules per thick filament is likely to be constant (6 per half thick filament) but the expression ratio of complaint (N2BA) to stiff (N2B) titin is instead variable(Cazorla et al., 2000). Because of the intimate relationship between the size of titin's I-band region and titin-based passive tension, with larger elastic I-band regions corresponding to lower passive tension, the titin isoform expression ratio in the heart is a crucial determinant of titin-based passive tension (Fig. 1C).

Differences in titin isoform expressions account for cardiac passive tension variability between species. More N2B titin is found in smaller mammals that have higher heart rates (Cazorla et al., 2000) with an expression ratio of N2B:N2BA titin of ~80:20 in the mouse as opposed to ~60:40 in humans. Importantly, variable titin expression ratios have been found in patients with cardiac disease. Patients with coronary artery disease (CAD) have been shown to express increased levels of N2BA titin that was accompanied by decreased stiffness at the myofibrillar level (Neagoe et al., 2002). Changes in titin isoform expression have also been found in patients with end-stage heart failure due to dilated cardiomyopathy (DCM) where the compliant N2BA isoform was up-regulated and associated with decreased passive stiffness and increased chamber compliance (Makarenko et al., 2004; Nagueh et al., 2004). The Nagueh et al. study(Nagueh et al., 2004) also suggested a physiological benefit of this change in titin expression via correlation between the titin isoform shift and improved exercise tolerance. Upregulation of compliant titin isoforms has also been found in patients with heart failure (HF) with preserved ejection fraction (HFpEF), a group of patients that accounts for about half of all HF cases and that is characterized by increased diastolic stiffness(Borbely et al., 2005; Borbely et al., 2009). An adaptive change in isoform expression towards increased expression of compliant titin isoforms also occurs in mice with pathological hypertrophy(Hudson et al., 2011) and rats with long-term hypothyroidism(Wu et al., 2007). Overall these studies suggest that up-regulation of the more compliant N2BA titin isoform is an important compensatory adaptation to counteract the increased stiffness of the extracellular matrix (Nagueh et al., 2004).

Posttranslational modification of titin's spring elements

Although expressing different isoforms is highly effective in altering passive stiffness, switching titin isoforms in the cardiac sarcomere is a slow process that requires days to possibly weeks to be completed. The cardiac myocyte has faster mechanisms available that revolve around posttranslational modifications (PTMs) on titin. One such mechanism involves binding of calcium to titin. Studies have suggested that E-rich PEVK motifs bind calcium and that this increases titin-based passive tension(Labeit et al., 2003). This mechanism is most prominent in skeletal muscle that express a large number of E-rich motifs whereas in N2B cardiac titin these motifs are absent and passive tension developed by N2B titin is therefore calcium insensitive(Fujita et al., 2004). However, N2BA titin does express E-rich PEVK motifs and passive tension of N2BA titin has indeed been shown to be increased by ~10% when calcium levels are increased from that in passive muscle to a level that causes maximal activation ([Ca2+] ~ 0.1 mM) (Fujita et al., 2004). Considering that the human heart co-expresses N2B and N2BA titins at similar levels(LeWinter et al., 2007), the maximal effect of calcium on passive tension is ~ 5%. An additional mechanism for altering passive tension is based on phosphorylation. Recent studies that include single molecule force spectroscopy on titin molecules have discovered that protein kinase phosphorylation significantly alters the stiffness of the PEVK and N2B spring elements of titin. This allows for rapid adjustment of titin stiffness and quick adaptations of cardiac performance to meet hemodynamic loads.

PKA and PKG phosphorylation of N2B spring

β-Adrenergic stimulation of cardiac muscle activates protein kinase A (PKA), which is well-known to phosphorylate proteins on the thin and thick filaments of the sarcomere. It has been demonstrated that titin is also phosphorylated by the β-adrenergic pathway(Yamasaki et al., 2002). Titin phosphorylation was observed after β-adrenergic receptor stimulation of intact cardiac myocytes and incubation of skinned cardiac myocytes with PKA. Mechanical experiments with isolated myocytes revealed that PKA significantly *reduces* passive tension. (For a schematic of the passive tension effect, see Fig. 1C.) It has also been shown that PKA reduces passive tension in cardiac mycoytes of rat and bovine (Yamasaki et al., 2002; Fukuda et al., 2005) and human cardiac myofibrils (Kruger and Linke, 2006). In vitro phosphorylation of recombinant titin fragments and immunoelectron microscopy showed that PKA targets a subdomain of the elastic segment of titin, the N2B spring element. β-Adrenergic receptor activation in intact rat ventricular trabeculae reduces diastolic force to a degree similar to that found in skinned preparations(Fukuda et al., 2005) providing further evidence that phosphorylation occurs not only in skinned muscle but is relevant in intact muscle and is therefore likely to be of physiological importance. A more pronounced PKA effect is present in skinned fibers when protein phosphatase 1 (PP1) de-phosphorylation was performed prior to PKA treatment, which shows that the basal level of phosphorylation plays an important role in determining passive tension levels(Kruger and Linke, 2006).

