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Roles of N2A Titin in Signaling and Muscle Mechanics

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ABSTRACT

Since the discovery of titin in the late 1970's, the N2A region has been of interest as a candidate locus for participation in cellular signaling and as a mechanical hub that mediates the transition from passive to active mechanical properties of skeletal muscles. The purpose of this review is to highlight our current understanding of the role of N2A titin in skeletal muscle mechanics and signaling. We review the known binding partners that interact with N2A titin and the evidence for calcium-dependent interactions between N2A titin and actin, as well as how these interactions might be stabilized in muscle sarcomeres. Evidence from co-sedimentation studies, atomic force microscopy and in vitro motility assays suggests that titin binds to actin at pCa = 6, before cross-bridges begin to produce force. This calcium-dependent N2A-actin binding eliminates low force straightening of the proximal tandem Ig domains, which would otherwise limit titin-based forces in actively stretched muscles. The idea that cross-bridges could account for the enhanced forces and energy stored during active stretch of skeletal muscles is incompatible with the much smaller strains experienced by cross bridges and the much shorter duration of their attachments to actin. A wide variety of experiments including eccentric contraction, residual force enhancement, and stretch-shortening cycles demonstrates a role for titin in force enhancement during and after stretching of active muscles. Based on the available data from different laboratories, we have developed a model in which two proximal tandem immunoglobulin domains, Ig80 and Ig83, act at the nucleation trigger for N2A-actin binding, with the binding of I83 providing the calcium dependence that has been associated with this interaction. While these domains appear necessary for N2A-actin binding, their binding affinities are likely not sufficiently strong to withstand the high forces experienced during stretch of active muscles, requiring other stabilizing interactions. The available evidence suggests that the proximal PEVK domains and CARP/MARP1 likely stabilize N2A – actin binding in active muscles, although neither of these interactions is itself calcium-dependent. The available biophysical and muscle mechanics data support the view that titin is a tunable viscoelastic material in muscles with calcium-dependent viscoelastic properties that modulate the response of muscles to passive and active stretch.

Keywords: active stretch, actin -titin binding, calcium dependence, residual force enhancement, stretch-shortening cycle effect, tandem Ig domains, UN2A unique sequence

Introduction

Since its discovery in 1976,[1] physiological roles for titin have expanded to include sarcomerogenesis, strain sensing and signaling,[2,3] in addition to a mechanical role in passive tension. Furthermore, research over the past decade has revealed that titin's function is not limited to passive force. Titin plays a significant role in active muscles as well. [4–10] The N2A region, which has been investigated predominantly in skeletal muscle, has been a particular focus of investigations on signaling and mechanical functions of titin in skeletal muscles.[2,11–13]

Due to its unique structural characteristics, the N2A region of the skeletal isoform is predisposed to interactions with a wide variety of ligands.[13] Major binding partners of N2A titin include: 1) members of the muscle ankyrin repeat family (MARP, CARP and Ankrd1);[2,14,15] 2) calpain 3;[16] 3) SMYD2 and HSP90;[17] and 4) the chaperone ab-crystallin,[18] as well as thin filaments[19,20] and calcium.[21] While proteases, stress-sensing, and chaperone proteins play important roles in muscle turnover and integrity, the focus of this review is on binding partners that likely contribute to the large changes in skeletal muscle viscoelastic properties upon calcium activation that contribute to the mechanics of active muscles.[6]

A large body of evidence demonstrates that titin contributes to the increased resistance of muscles to active stretch (Table 1). This property is evident in phenomena that include eccentric contraction,[7], residual force enhancement,[22] and the stretch-shortening cycle effect (SSC effect).[23,24] The energy stored when active muscles are stretched[25] can be recovered to increase the power of active shortening.[24] Because titin is a viscoelastic material,[26] its resistance to stretch includes not only elastic but also viscous forces that increase with stretch and activation.[6] The length-and activation-dependent viscoelastic properties of muscles are crucial for dynamic function because they enable muscles to respond rapidly to unexpected perturbations[27–30] without incurring the delays associated with proprioceptive feedback and excitation-contraction coupling.[31] Current muscle models perform poorly at predicting *in vivo* forces[32–34], in large part because they fail to capture these properties.

The purpose of this review is to highlight our current understanding of the role of N2A titin in skeletal muscle mechanics and signaling. We focus on the evidence for calcium-dependent interactions between N2A titin and actin, as well as how these interactions might be stabilized in muscle sarcomeres. By critically evaluating the available data, we attempt to reconcile perceived discrepancies among results from different laboratories.

Table 1: Types of experiments and associated observations that provide evidence for N2A binding to actin in active muscles. BDM = Butanedione monoxeme.

