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COMMENTARY

Unraveling the mysteries of the titin–N2A signalosome

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The muscle protein titin is best known for its mechanical role in myocytes. Titin filaments span one half of the sarcomere, the contractile unit of striated muscles, and are extensible within the I-band region, where they link myosin-based thick filaments and actin-based thin filaments as viscoelastic springs (Fig. 1, A and B). These titin springs provide nearly all stiffness and tension in resting myofibrils (reviewed by Linke, 2018). The composition of these molecular springs depends on alternative splicing of I-band titin exons, giving rise to different titin isoforms known as "N2A" in different skeletal muscles that differ in stiffness (Prado et al., 2005). The I-band region of the N2A isoforms is comprised of distinct in-series spring elements, tandem Ig-domain segments located near the Z-disk (proximal) and A-band (distal), which straighten out at low stretch forces and also unfold/refold individual domains (Rivas-Pardo et al., 2016), and the proline-glutamate-valine-lysine-rich (PEVK) segment, which extends at higher stretch forces (Linke et al., 1996). The proximal-Ig and PEVK segments flank the N2A region, which consists of four Ig domains and a unique insertion sequence called UN2A (Fig. 1 B; Labeit and Kolmerer, 1995). In an earlier issue of *JGP*, van der Pijl et al. (2021) address a novel, intriguing mechanical function of the titin–N2A region.

Apart from being a viscoelastic element, I-band titin is also thought to be a signaler of myofibrillar stress in healthy and diseased muscle, with a critical sensor region at the N2A region, the "N2A signalosome" (Linke, 2018; Nishikawa et al., 2020). At the N2A signalosome, titin complexes with no less than 10 other proteins (Fig. 1 C), including the protease calpain 3 (Sorimachi et al., 1995), the methyltransferase SMYD2, which recruits the chaperone HSP90 to I-band titin (Donlin et al., 2012), and muscle ankyrin repeat protein (MARP) family members MARP1 (CARP/Ankrd1), MARP2 (Arpp/Ankrd2), and MARP3 (DARP/Ankrd23), which also target myopalladin to the N2A region (Miller et al., 2003). This protein cluster is not stagnant, as proteins swap in and out under different stress conditions, especially during myopathy (Swist et al., 2020). For example, chaperones such as αβ-crystallin (αβ-C) and HSP90 translocate from the Z-disc or cytosol to the N2A region in myopathic muscle, presumably to play a protective role on the titin spring (Kötter et al., 2014; Unger et al., 2017). van der Pijl et al. (2021) now focus on the MARP1 protein, which in healthy skeletal muscle usually is present in trace amounts but is up-regulated in diseased muscle (Swist et al., 2020; Wette et al., 2017; van der Pijl et al., 2019). MARP1 was recently found to increase passive myofiber force when added ex vivo (Fig. 2 A), and this was explained by MARP1 cross-linking of actin and the N2A region of titin (Fig. 2 B; Zhou et al., 2021). van der Pijl et al. (2021) confirm and extend these findings, and, for the first time, place them in a pathophysiological context.

Previously, van der Pijl et al. (2018) found that, after unilateral diaphragm denervation in mice, MARP1 was up-regulated and localized in the I-band. In their new study, van der Pijl et al. (2021) carefully characterize the mechanical implications of MARP1. Their most striking findings are that (1) MARP1 does not only localize and bind to the N2A region in in vitro preps, but also in vivo in the diaphragm of mechanically ventilated mice and humans, and (2) by measuring I-band titin extension profiles with sarcomere stretch, they provide strong evidence that the MARP1-mediated titin–to–thin filament binding at the N2A region functionally shortens titin's free spring length, leading to a seemingly permanent increase in passive force and stiffness. The authors conclude that the up-regulation of MARP1 in muscle primarily increases resting fiber tension, protecting against overstretch and stabilizing the sarcomere. The demonstration of an I-band titin–thin filament connection in vivo is remarkable, as it advances several long-held debates in muscle mechanics, of which we discuss three below.