Similar to PKA, protein kinase G, which is a cGMP-dependent protein kinase that is part of signaling cascades initiated by nitric oxide (NO) and natriuretic peptides (NPs), also phosphorylates the unique sequence of the N2B element and reduces passive tension. PKG phosphorylates the same residue as targeted by PKA (Kruger et al., 2009). Using site-directed mutagenesis, serine residue S469 (S4185 in the full titin sequence) near the COOH terminus of the cardiac N2B –Us was identified as a PKG and PKA phosphorylation site(Kruger et al., 2009). Unlike the identified PKC sites in the PEVK region (see below), S469 is not well conserved and in addition to in human is only found in non-human primates and in pig but is absent in other species (Fig. 2A and B). It seems likely that in these other species different sites are present in the N2B element that can be phosphorylated. The effect

of PKG on the passive tension of skinned muscle from human donor hearts has been studied and a significant reduction in passive tension following PP1 treatment was found. Single molecule data suggests that PKG phosphorylation increases the bending rigidity of the N2B-us, which is consistent with the reduced passive tension measured in muscle mechanics experiments (Kruger et al., 2009).

In summary, the cardiac-specific N2B spring element is a substrate for both PKA and PKG and phosphorylation by either protein kinase results in a reduction in passive tension. The reduction is larger when the muscle is PP1 pretreated suggesting that under normal conditions there is a basal phosphorylation level of titin.

PKCα phosphorylation of PEVK spring

The PEVK spring element has been found to be phosphorylated by protein kinase C (PKC). PKC is activated by the α1-adrenergic signaling pathway that is a key mediator of physiological and pathological adaptation of cardiac function. It was found that PKCα, the predominant isozyme in the heart and a key player in contractile dysfunction and heart failure (Molkentin and Dorn, 2001; Belin et al., 2007), phosphorylates the PEVK spring element(Hidalgo et al., 2009). Furthermore, mass spectrometry in combination with site-directed mutagenesis identified 2 sites in the PEVK region that are phosphorylated by PKCα (S11878 and S12022, or S26 and S170 in the minimal PEVK sequence in the N2B cardiac isoform); when these 2 sites are mutated to alanine, phosphorylation is effectively abolished. Mechanical experiments with skinned ventricular myocardium revealed that PKCα significantly increases titin-based passive tension, an effect that is reversed by protein phosphatase 1 (see Fig. 3 in (Hidalgo et al., 2009)). The identified PKCα sites are constitutively expressed in all titin isoforms and are conserved in a wide range of species (Fig. 2C). Single molecule force-extension curves show that PKCα decreases the PEVK persistence length, without altering the contour length, and a physical model of titin's extensible region (a serially-linked wormlike chain model(Watanabe et al., 2002)) predicts increases in titin-based passive force that are similar to those measured in skinned myocardium after PKCα phosphorylation (Hidalgo et al., 2009). The link between PKCα, PEVK phosphorylation, and passive tension was further established by a study that showed that PKCα had no effect on passive tension in mice that had the PEVK sites genetically removed (Hudson et al., 2010). Thus, the combination of techniques including single molecule force spectroscopy and novel mouse models have established that post-translational modifications of titin via PKCα directly influence titin-based passive tension.

In summary, the two main spring elements in cardiac titin can each be phosphorylated but the effect on passive tension are in opposite directions with the N2B element decreasing passive tension following phosphorylation (PKA/PKG) and the PEVK increasing passive tension following phosphorylation (PKC), see Figure 1C. It is also important to note that the effect on passive tension is larger for the N2B isoform than the N2BA isoform(Fukuda et al., 2005) which can be explained by the difference in contour length of the two isoform(Fukuda et al., 2005).