Experiment	Observation	Reference(s)
Co-sedimentation of single titin molecules	Titin N2A constructs bind to actin at pCa = 6.0	Dutta et al. 2018
In vitro motility with single titin molecules	Titin molecules and N2A constructs bind to actin at pCa = 6.0	Kellermayer and Granzier 1996a Dutta et al. 2018
Atomic force microscopy of single titin molecules	Titin N2A constructs bind to actin at pCa = 6.0	Dutta et al. 2018
Stretch of single wild-type myofibrils	When stretched beyond overlap of thick and thin filaments to eliminate cross-bridge forces, force rises faster with length in active than in passive myofibrils	Leonard and Herzog 2010 Powers et al., 2014
Stretch of single *mdm* myofibrils	When stretched beyond overlap of thick and thin filaments to eliminate cross-bridge forces, force rises at the same rate with length in active and passive myofibrils	Powers et al. 2016
Stretch of single myofibrils in skeletal and cardiac muscles	Residual force enhancement depends on the isoform of titin expressed in different muscles	Shalabi et al. 2017
Antibody double-labeling of single myofibrils	The entire I-band region of titin extends when single myofibrils from skeletal muscles are stretched passively, whereas only the PEVK region extends in calcium-activated myofibrils	Desai et al. 2025 Sekhon 2025
Stretch of single intact muscle fibers	Stiffness rises before force during fast stretch, is unaffected by BDM but is affected by calcium release inhibitor dantrolene, higher in fast than slow fibers	Bagni et al. 1994, 2002, 2004 Colombini et al. 2016 Nocella et al. 2012, 2013, 2014
Stretch of single skinned muscle fibers	Residual force enhancement is observed at short sarcomere lengths on the ascending limb of the force-length curve	Peterson et al. 2004
Stretch of single skinned muscle fibers with x-ray diffraction	In the force-enhanced state, muscles exhibit a distinct structure that includes increased thick filament strain and decreased lattice spacing. This structure is impaired in titin-cut transgenic and *mdm* mouse muscles.	Hessel et al. 2024

Experiment	Observation	Reference(s)
Stretch of single skinned muscle fibers with cross bridges inhibited using BDM	Eccentric force is preserved compared to the pre-stretch isometric force when cross-bridge cycling is inhibited	Fukutani et al. 2022
Stretch of single skinned fibers with cross bridges inhibited using BDM	Non-crossbridge stiffness increases linearly with muscle length	Tomalka et al. 2017
Stretch of single skinned muscle fiber bundles from slow and fast muscles	Cross bridges alone cannot explain the linear increase in force with increasing velocity during stretch. This phenomenon requires velocity-dependent non-XB forces that may arise from viscoelastic titin.	Weidner et al. 2022 Weidner et al. 2024
Stretch of single skinned muscle fibers	After stretch of active muscle fibers and myofibrils, force remains elevated for seconds after muscle deactivation	Joumaa et al. 2007, 2008 Herzog 2019 Liu et al. 2022
Stretch of single *mdm* skinned fibers	No residual force enhancement in single fibers from *mdm* muscles	Mishra and Nishikawa 2022
Stretch of in intact wild-type and *mdm* muscles	Residual force enhancement increases with length in intact wild-type soleus muscles but not in soleus muscles from *mdm* mice	Tahir et al. 2020
Stretch-shortening cycle in single skinned fibers with cross bridges inhibited using BDM	Enhanced forces and mechanical work during the concentric phase of SSCs compared to shortening contractions with and without BDM	Tomalka et al. 2021 Fukutani & Herzog 2018, 2020 Fukutani et al. 2022 Hessel et al. 2021
Stretch-shortening cycle with cross bridges inhibited using blebbistatin	Increased force during shortening persists after cross bridge inhibition and is relatively larger in blebbistatin vs. control fibers.	Tomalka et al. 2020 Tomalka et al. 2024
Stretch-shortening cycle in intact soleus muscles	In SSCs in wild type muscles, a doublet stimulus led to an increase in peak force and work per cycle. *Mdm* muscles showed neither doublet potentiation, nor phase-dependence of activation.	Hessel et al. 2021
Rapid unloading experiments in intact muscles	Active wild-type muscles recoil to a distance that is 15-20% shorter than in passive muscles, but *mdm* muscles do not	Lappin et al. 2006 Monroy et al. 2017

Titin structure, N2A domain organization, and N2A binding interactions with actin:

Titin is a giant, modular protein whose I-band elasticity is dominated by tandem Ig segments and the PEVK region.[35–39] In skeletal muscles, a unique N2A segment sits between the distal tandem-Ig segments and PEVK [40]. Cardiac N2BA titin also contains N2A, whereas the N2B isoform does not. The location of N2A within the I-band places it in an ideal position to sense strain and to coordinate binding events that couple mechanics to signaling.[11,12,41]

Domain architecture and UN2A: The N2A segment comprises four Ig-like domains (I80–I83) immediately N-terminal to the PEVK region.[42] Between I80 and I81 is a unique sequence (UN2A) that is largely disordered but contains a compact Tri-Helix Bundle (THB) flanked by flexible linkers. The UN2A THB is structurally analogous to a similar Tri-Helix Bundle in myosin-binding protein C (MyBP-C) (Fig. 1).[15,43] In MyBP-C, the THB is found between the C1 and C2 domains, which is the region associated with F-actin binding.[44–46] The C2 domain is the nucleation point for MyBP-C/F-actin binding, but the affinity increases when the M-domain (the name for the THB region) is included. A similar domain architecture is found in other actin binding proteins, like myopalladin and palladin,[43,47,48] but they lack a THB region. This suggests that there could be a recurring "compact-core/IDR-linker" architecture associated with actin binding, potentially to help orient the immunoglobulin domains to the F-actin to facilitate binding.