First, the concept of an I-band titin–thin filament interaction, regardless of potential intermediaries, is highly debated. In skeletal muscle, there are several unsolved mechanical properties, such as the history dependence and length dependence of $[Ca^{2+}]$ sensitivity, which could be explained by an activation-dependent binding of titin to the thin filament at the N2A signalosome (Nishikawa et al., 2020). However, there is no direct in vivo evidence of this (Linke, 2018). Although various I-band

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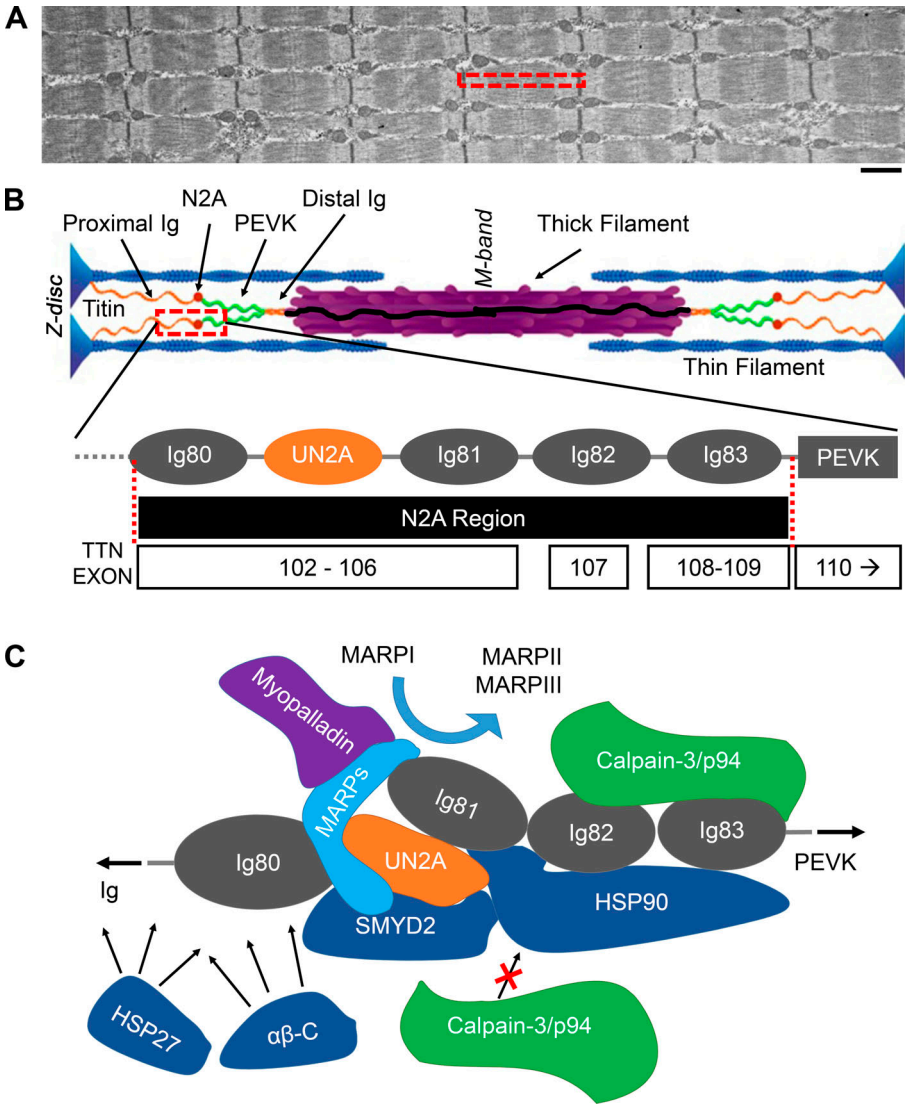

Figure 1. **The N2A signalosome of skeletal muscle titin. (A)** Electron micrograph of skeletal muscle tissue made up of repeating contractile units, the sarcomeres (red box, scale bar, 1 µm). **(B)** Layout of N2A–titin isoform in the sarcomere. Each titin molecule is bound to the thin filament (blue) in the Z-disc and to the thick filaments (purple) in the A-band. The N2A segment (red) is located between the proximal tandem Ig segment (orange) and the PEVK segment (green). The N2A region is comprised of four Ig domains and a unique insertion sequence (UN2A). **(C)** The N2A signalosome is made up of many proteins for reasons that are poorly understood. Images used and modified with permission from Nishikawa et al. (2020).

titin fragments (notably PEVK fragments) have been found to bind thin filament proteins in vitro (Dutta et al., 2018; Nagy et al., 2004; Zhou et al., 2021; Linke et al., 2002), this does not guarantee the same in vivo. We recently presented evidence of a titin–thin filament interaction in permeabilized fibers, where I-band titins were controllably cleaved close to the A-band, and titins got stuck on thin filaments while recoiling toward the Z-disc, showing that titin–thin filament binding is possible per se (Li et al., 2020). However, numerous experiments have been conducted on passive fibers and single myofibrils, where I-band titin extension during sarcomere stretch was not obviously impeded by an interaction with the thin filament, although no specific experiments have been designed to assess low-level titin–thin filament interactions. A single result suggesting the opposite, based on experiments with actively contracting myofibrils, was inconclusive (DuVall et al., 2017) because antibodies were used to track titin extension, which likely caused cross-linking of titin with other (including contractile) proteins. Therefore, the van der Pijl et al. (2021) finding that mechanically ventilated mouse and human diaphragm muscles produce MARP1, subsequently linking titin to the thin filament, is extraordinary. Unfortunately, it should be noted that this seemingly strong and permanent binding does not provide a mechanism for an activation-dependent titin–thin filament interaction, as MARP1 is sparse in healthy muscle and binds well in resting and contracting muscle. However, a pathway for titin–thin filament anchoring is now demonstrated through the N2A signalosome in myopathy—so why not also in healthy muscle?