ERK2 and CaMKII phosphorylation of titin

A recently discovered novel phosphorylation pathway involves the extracellular-signal-regulated kinase- 2 (ERK2) that phosphorylates the N2B-Us at 3 serines(Raskin et al., 2012). One of these sites is well conserved (S4010) but the other two are not (Fig. 2A and B). It was surmised that this ERK2-based phosphorylation lowers titin-based passive tension (increased compliance), but experimental evidence for this proposal is still required. Furthermore ERK2 phosphorylation was shown to be inhibited by binding of the 4 and a half LIM protein 1 (FHL1) to the N2B-Us(Raskin et al., 2012). FHL1 has previously been

shown to assemble a stretch sensing signalosome that consists of components of the mitogen activated signaling pathway(Sheikh et al., 2008) and the findings suggest a possible link between stretch sensing and phosphorylation-based regulation of passive stiffness. Another novel pathway involves CaMKII, a Ca^{2+} and calmodulin dependent serine/threonine kinase that is activated by increases in cellular Ca^{2+}. Four isoforms have been described (α, β, γ, and δ) of which CaMKIIδ is the predominant isoform in the heart(Couchonnal and Anderson, 2008). Hidalgo and colleagues have shown that CaMKIIδ phosphorylates titin in mouse LV skinned fibers and that the titin N2B and PEVK spring elements, but not Ig domains are phosphorylated by CaMKIIδ (Hidalgo et al., 2012). Furthermore, the phosphorylation sites overlap with the PKC sites (including the PKC sites S26 and S170 of the PEVK element, see Fig. 2B)(Hidalgo et al., 2012). Although mechanical studies have not been completed, it is to be expected that the effect of CaMKIIδ phosphorylation of the PEVK sites will be similar to that reported for PKC phosphorylation, which is an increase in passive tension. These intriguing new phosphorylation pathways (ERK2 and CaMKIIδ) warrant additional research focused on their possible role in physiology and pathophysiology.

Oxidation of N2B spring element

The mechanical properties of the N2B-Us can be altered by more than just phosphorylation. For example, there are six cysteine residues in the human N2B-Us (cysteines are absent from the PEVK spring element) that have the potential to form disulfide bonds with one another, depending on the oxidative state within the sarcomere. A disulfide bond reduces the contour length of the sequence and changes its mechanical response to stretch. The effect of cysteine cross-linking on the mechanics of the N2B-Us was shown at both the single molecule level(Grutzner et al., 2009) and the tissue level where oxidative stress increased passive tension and hysteresis in wildtype tissue(Nedrud et al., 2011) but had an attenuated effect in tissue from a mouse model where the entire N2B element was removed (Nedrud et al., 2011). The study of oxidative conditions and changes in passive tension is important considering that oxidative stress is elevated in heart failure patients and has been correlated with myocardial dysfunction(Grieve and Shah, 2003).

Phosphorylation of Z-disk and M-band unique sequences

Although phosphorylation events within titin's extensible region have received in recent years extensive scrutiny there are also phosphorylation sites in titin's non-extensible regions. At both the N-terminal (Z-disk) and C-terminal (M-band) ends of the titin molecule the normal pattern of serially-linked immunoglobulin (Ig)-like domains and fibronectin type III (Fn3)-like domains is interrupted by unique sequences. These unique sequences are referred to as interdomain segments (*is*); there are nine interdomain segments in the Z-disk (Z*is*1-9) and seven in the M-band (M*is*1-7). It has been suggested that these inter-domain segments are sites for protein-protein interactions and that their phosphorylation status might regulate these interactions. Indeed several interdomain segments contain consensus sequences for specific protein kinases. At the N-terminal region of titin serine/proline xSPxR rich sites exist in Zis1 and Zis 5 and these can be phosphorylated in vitro by erk (extracellular-signal-regulated kinase) and cdc2 (cyclin-dependent protein kinase-2) (Sebestyen et al., 1995; Gautel et al., 1996). The C-terminal region of titin's Mis4 contains four KSP repeats and in vitro phosphorylation assays have provided evidence that these motifs can be phosphorylated by cdc2 in developing but not in differentiated muscle(Gautel et al., 1993). Thus phosphorylation sites exist at both ends of the titin molecule. The phosphorylation status of these sites may regulate the binding of titin to many Z-disk and M-band proteins and regulate thereby assembly and turnover of Z-disk and M-band proteins. The finding that

these phosphorylation motifs are well conserved in a wide range of species (Fig. 2C) supports that they perform important functions.