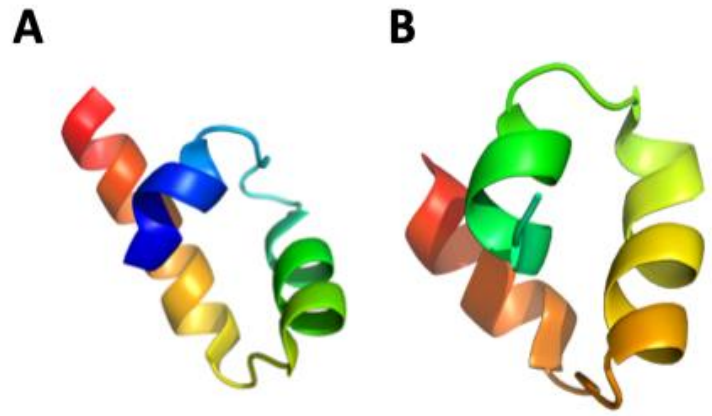

Figure 1: Structure of tri-helix bundles from the N2A region of titin (A, PDB: 7NIP) and Myosin Binding Protein-C (B,PDB: 2LHU)

Ig-domain contributions: The interactions among Ig domains and F-actin differ because each Ig domain is unique. Domains in the proximal Ig region produce weak binding interactions with actin. However, multiple lines of evidence indicate that Ig domains in the N2A region are essential for actin binding. Calcium enhances the interaction between N2A constructs and F-actin in vitro,[20] with I83 emerging as the likely calcium sensor based on truncation and mutation studies.[49,50] In stability measurements, I81 is the most stable and I83 the least stable (I81 > I82 > I83) of the three Ig domains.[21,49] Ca^{2+} binding increases I83 stability and correlates with stronger actin engagement by larger N2A constructs containing I80–I83.

A series of truncations that covered all the possible combinations of the domains in the N2A region have been tested for their ability to bind F-actin using co-sedimentation.[50] The only truncations that demonstrated F-actin binding activity were constructs containing I80.[50] This points to the I80 domain as a necessary element for detectable F-actin binding by N2A. Expression of the I80 domain by itself has been challenging, which might suggest some unique characteristics that make it important for binding. Stronczek et al.[51] found no calcium-dependent binding of an I81–I83 construct to F-actin, similar to findings by Dutta et al.[20] and Tsiros et al.[50] that only constructs that include I80 showed calcium-dependent F-actin binding. However, they did find non-calcium-dependent binding between I81–I83 constructs when CARP/MARP1 is present.[51–53] Based on concentrations required in co-sedimentation and motility assays, we estimate an apparent K_d for N2A–F-actin in the high-µM range under standard buffers, which is consistent with a transient, load- and context-sensitive tether. Taken together, these data provide a straightforward mechanistic link between calcium activation and titin–thin filament interactions in active muscles.

One useful animal model is the *mdm* mouse line,[41] which contains a short deletion at the N2A–PEVK junction, and highlights the functional relevance of this region. The *mdm* deletion results in the loss of 83 amino acids from the titin protein, 33 in exons 106-107 that code for I83 and 50 in exons 108-109 that code for the N-terminal PEVK region. The same 50 PEVK amino acids that are deleted in *mdm* were also removed by genetic engineering in the *Ttn* delta 112-158 transgenic mouse,[54] which has a normal phenotype, demonstrating that the *mdm* phenotype results from deletions in I83. The deletion of 33 amino acids from the I83 domain removes two of the b-sheets that stabilize this domain, making it unable to form a stable structure, and highlighting the importance of proper folding of this domain (unpublished data from the Gage lab).

Mdm muscles exhibit altered titin-based passive stress in single fibers and intact muscles,[22,55] and broad post-transcriptional splicing changes across the elastic I-band including Z-repeats, PEVK, and MEx5 that shorten titin's elastic I-band and plausibly increase titin-based stiffness,[56] pointing to the N2A-PEVK junction as a control point for mechanical tuning. Functionally, progressive trypsin → KCl → KI extraction in skinned soleus bundles shows a larger drop in passive stress in *mdm* than WT (titin-based component) and a higher collagen-based residual tension.[55,57] Hettige *et al.* (2022) illustrates both the magnitude of these changes and their separation into titin- versus collagen-derived contributions (see Fig. 6 in Hettige et al[57]). Together, these results argue that the N2A–PEVK region is a locus where molecular composition (splicing, local deletions) and environmental cues (Ca^{2+}, pH/ionic strength) converge to regulate titin–thin filament interactions and whole-muscle passive mechanics.

Adjacent PEVK region: Although N2A itself is not an intrinsically disordered region (IDR), it is directly coupled to the disordered, charge-dense PEVK region whose conformational properties are sensitive to the ionic environment. The PEVK region shows non-calcium-dependent binding to actin along its entire length.[58,59] The affinity of actin binding is greatest in the middle third (PEVKII), intermediate in the N-terminal third (PEVKI), and least in the C-terminal third of PEVK (PEVKIII) and appears to be associated with glutamate rich regions.[58] It has been suggested that this interaction might lead to viscous forces that resist rapid shortening and may contribute to muscle thixotropy.[59] Given the proximity to the N2A region, it seems possible that non-calcium-dependent PEVK-actin binding may also stabilize calcium-dependent interactions between N2A and actin. A similar pattern of differences is found in the flexibility of the adjacent PEVK subsegments, with PEVKI having the

shortest persistence length and PEVKIII having the longest persistence length,[60] although the functional significance of this observation remains unclear.