Second, do the other proteins at the N2A signalosome play a role in titin–thin filament tethering? Among these proteins, myopalladin is an actin-associated scaffold that binds directly to MARPs (1 and 2; Miller et al., 2003), and so may stabilize the MARP1/titin–thin filament interaction, but this is speculative. As pointed out by van der Pijl et al. (2021), while MARP3 is dissimilar to MARP1, MARP2 is highly similar, and so an ability to bind the thin filament is probable; however, no binding assays have yet been attempted. What we can be sure of is that titin-extension characteristics in resting healthy muscle (myopalladin/MARP2 dominating) do not suggest a strong titin–thin filament interaction. Whether this is also true during contraction in the presence of high [Ca^{2+}] remains to be explored. Other

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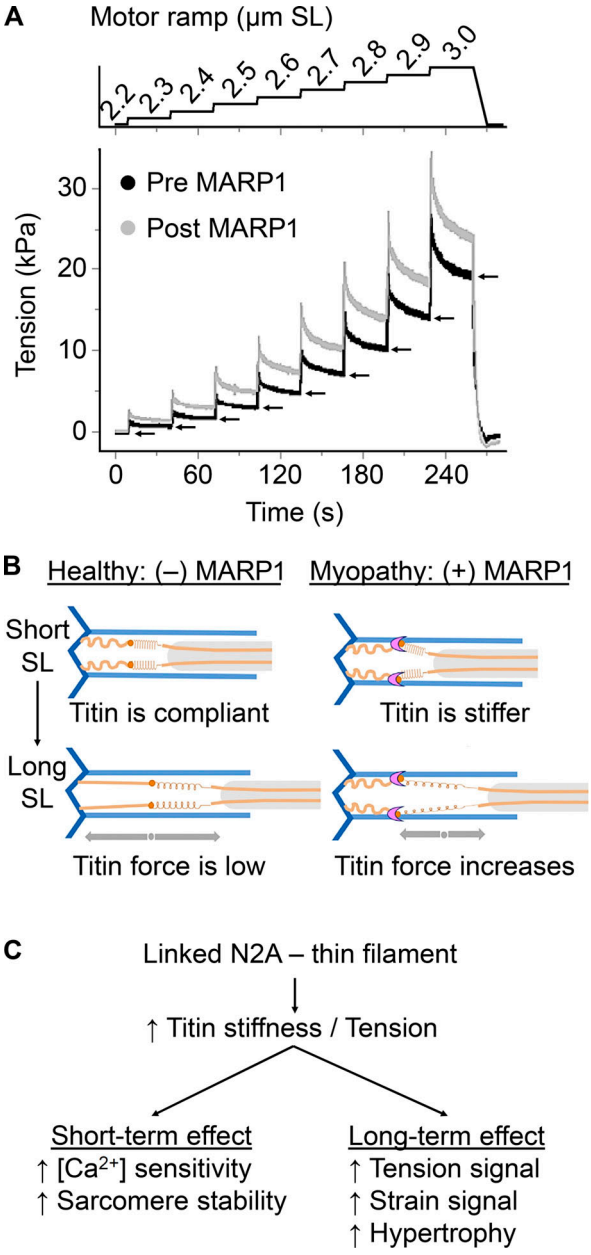

Figure 2. **The function of MARP1 in skeletal muscle. (A)** Permeabilized muscle fibers were subjected to a passive ramp-hold protocol before (Pre MARP1) and after (Post MARP1) incubation with a recombinant MARP1 fragment. The addition of MARP1 increased fiber passive tension. **(B)** Recent evidence (Zhou et al., 2021; van der Pijl et al., 2021) suggests that MARP1 tethers titin to the thin filament, functionally shortening titin's free lengths to the stiffer PEVK region, and thus producing more passive tension upon sarcomere stretch. **(C)** MARP1's function may not only be to increase passive tension, but also to enhance hypertrophic signaling. A and B used with permission from Zhou et al. (2021).

proteins localized to the N2A region, such as Smyd2-HSP90, HSP27, or αβ-C, appear to have little, if any, effect on the titin extension–sarcomere length relationships in resting muscle (Unger et al., 2017), and so do not seem to play a role in the titin–thin filament interaction; however, just a few data are available, and none measured during contraction. In summary, apart from MARP1 and myopalladin, the other identified N2A signalosome proteins either show no prevalence to thin filament binding in vivo or have not yet been evaluated.