Posttranslational modifications and disease

Whether the basal phosphorylation level of titin's PKA/PKG sites is altered in cardiac disease has been addressed in several recent studies. One of the first studied compared end-stage dilated cardiomyopathy (DCM) patients with non-failing donor hearts and this revealed a trend towards a reduced basal level of phosphorylation of the PKA/PKG sites (Kruger et al., 2009), which is predicted to increase passive tension. A focus has also been on HFpEF patients because of the elevated diastolic stiffness that is a hall mark feature of HFpEF(Zile et al., 2011). High diastolic stiffness can be detected in intact muscle strips(Selby et al., 2011) and skinned single cardiomyocytes obtained from the left ventricular myocardium of HFpEF patients(Borbely et al., 2005). Evidence for hypo-phosphorylation of titin was obtained in both HF patients with a reduced ejection fraction (HFrEF) and HFpEF patients; mechanical experiments revealed increased passive tension of cardiac myocytes that was partially normalized after PKA or PKG treatment of the cells(Borbely et al., 2009). A recent study showed low PKG activity in patients with HFpEF and this reduced activity was associated with increased cellular stiffness and hypophosphorylation of PKA/PKG sites on titin(van Heerebeek et al., 2012). High diastolic stiffness responds favorably to increased PKG activity by in vivo administration of sildenafil and administration of sildenafil to old hypertensive dogs lowered diastolic LV stiffness through restored phosphorylation of the N2B segment of titin(Bishu et al., 2011).

Although evidence is mounting that hypo-phosphorylation of PKA/PKG sites on the N2B element plays an important role in the elevated passive stiffness of HFpEF patients, several studies have shown that cellular passive tension of HFpEF patients is not fully normalized by either PKA or PKG phosphorylation(Borbely et al., 2009). Instead passive tension remained higher than in controls (considering the titin isoform shift toward the more compliant N2BA isoform in HFpEF patients (see above) passive tension was expected to be *lower* than in the controls(Borbely et al., 2005; Borbely et al., 2009)). This higher passive tension following normalization of the PKA/PKG phosphorylation sites of titin could be explained by a change in the basal phosphorylation level of the PKC sites found in the PEVK spring, but this was not investigated.

That titin's PKC sites might play a role in pathological changes in stiffness is suggested by a study of transverse aortic constriction (TAC) induced heart failure in mice(Hudson et al., 2011). Mechanical studies on skinned left ventricle myocardium measured total and titin-based and extracellular matrix-based passive stiffness. Total passive stiffness was increased in HF mice, and this was attributable to increases in both extracellular matrix-based and titin-based passive stiffness, with titin being dominant. Protein expression and titin exon microarray analysis revealed increased expression of the more compliant N2BA isoform at the expense of the stiff N2B isoform in HF. These changes are predicted to lower titin-based stiffness. Back phosphorylation and Western blot assays with novel phospho-specific antibodies showed hyperphosphorylation of PKA sites and the PEVK S26 PKC site, but hypophosphorylation of the PEVK S170 PKC site. Protein phosphatase I abolished differences in phosphorylation levels and normalized titin-based passive stiffness levels between control and HF myocardium. Thus, TAC-induced HF results in increased extracellular matrix-based and titin-based passive stiffness. Changes in titin splicing occur, which lower passive stiffness, but this effect is offset by hyperphosphorylation of residues in titin spring elements, particularly of PEVK S26. Thus, complex changes in titin occur that combined are a major factor in the increased passive myocardial stiffness in HF.

In summary, titin-based myocardial stiffness is determined by the titin isoform composition and the phosphorylation status of titin's elastic I-band, with different protein kinases affecting titin elasticity in disparate ways. Comprehensive studies of titin isoform expression and their phosphorylation status are required for determining the mechanisms through which titin stiffness changes during acute and chronic disease. Changes in post-translational modification, especially hypo-phosphorylation, are increasingly recognized as contributors to the pathophysiology of acquired heart disease. These new insights in titin provide novel therapeutic modalities for normalizing diastolic function in heart failure patients.