In peptides derived from titin's glutamate rich, poly-E motif, we have demonstrated a reproducible pH-dependent compaction with a midpoint near pH 5–6, in contrast to PPAK peptides that show little pH sensitivity over the same range.[61–63] These data support a model where decreases in pH reduce electrostatic repulsion among glutamates, allowing poly-E–rich segments to collapse and sample compact states. Mechanistically, the intervening uncharged residues in poly-E strongly tune this response using aromatic side chains to promote compaction (even at neutral pH), while proline content appears to dampen pH sensitivity.[62] Although the measured transition midpoint for 15–28mer poly-E peptides is slightly more acidic than typical activity-induced muscle acidification, longer motifs and local sequence context can shift the sensitivity window.[61] The key point for N2A is that the mechanical landscape at the N2A–PEVK junction will change with activation-related ionic and pH shifts.

Interactions with ankyrin repeat proteins: In addition to PEVK, members of the ankyrin family of proteins are stress-responsive transcription factors of striated muscles that bind to N2A titin and mediate signaling functions in processes including muscle hypertrophy.[12,15,53,64] Cardiac ankyrin repeat protein (CARP, also known as MARP1 and Ankrd1) binds to the tri-helix bundle (THB).[65] CARP/MARP1 binds to F-actin, as well as to N2A titin.[52] In single myofibrils, addition of CARP/MARP1 protein to myofibrils from transgenic CARP/MARP1 knockout mice increased their passive tension, specifically by reducing the extension of the PEVK region. However, the presence of CARP/MARP1 did not increase N2A actin binding, nor was it calcium dependent. In addition to potential protective function of the increase in passive tension with stress and disease, CARP/MARP1 also appears to play a role in signaling pathways associated with muscle hypertrophy. [66]

Role of N2A in active muscle mechanics

During natural movements, skeletal muscles exhibit low passive stiffness when stretched by antagonistic muscles, and high stiffness during active contraction to produce positive work and power. Thus, skeletal muscles require tunable stiffness that enables large changes in viscoelastic properties during every cycle of movement.[6] Changes in muscle stiffness with activation have been observed in a variety of experiments involving active stretching. Eccentric contraction refers to the stretching of active muscles.[7] The "stretch-shortening cycle (SSC) effect, first observed in frog muscles by Cavagna et al.,[23] refers to the observation that muscles produce higher force, work and power during shortening when that shortening is preceded by active stretching (a stretch-shortening cycle), compared to pure shortening that is not preceded by active stretching. A related phenomenon, termed residual force enhancement, is defined as the extra force observed upon stretching active muscle fibers compared to their isometric force at the same final length.[67] Active muscles store up to 56% of the energy expended in stretching them [25]. During active stretching, muscles produce more force at a lower energy cost than during isometric or concentric contraction,[68] and the energy cost of SSCs is much lower than that of a shortening contraction without prior stretch.[69]

Historically, cross bridges alone were thought to be responsible for active muscle stiffness.[70] While early investigations interpreted cross bridges as contributing to elastic energy storage,[70] muscle force during active stretch,[71] and even residual force enhancement,[72] these inferences were based on assumptions that no longer thought to be accurate. We now know that less than 20% of cross bridges are attached to actin at any given time,[73] that cross bridges remain attached to actin for short periods of time (~25 ms,[74] and that they remain attached only over very short distances (~10-12 nm).

The extra force produced by muscles during active stretching (i.e., eccentric contraction, residual force enhancement), and the SSC effect require the existence of a structure in muscle sarcomeres that increases its stiffness in calcium-activated muscles, extends over hundreds of nanometers (exceeding 20% L0), and stores substantial amounts of energy for up to several seconds.[75] The small strains (< 1% L0[70] and rapid cycling (~40 Hz[76] of cross bridges severely limit their ability to store elastic energy during active stretch, with an estimated contribution of no more than 12%.[25] Because the PEVK domain of titin alone possesses the properties required to explain the large strains, high energy storage, and slow relaxation[19,37] observed during stretch of active muscles, isolating this domain from the compliant regions of I-band titin is required.

Calcium-dependent N2A binding to actin in active muscles[6,7,20,77] isolates the PEVK region from the entropic tandem IG domains[37] that would otherwise severely limit the stiffness and energy storing capacity of I-band titin at sarcomere lengths below 2.8 um (~1.15 L0). Because the tandem Ig domains extend under low force at short sarcomere lengths < 2.8 um, they dissipate rather than store energy. So long as the entropic tandem Ig domains are in series with the PEVK domain, especially at sarcomere lengths below 2.8 um, straightening of the tandem Ig domains at low force will dissipate rather than store elastic energy during stretching. Thus, without calcium-dependent binding to actin, titin cannot explain the changes in stiffness and energy storage observed in active muscles.

