

COMMENTARY

# Titin-N2A: More than a signaling node?

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The smallest contractile unit of striated muscle, the sarcomere, is comprised of three major filaments: the myosin-based thick filaments, the actin-based thin filaments, and the giant filamentous protein titin. Thin and thick filaments interact to produce active force and the titin filaments produce passive force upon sarcomere stretch. This passive force is essential for maintaining the structural integrity of the contracting sarcomere, limiting sarcomere length inhomogeneity along myofibrils, and regulating the level of active force during contraction (Horowitz et al., 1986; Fukuda et al., 2005; Brynne et al., 2018). In skeletal muscle, the passive force of titin is generated by the extension of its tandem Ig section and the so-called PEVK segment—rich in proline (P), glutamate (E), valine (V), and lysine (K; Trombitás et al., 1998). At the N-terminal end of titin’s PEVK segment is another component, the N2A element (Labeit and Kolmerer, 1995a), the structure and function of which has been understudied and, therefore, has remained elusive. In this issue of the *Journal of General Physiology*, Stronczek et al. (2021) reveal the unique structure of titin-N2A and how this facilitates the binding of signaling proteins. The authors also aimed to establish whether titin-N2A directly interacts with the actin-based thin filament, a mechanism which might affect the passive force generated by titin as sarcomeres change length. However, the data obtained do not support such interaction, and the implication of this finding is highlighted below.

## Titin provides passive stiffness to striated muscle

Titin is the largest mammalian protein known to date (3–4 MD) and spans the entire length of the half-sarcomere from Z-disc to M-band (Labeit and Kolmerer, 1995b). As in the Z-disc, where titin filaments from opposite sarcomeres overlap, titin filaments from opposite half-sarcomeres are thought to overlap within the M-band, where they are interconnected by M-band proteins. Thus, titin filaments with opposite polarity overlap in both the Z-disc and M-band, forming a contiguous filament along the myofibril. This layout of titin within the muscle’s sarcomere makes it ideally suited to sense changes in mechanical loading. Indeed, mechanosensing by titin involves titin-binding proteins,

of which several are intimately involved in atrophy/hypertrophy signaling. These titin-binding proteins are not randomly distributed along the titin molecule but are restricted to “hot spots”: one near the Z-disc, one in the M-band region, and another in the central and elastic I-band region (van der Pijl et al., 2019). One of these I-band hotspots localizes to the N2A element. The N2A element contains four Ig domains (I80–83) and several unique sequences, of which the 104-residue unique sequence (UN2A) with flanking Ig domains I80 and I81 is a major component (Labeit and Kolmerer, 1995b). Several proteins interact with the N2A element, such as SMYD2, P94/calpain3, and the muscle ankyrin repeat proteins (MARPs). Titin-N2A might also interact with the actin-based thin filament, presumably in a Ca<sup>2+</sup>-dependent manner (Dutta et al., 2018). This locking of the titin-N2A element to the thin filament might have significant mechanical effects by preventing the proximal tandem Ig segment from extending as sarcomeres are stretched, thereby forcing the PEVK segment to increase its extension, and therefore generate higher passive force. However, to date, strong evidence for this locking mechanism has been lacking.

## Titin-N2A does not directly interact with F-actin

To gain more insights into the structural basis of titin-N2A binding to actin filaments, Stronczek et al. (2021) revealed the atomic structure of I81–I83 by x-ray crystallography. Although the authors elucidated unique topographic features, they could not identify specific Ca<sup>2+</sup>-binding sites in the Ig domains. The authors aimed to confirm these findings in intact sarcomeres, by assessing the interaction between titin-N2A and F-actin in C2C12 cells transfected with fluorescently labeled titin-N2A constructs. Based on a diffuse localization of the constructs rather than a localization in a striated pattern, titin-N2A appears to not colocalize with actin. The authors concluded that these findings support the absence of interactions between titin-N2A and F-actin. However, caution is warranted as cytosolic [Ca<sup>2+</sup>] is low in these C2C12 cells, and therefore Ca<sup>2+</sup>-mediated interactions between titin-N2A and F-actin cannot be assessed by this approach. Furthermore, the structure of (differentiated) C2C12

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This work is part of a special collection on myofilament function and disease.

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cells is immature, which might have confounded the outcomes. Future studies should address this question by transfecting intact, mature muscle fibers. Fortunately, the authors performed cosedimentation assays, which supported the absence of interactions between titin-N2A and F-actin, both in the presence and absence of  $Ca^{2+}$ . Thus, although the study by Stronczek et al. (2021) did not reveal a new mechanistic role for titin-N2A, the results presented are important as they increase our understanding of titin's structure and its role in sarcomere mechanics.