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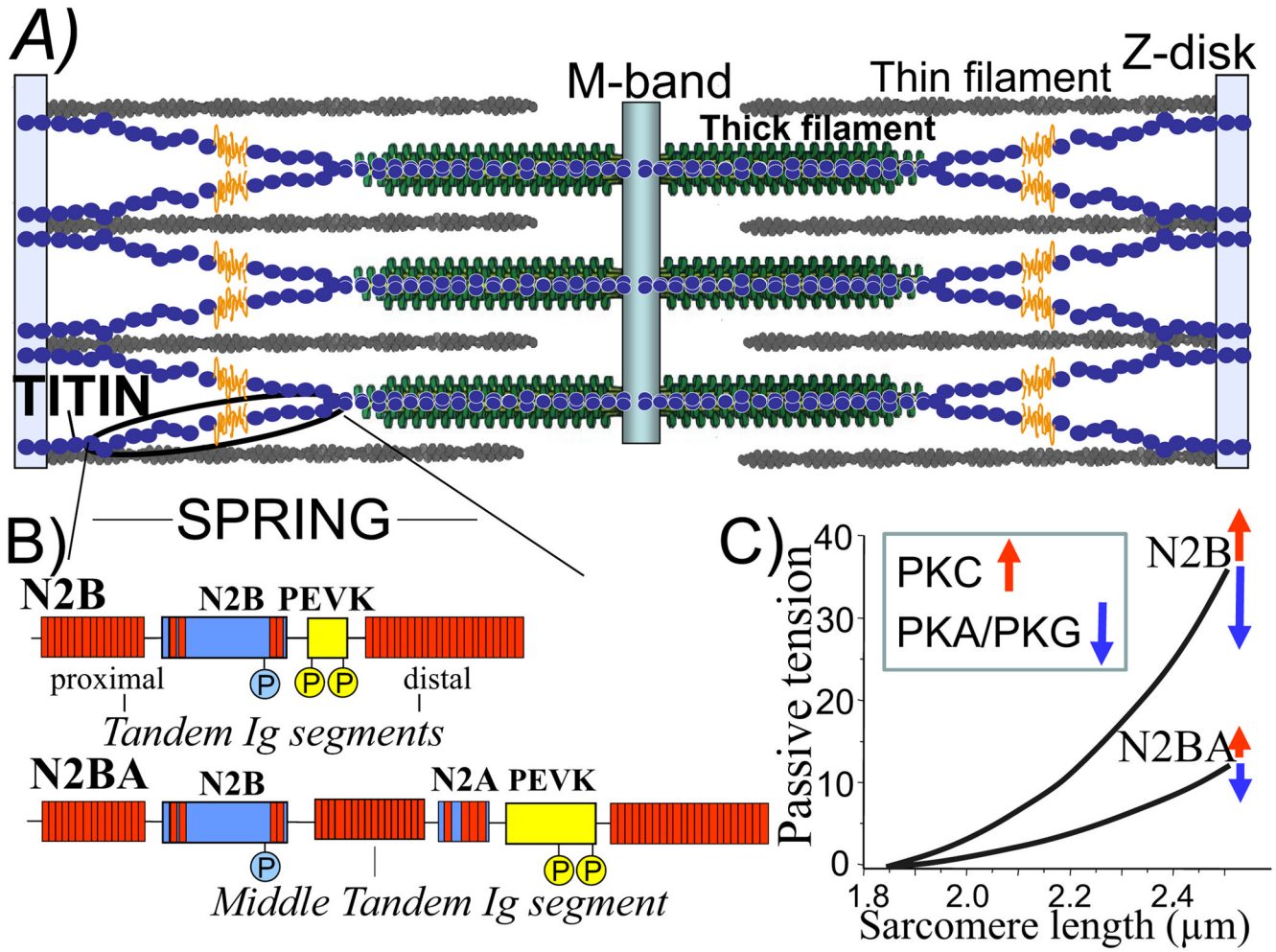

Figure 1.

A) Schematic of titin in the cardiac sarcomere. Single titin molecules (shown in blue and yellow) span from Z-disk (N-terminus) to M-band (C-terminus).

B) Composition of extensible I-band region of the N2B and N2BA titin isoforms (found in adults). Red blocks denote Ig-like domains, blue is unique sequence and yellow is PEVK sequence. Also indicated are known phosphorylation sites for PKA/PKG (blue) and PKCα (yellow).