Overwhelming evidence from a wide variety of ex vivo experiments and preparations supports the existence of activation-dependent titin-actin interactions in active muscles (Table 1). Conditions that have been tested (described in more detail below) include single titin molecules,[20,26] single myofibrils,[78–81] intact[82] and skinned single muscle fibers,[10,83] and intact muscles.[22,84,85]

Evidence from single titin molecules: Early studies suggested that titin binds to actin in motility assays at a minimum pCa of 6.0, 30 times higher than the calcium concentration required to achieve maximum isometric force.[19] Although systematic efforts were made to test titin constructs from titin's entire I-band for calcium-

dependent actin binding using co-sedimentation experiments, the results were largely negative.[58–60,86] The results did, however, reveal non-calcium-dependent binding in the PEVK segments of both skeletal and cardiac muscles.[58,59,86] Surprisingly, a small region (115 amino acids) of I-band titin at the N2A-PEVK junction, including 83 amino acids deleted in the *mdm* mutant,[41] had been overlooked.[20] This region showed calcium-dependent binding to actin in co-sedimentation assays, in vitro motility assays, and atomic force microscopy experiments (Table 1).[20]

Evidence from stretch of single myofibrils: Evidence for a large activation-dependent increase in titin stiffness was first provided by Leonard & Herzog[81] and others,[80] who stretched single myofibrils beyond overlap of the thick and thin filaments, demonstrating that titin stiffness increases with calcium activation in the absence of cross-bridge forces. These studies also demonstrated that the increase in titin stiffness upon activation is impaired in *mdm* muscles.[55,79] In addition, residual force enhancement in single myofibrils depends on the titin isoform they express.[87] Rabbit papillary cardiac muscles that do not express N2A exons failed to show residual force enhancement, whereas skeletal muscles that express N2A exons showed residual force enhancement as expected. These experiments unambiguously suggest that the N2A region of titin binds to actin in calcium-activated myofibrils.

Time course of stiffness vs. force: The rise of force in isometrically contracting single intact muscle fibers is preceded by a rise in stiffness that closely follows the time course of calcium release.[82,88–93] The delay between the rise in cross-bridge force and the rise in muscle stiffness implies that titin stiffness increases prior to cross-bridge force, because cross-bridge force and cross-bridge stiffness must rise simultaneously. Likewise, when muscle fibers are deactivated, their tension remains elevated for up to 19 seconds,[9,94–96] which implies that elastic forces stored in titin take much longer to dissipate than cross-bridge forces. Recent studies suggest that titin stiffness increases 100-fold upon activation [4,5] with a time course that precedes thick filament activation.[8]

Evidence from eccentric contraction, residual force enhancement and stretch-shortening cycles in intact and skinned single muscle fibers: Numerous studies (Table 1) have demonstrated that titin contributes to the extra force produced by muscles in eccentric contraction, residual force enhancement, and stretch-shortening cycles. Following active stretch, sarcomeres exhibit a distinct structure that includes increased thick filament strain and decreased lattice spacing.[10] These features disappear when titin is cleaved in titin-cut transgenic mouse muscles and in muscles from *mdm* mice, which exhibit no residual force enhancement when stretched.[22]

Because calcium activation of cross-bridges and calcium-dependent titin - actin binding are currently the only mechanisms that can account for the increase in stiffness of skeletal muscles upon activation, experiments using butanedione monoxeme (BDM)[83,97–102] or blebbistatin[102] to inhibit cross bridges during residual force enhancement and stretch-shortening cycles consistently demonstrate that titin contributes to the extra force produced when active muscles are stretched. The extra force after stretching persists even after inhibition of crossbridge cycling, implying a role for PEVK titin. Although the magnitude of residual force enhancement increases with stretch amplitude (Fig. 2D,E,F),[22] residual force enhancement is nevertheless consistently observed in single fibers of frog lumbrical muscles even at short sarcomere lengths corresponding to 90% L_0[103], lengths at which low force straightening of the proximal tandem Ig domains[37] would preclude force enhancement.

While previous studies have emphasized the importance of the increase in titin stiffness with activation,[6,10,81,104] it is clear that not only titin stiffness but also titin viscosity increases with activation (Fig. 2B, C, D, F).[22,29] In ramp stretch experiments (Fig. 2A, B, C), the force response is the sum of conservative elastic forces that persist over time, and dissipative viscous forces that decay over time. The results of ramp stretch experiments clearly show that not only elastic but also viscous forces increase in active compared to passive muscles (Fig. 2E, F).[22] Furthermore, because cross-bridge forces decline with increasing velocity,[74] the rise of muscle power with increasing stretch velocity of single skinned muscle fibers in stretch-shortening cycles is also inconsistent with cross-bridge mechanisms[24,105] and likely involves a role for titin viscosity.[26]

The observations that activation-dependent stiffness,[79] residual force enhancement and depression,[22] and the change in resting length of rapidly unloaded muscles[85] are impaired in muscles from *mdm* mice support the hypothesis that calcium-dependent binding of Ig83 to thin filaments is necessary for titin actin binding in active muscles. This is consistent with the truncation data described previously in this review, showing that removal of the I83 domain eliminated the Ca^{2+} dependence of F-actin binding. However, I83-thin filament interactions are not sufficient for binding since I83 does not bind independently of other domains in the N2A region.[50] Therefore, a variety of additional mechanisms may contribute to stabilizing titin-thin filament interactions *in vivo*.