Third, is there enough MARP1 in vivo, during disease, to make a meaningful change to passive force and protect sarcomere overstretch? The reported ∼76-fold increase in MARP1 in mechanically ventilated human diaphragms is impressive (van der Pijl et al., 2021), but it is important to remember that MARP1 is present in trace amounts in resting skeletal muscle, and the increase in MARP1 may not saturate all available titins. A quick estimation is tricky because the amount of MARP1 seems to vary between muscles and is not necessarily localized to the sarcomeres only. Wette et al. (2017) showed that, in healthy human vastus lateralis, MARP2 is 150-fold more abundant than MARP1 (only trace amounts), and of the available MARP2, only ∼15% are bound within the cytoskeleton, which Wette et al. estimated to cover ∼25% of total titins. If we assume the MARP1-titin binding distribution is similar to MARP2 (Wette et al., 2017), then MARP1 would cover ∼0.2% of titins in healthy muscle (i.e., functionally inconsequential). With the measured 76.1-fold increase in diaphragms of mechanically ventilated humans by van der Pijl et al. (2021), this would cover ∼15% of total titins, and so we predict only 15% of titins are linked to the thin filament, and thus change their extension characteristics. However, the confocal images of van der Pijl et al. (2021) do not support the idea that a large fraction of titins are unbound, producing a "normal" titin extension pattern. This discrepancy could be because confocal imaging, even at the super-resolution level, may not resolve these subpopulations, and/or MARP1 has a larger binding distribution to titins than reported for MARP2 (Wette et al., 2017), potentially increasing the fraction of bound titins to a maximum of 85% if all MARP1s bind. It would be worthwhile to repeat the experiments of Wette et al. (2017), with control and mechanically ventilated human diaphragms, to get a clearer picture of MARP1 levels and binding patterns. Furthermore, immunoelectron micrographs may provide a clearer visual of any titin-extension subpopulations.

Regardless of how many MARP1s bind titin in critically ill patients, an increase in myofibril passive tension is measured (Fig. 5 in van der Pijl et al., 2021), but it is not yet clear whether these increases in passive tension (5–10 kPa) are important for sarcomere stability or over-stretch protection during contraction (active tension >100 kPa). Why would increased titin-based force and stiffness be advantageous? The first theory is to stimulate hypertrophy through protein sensors associated with (full-length) titin, as discussed in van der Pijl et al. (2021, 2019, 2018). Only passive overstretch is needed to up-regulate N2A signalosome proteins, leading to their titin localization (van der Pijl et al., 2021, 2018), and subsequently sparking a hidden signal cascade for muscle hypertrophy. MARP1 linking the N2A region to the thin filament leads to relatively more extension of PEVK, and enhanced titin-based forces, with sarcomere stretch, compared with healthy tissue. These changes could impact the proteins bound to mechanosensory titin regions, triggering a pro-hypertrophic signal cascade. It is also possible that MARP1's impact on titin-based force is not meant to be a sensor at all; instead, the purpose of it is to increase [Ca^{2+}] sensitivity. Titin-based forces strain the thick filament, which has been shown to

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reorient myosin heads into a more active ("on") state, enhancing Ca^{2+} sensitivity and improving active force production (Ait-Mou et al., 2016). This mechanism could be advantageous for a muscle: if the muscle is being overstretched by the applied load, why not improve force production through the MARP1 pathway to protect overstretch in the short term, while also increasing muscle size in the long term? These types of ideas are already generating experiments that should be completed over the next few years.

In conclusion, van der Pijl et al. (2021) provide concrete evidence that skeletal muscle can react to muscle disease through an up-regulation of the N2A signalosome protein MARP1, which links titin to the thin filament, functionally increasing titin-based stiffness and force. Although the purpose of this is not yet clear, it is likely related to (1) an "override" of the normal hypertrophic signaling pathway, as well as (2) a short-term measure that both protects against overstretch and enhances active force generation until muscle turnover is complete. Clearing up these questions, as well as others (such as how MARP1 is removed), will keep the field busy for many years; we are only observing the tip of the iceberg for this important topic.

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