C) Schematic of force-extension curves of titin isoforms and the effects of phosphorylation on passive tension.

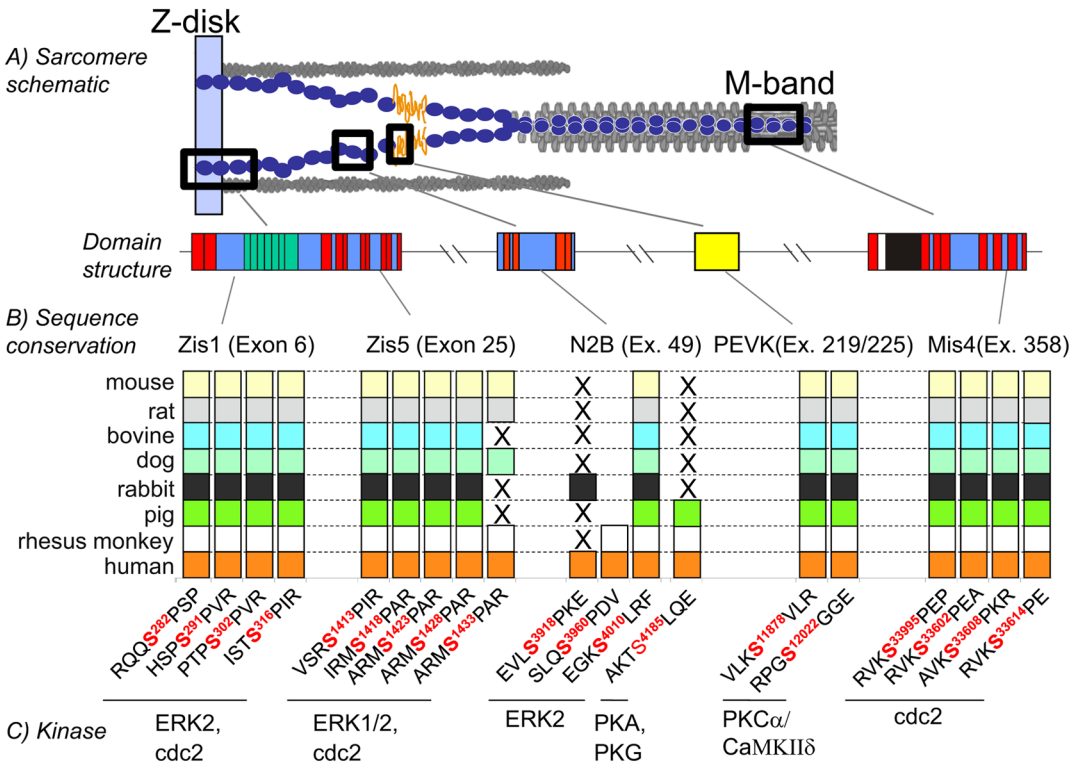

Figure 2.

A) Schematic of titin in the cardiac sarcomere with at bottom the domain structure of regions within titin that are phosphorylated. There are two unique sequences in the Z-disk (Zis1 (exon 6) and Zis5(exon 25)) that are phosphorylated by ERK1/2 and cdc2 kinase(Sebestyen et al., 1995; Gautel et al., 1996); the large unique sequence in N2B element (exon 49) is phosphorylated on 3 sites by ERK2(Raskin et al., 2012) and on one by PKA and PKG(Kruger et al., 2009); the PEVK is phosphorylated by PKCα and CaMKIIδ (exons 219 and 225)(Hidalgo et al., 2009; Hidalgo et al., 2012) and a unique sequence in the M-band region (Mis4(exon 358)) is phosphorylated by cdc2(Sebestyen et al., 1995).

B) Conservation of titin sequence that can be phosphorylated. Bottom shows the human titin sequence with in red the residue that is phosphorylated (number in superscript is based on Q8WZ42-1). (Rhesus monkey F7FBM0, bovine F1N757, pig F1RZC8, dog F1PV45, rabbit G1U9S3, rat F1M7S9, and mouse E9Q8N1 all in UnitProKB Protein Knowledgebase). Each row indicates a different species and each column a different phospho site in the human titin sequence. Sites that are absent are indicated by a cross. Except for the sites in the N2B unique sequence where conservation is limited all sites are highly conserved.

C) Protein kinases that have been shown to phosphorylate the indicated sites.