Evidence from rapid unloading experiments: If N2A titin binds to actin upon calcium-activation of muscle sarcomeres, then active muscles must recoil to a shorter resting length than passive muscles.[77] The change in rest length should be approximately equal to the length of the proximal tandem Ig domain. When muscles were stimulated isometrically to a given load and then rapidly unloaded to a smaller load, the recoil distance was 15-20% shorter in active muscles compared to passive muscles when starting and ending at the same loads.[84] This experiment demonstrates that the resting length of the muscles decreases upon activation, consistent with N2A binding to thin filaments. Furthermore, *mdm* muscles with a deletion in I83, which show no increase in titin-based stiffness in myofibrils,[79] also show no change in equilibrium length in unloading experiments.[85]

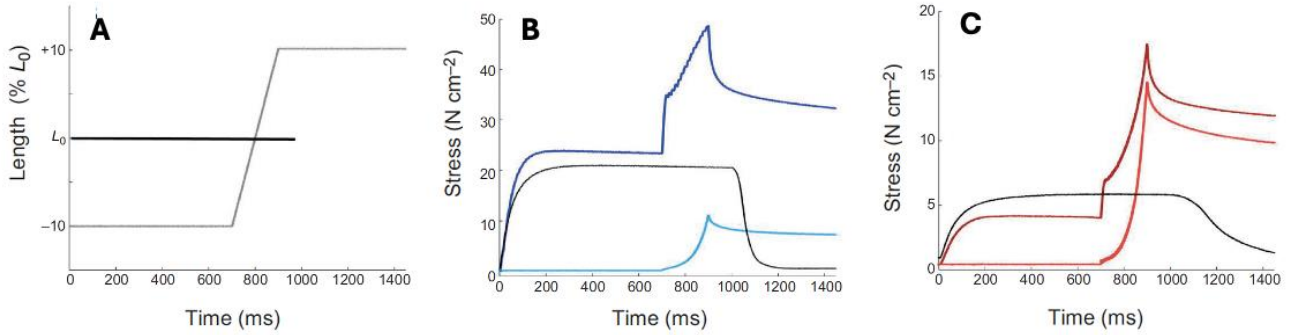

Figure 2: Results of ramp stretch and isometric experiments in wild-type (blue) and *mdm* (red) soleus muscles. A) Muscle length vs. time: isometric (black) and ramp stretch (gray). **B**) Force traces corresponding to length changes for wild-type soleus: isometric (black), passive stretch (light blue) and active stretch (dark blue). The passive stretch response is mostly elastic, whereas the active force is mostly viscoelastic. **C**) Force traces corresponding to length changes for *mdm* soleus: isometric (black), passive stretch (light red) and active stretch (dark red). Note that the passive and active stretch responses are mostly elastic. Adapted from Tahir et al. (2020) with permission from the Company of Biologists.

Holistic model for N2A- actin interactions

Skeletal muscles require tunable viscoelastic properties to function efficiently during movement, with low force and stiffness permitting passive stretch by antagonistic muscles but high active force and stiffness enabling energy storage during active stretch. Before the discovery of titin, cross bridges were understandably assumed to underpin changes in muscle properties with activation. At present, however, a large body of evidence provides strong support for the hypothesis that titin possesses calcium-dependent viscoelastic properties that are mediated at least in part by calcium-dependent N2A-actin binding. The theoretical basis for this inference relies on both the entropic, energy dissipating properties of the proximal tandem Ig domain,[37] as well as the experimental evidence that the resistance of skeletal muscles to active stretch persists after inhibition of the cross bridges.