### Correction: Titin-N2A actually does interact with F-actin, if assisted by a third party

So is there no role for titin-N2A in mediating sarcomere mechanics? On the contrary. In a recent follow-up paper by the Mayans group (Zhou et al., 2021), they showed that a third protein is at play: MARP1 (also known as ANKRD1/CARP). MARP1 interacts with the UN2A-Ig81 fraction of N2A (Miller et al., 2003), but its effect on titin mechanics was unknown. The Mayans group revealed that MARP1 peptide 106-319 (encoding the ankyrin repeats) binds to titin-N2A and then locks it to F-actin. Sarcomeres exposed to MARP1 had elevated passive force (Zhou et al., 2021). Another independent study by Van der Pijl et al. (2021) confirmed this locking mechanism with full length MARP1, and super-resolution microscopy showed that the large effect on passive force was caused by increased extension of the PEVK segment during sarcomere stretch. In skeletal muscle, this newly identified mechanism might have large significance during conditions of muscle stress. Under normal conditions, MARP1 is expressed at very low levels. However, during periods of muscle stress, such as diaphragm inactivity in mechanically ventilated patients in the intensive care unit, MARP1 is massively up-regulated (Van der Pijl et al., 2021). This MARP1-induced locking of titin-N2A to actin and the resulting increase in passive force might be a mechanism to stabilize sarcomeres and protect them from mechanical stress.

### Future directions

First, future studies should address whether in cardiac muscle MARP1 plays a similar role as in skeletal muscle. Compared with skeletal muscles, the role of the locking mechanism might be more finely tuned in the heart. The heart has easily detectable MARP1 levels, which increase in cardiomyopathies (Arber et al., 1997; Bang et al., 2014). Thus, titin-N2A locking to F-actin in cardiomyocytes might significantly impact diastolic function by affecting ventricular filling. However, in human left ventricles, ~60% of the titin molecules do not contain the N2A element (Cazorla et al., 2000), which might limit the effect of this mechanism on ventricular structure and function. Second, are there other "third parties" that can lock titin-N2A to F-actin? Future studies should focus on MARP2 (ANKRD2/ARRP), which shares  $\geq 50\%$  homology with MARP1. Similar to MARP1, MARP2 can bind to titin-N2A and in unstressed muscles the levels of MARP2 are much higher than those of MARP1 (Wette et al., 2017). Whether MARP2 is also capable of locking titin-N2A to the sarcomeric thin filament should be tested. Third, the mechanical relevance of the interaction between titin-N2A and F-actin should be established. Does this interaction indeed



Figure 1. **Schematic representation of MARP1 locking titin-N2A to the thin filament.** In the "free state" the N2A-segment (region spanning Ig-like [I; red domains] domains 80–83) is unbound to the actin-based thin filament. Under stress conditions, muscles up-regulate MARP1 (black, coiled-coil domain; orange, ankyrin repeats), which binds to the N2A unique sequence (Us; blue) and I81. The N2A-MARP1 complex forms a molecular "glue" that binds to the actin-based thin filament (magenta), locking part of titin's I-band spring. This "locked state" increases the extension of titin's PEVK domain (in yellow, flanked by I83 and I84) during sarcomere stretch, thereby increasing the passive force generated by titin.

stabilize sarcomeres and prevent damage? Are muscles in which this mechanism is abolished (for example, by deletion of titin-N2A and/or MARP1) more susceptible to damage caused by muscle stress such as inactivity, denervation, or ventricular volume overload?

In summary, the manuscript by Stronczek et al. (2021) provides important new information on the ever-expanding role of titin in striated muscle. Titin-N2A might play an important mechanical role in striated muscle by locking to F-actin and increasing the extension of the PEVK segment, and thereby passive force, during muscle stretch. In combination with the data from Zhou et al. (2021) and Van der Pijl et al. (2021), the current manuscript reveals that the titin-N2A is unlikely to interact with the actin-based thin filament, but that with the help of a third protein (MARP1) it can effectively lock titin to the thin filament (Fig. 1).

### Acknowledgments

Henk L. Granzier served as editor.

Research reported in this work was supported by a grant from the National Institutes of Health/National Heart, Lung, and Blood Institute (R01HL121500, to C.A.C. Ottenheijm).

The authors declare no competing financial interests.

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