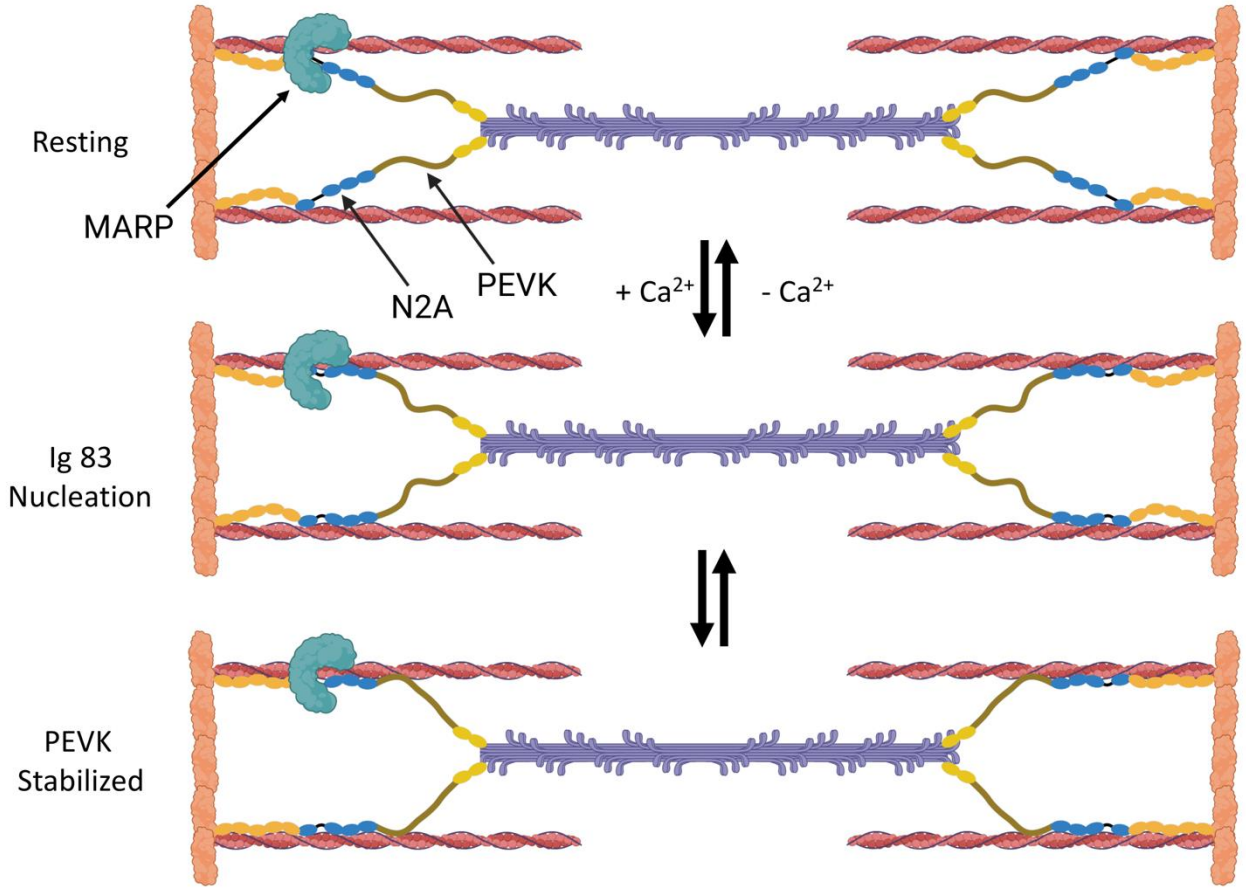

Figure 3: Proposed model for N2A binding to F-actin. In the resting state (top panel) the I80 Ig domain in the N2A region weakly associates with the F-actin to keep titin close to the thin filament. The calcium influx associates with activation results in the I83 domain associating with thin filament (middle panel), nucleating the binding. This binding is then stabilized by association of other proximal Ig domains and the PEVKI portion of the PEVK region, fully tethering the N2A region to the thin filament. Prepared using BioRender.

Direct and indirect evidence further suggests that calcium-dependent N2A-actin interactions require a properly folded I83 domain. Numerous in vitro assays demonstrate calcium-dependent binding of I83 to actin,[19,20] and many ex vivo experiments demonstrate that *mdm* muscles lack the structural and functional markers of increased active force and stiffness (Table 1). These data suggest that calcium-dependent binding of I83 to actin is necessary for titin-actin binding and increased active force and stiffness of skeletal muscles. However, it is also clear that calcium-dependent binding of I83 to actin alone is not sufficient to account for the increased active force and stiffness of skeletal muscles.[20,50,51] In vitro binding studies from multiple laboratories demonstrate that not only I83 but also I80 is required for N2A binding to actin.

The observation of calcium-dependent binding of N2A to F-actin in some studies[20,50] and not others[51] are not in conflict when the constructs used in the various studies are carefully examined. The I81-I83 construct did not show binding in either study. The truncation study[50] did not investigate potential stabilizing interactions between MARP1 and the I81-I83/F-actin complex, while the MARP1 study[51] did not test the I80-I83 construct, which is includes the I80 domain that appears to be necessary for binding of N2A to F-actin in vitro.[50] It should be noted that these in vitro studies have been conducted using fragments of the entire titin protein, which eliminates potential non-specific interactions that could help stabilize this interaction.

We suggest a model in which N2A binds weakly to actin via I80 in the absence of calcium (Fig. 3). Upon Ca^{2+} influx, I83 also binds weakly to actin. While I83 binding is required for the calcium-dependent titin-actin interaction, PEVKI[58,59] and/or CARP/MARP1[51,52] proteins may enhance residence time on the thin filament. There is also the potential for domains in the proximal Ig region to interact with the thin-filament, adding stabilization to the N2A/F-actin interaction. We propose a model in which I83 provides the nucleation site for calcium-dependent titin/thin-filament interactions, with other factors stabilizing this association, enabling physiologically relevant residence times during activation without "locking" the filament.

The N2A region of titin is not only a mechanical switch in skeletal muscle, but also a signaling hub, with interactions including MARP family proteins, S100A1, proteases and chaperones[2,13]. These signaling functions are critical for maintaining sarcomere integrity and responding to stress. MARP family proteins in general and MARP1 in particular bind to actin and increase passive muscle tension,[52] presumably to protect muscles from overstretch. Over longer timescales, MARP1 is also involved in signaling pathways associated with longitudinal hypertrophy that occurs during development and in response to overstretch. MARP1 is highly upregulated in *mdm* muscles,[106–108] yet this upregulation rescues neither the blunted force response to active stretch,[10,22,79,85] nor the reduction in developmental hypertrophy that prevents normal growth after weaning in the *mdm* phenotype.[41,109]

The protease calpain/p94 also binds to multiple regions of N2A titin, including the N-terminal Ser8934 residue which is located in the *mdm* deletion.[110] Mutations of calpain/p94 lead to a class of muscular dystrophies, termed calpainopathies, which share phenotypic characteristics with *mdm*, including vertebral kyphosis, muscle weakness and toe-walking. Yet, the *mdm* mutation does not abolish binding of calpain/p94 to N2A titin, presumably because calpain/p94 can bind to other sites in N2A titin. In wild-type muscles, binding of calpain/p94 to N2A titin prevents autolysis of calpain/p94 which is necessary for normal muscle function. It also prevents calpain/p94 proteolysis of N2A titin and MARP2, which likely changes its function.

While there is no doubt that the *mdm* mutation is complex, including the failure of developmental muscle hypertrophy which might be related to changes in MARP1 signaling,[52] it is also true that *mdm* soleus muscles show none of the structural or functional signatures of force enhancement [10] even at 24 days of age before obvious changes in sarcomere structure have developed.[108,111] Furthermore, *mdm* muscles also exhibit increased titin-based passive tension associated with altered exon splicing in PEVKI and II.[57] In terms of muscle mechanics, they exhibit only a decrease in active tension in their force response to active stretch. It therefore seems unlikely that the absence of force enhancement and related mechanical properties results from signaling per se, but rather from altered N2A binding.

Critical Experiments

As has been demonstrated throughout this review, there is significant evidence supporting the importance of N2A binding to F-actin. However, models vary in how the N2A/F-actin interaction is formed and stabilized, so further work is necessary to clarify the specifics of this interaction. One of the major differences among models is the necessity of MARP1 in regulating this interaction. It has been suggested that MARP1 is required to stabilize the N2A/F-actin interaction, and that the measured interaction of N2A to F-actin is too weak to resist forces that would be applied during contraction in vivo. However, our working model proposes that N2A is the nucleation site for this binding and that the interaction is stabilized by interactions of the N-terminal region of the PEVK domain as well as the proximal Ig domains with F-actin.

We propose several experiments that could provide additional clarity to the importance of MARP in the N2A/F-actin binding process. First, the binding constant for N2A binding to F-actin has not been determined. It is assumed to be in the low μM range based on the co-sedimentation and *in vitro* motility assays but determining an accurate binding constant would assist in modeling the binding strength versus force relationship. Second, our model suggests that binding would be strengthened by non-specific interactions involving surrounding domains, which could be tested by measuring binding with longer constructs. If our model is accurate, we would expect to see stronger binding as we add regions of the PEVK and proximal Ig domains.

These experiments would test the direct binding between titin and F-actin but do not address whether the binding

is physiologically relevant. This could be addressed using myofibrils or skinned fibers. Forces could be measured in the presence or absence of various N2A constructs, which would potentially compete with N2A binding to F-actin. If our model is accurate, we should see decreased forces if we add exogenous N2A since it would reduce nucleation. A fluorescently labeled N2A construct would allow visualization of binding to verify that the exogenous N2A was binding to F-actin. To test the role of MARP, a similar experiment could be conducted by inhibiting MARP binding to titin. This is a more challenging experiment as there are currently no known inhibitors for this interaction, but it might be possible to use anti-MARP antibodies to inhibit binding.

Obviously, an ideal experiment would be to directly measure N2A binding to F-actin in a sarcomere and to show that it remains in the same location during an eccentric contraction. Recent work in the Herzog lab using double-labeling of N2A and the distal PEVK region (F146) provides evidence for stabilization of N2A[78,112] in active muscle fibers. However, labeling with fluorescent secondary antibodies results in artifacts due to antibody cross-linking, which increases the experimental variability and the force required to extend the sarcomeres,[113] so using labeled primary antibodies instead of labeled secondary antibodies is essential for understanding N2A - F-actin binding in vivo.

Conclusion

Recent studies have elaborated on known functions and discovered new functions of N2A titin in stress and hypertrophic signaling, protein breakdown and turnover, and mechanical switching from low passive viscoelasticity to high active viscoelasticity in skeletal muscles.[2,13,52,66] While the N2A region is clearly a multi-functional signaling hub with numerous binding partners, it is also clear that these processes take place over widely different spatial and temporal scales. Signaling processes that regulate gene expression occur over hours to days. Changes in titin's viscoelastic properties follow the time course of calcium release and reuptake rather than the course of cross-bridge activation and deactivation, and titin-based viscoelastic forces persist over time scales and distances far too large to be borne by cross-bridges alone. While N2A interactions with F-actin appear to require both I80 and I83, it is likely that this interaction is stabilized in vivo by the proximal PEVK region as well as other proteins including CARP/MARP1. Much challenging work remains to elucidate the mechanisms of N2A-F-actin binding in a physiologically relevant experimental setting.

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