The N2A region of titin has a unique structural configuration

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The N2A segment of titin is a main signaling hub in the sarcomeric I-band that recruits various signaling factors and processing enzymes. It has also been proposed to play a role in force production through its Ca^{2+}-regulated association with actin. However, the molecular basis by which N2A performs these functions selectively within the repetitive and extensive titin chain remains poorly understood. Here, we analyze the structure of N2A components and their association with F-actin. Specifically, we characterized the structure of its Ig domains by elucidating the atomic structure of the I81-I83 tandem using x-ray crystallography and computing a homology model for I80. Structural data revealed these domains to present heterogeneous and divergent Ig folds, where I81 and I83 have unique loop structures. Notably, the I81-I83 tandem has a distinct rotational chain arrangement that confers it a unique multi-domain topography. However, we could not identify specific Ca^{2+}-binding sites in these Ig domains, nor evidence of the association of titin N2A components with F-actin in transfected C2C12 myoblasts or C2C12-derived myotubes. In addition, F-actin cosedimentation assays failed to reveal binding to N2A. We conclude that N2A has a unique architecture that predictably supports its selective recruitment of binding partners in signaling, but that its mechanical role through interaction with F-actin awaits validation.

Introduction

Titin contains several mechanosignaling hubs that act to scaffold and organize mechanoresponsive signaling elements (Henderson et al., 2017). One of these, the N2A component, is located within the I-band region of titin, immediately N-terminal to the PEVK spring (Fig. 1 A; LeWinter and Granzier, 2010). Titin is differentially spliced into different isoforms with and without the N2A component. The titin N2A isoform, which takes its name from this element, is the predominant isoform in skeletal muscle cells. However, the N2A element is also present in the titin N2BA isoform expressed in cardiac muscle (Cazorla et al., 2000). The N2A region is composed of four Ig domains (I80, I81, I82, and I83) and a unique sequence of ~100 residues (UN2A) inserted between domains I80 and I81 (Fig. 1 A). The UN2A insert has been found to have an α-helical structural content (Zhou et al., 2016; Tiffany et al., 2017). Titin N2A acts as a protein interaction node that recruits several regulatory proteins and stress response factors to the sarcomere. Of these, the interaction with muscle ankyrin repeat proteins (MARPs) is the best characterized to date, in particular with the cardiac ankyrin repeat protein (CARP) member of this family, which has been shown to bind the UN2A-I81 fraction of N2A (Miller et al., 2003; Lun et al., 2014; Zhou et al., 2016). Additionally, the SET and MYND-containing lysine methyltransferase 2 (SMYD2) has also been identified as a UN2A binder (Donlin et al., 2012). The P94/calpain3 protease binds I80-UN2A and also across I82-I83 stretching into the PEVK region (Hayashi et al., 2008). Currently, the molecular basis of these titin interactions and their regulatory principles remain poorly characterized.

Titin N2A is also suspected to play a mechanical role in the sarcomere. The titin protein is known to contribute passive force to the sarcomere and to maintain its structural integrity (Granzier and Labeit, 2004). Titin’s role in passive force resides in the extensibility of its I-band region, where first its tandem-Ig section and then its PEVK region extend serially in function of stretch (Trombitás et al., 1998). More recently, titin has been thought to also play a role in active force development, possibly by intervening in length-dependent activation in a calcium-dependent manner (Hessel et al., 2017). The exact mechanisms by which titin might lead to an increase in length-dependent...
active force production remain a subject of debate. In this re-
spect, titin-based passive stiffness has been shown to affect the
calcium sensitivity of force through myofilament lattice spacing
effects, whereby the structure of the thick filaments is altered,
affecting the activation of cross-bridges and the production of
active force (Cazorla et al., 2001; Lee et al., 2013). The molecular
mechanisms underlying this phenomenon are not fully under-
stood, although it has been proposed that titin could form a
calcium-dependent association with actin to oppose shortening
during contraction (Hessel et al., 2017). In this regard, the
calcium-dependent association of titin with both filamentous
actin (F-actin) and reconstituted thin filaments has been ob-
served (Kellermayer and Granzier, 1996). Molecularly, titin’s
PEVK region has been shown to associate with F-actin, thereby
elevating passive tension in skinned myofibrils (Yamasaki et al.,
2001). PEVK is highly charged and minimally structured, being
known to bind calcium, which regulates its molecular spring
properties (Labeit et al., 2003). Lately, the titin N2A component
has also been proposed to form a calcium-enhanced association
with F-actin based on in vitro
, single-molecule, atomic force
spectroscopy data (Dutta et al., 2018; Nishikawa et al., 2019).
This association could affect the extensibility of titin’s I-band
region, in particular its proximal Ig-tandem, by pinning the
N2A to the thin filament. In view of a recent biochemical study
that showed domain 183 to be poorly stable but to become sta-
bilized by calcium (Kelly et al., 2020), it was then hypothesized
that 183 might be central to mediate the calcium-regulated titin-
N2A/F-actin interaction (Nishikawa et al., 2020). A potential
role of 183 in active force production has been further supported
by data from the mdm mouse. These mice contain a deletion that
removes the final two β-strands of domain 183 as well as a small
portion of the PEVK region (Garvey et al., 2002). This results in a

Figure 1. N2A location and Ig domain components of titin N2A with their unique loop features. (A) Left: Location of N2A in the sarcomere. Thin filaments shown in blue; thick filaments in yellow; Z-line grey rectangle. Titin’s tandem domains are orange and the PEVK is grey. Right: Domain composition of the N2A region. (B) Alignment of human titin sequences of N2A Ig domains (180-183) and representative domains from constitutive (9-11) and skeletal (167-169) tandems. BC loops are highlighted in blue, FG β-hairpin in orange, and CD loops in shades of green. (C) Ig domain components of the N2A region. 181, 182, and 183 domains are as elucidated crystallographically in this work; 180 was modeled using i-TASSER (Yang and Zhang, 2015). Ig domains are shown in cartoon representation, with BC, CD, and FG loop features colored as in B. C, C terminus; N, N terminus.
severe muscle dystrophy phenotype (Garvey et al., 2002), with a three- to fourfold decrease in force production during active stretch (Powers et al., 2016; Tahir et al., 2020). The mice also show disruption of the N2A signaling pathways (Garvey et al., 2002; Witt et al., 2004; Huebsch et al., 2005). Given these observations, a need now exists to unravel the molecular basis of titin N2A’s contributions to sarcomere signaling and mechanics.

To determine how the seemingly innocuous, highly conserved Ig domain components of titin N2A can function as a selective protein interaction node, and how this could contribute to the mechanical function of titin, we elucidated the atomic structure of the I81-I83 Ig tandem and examined the model to probe its capability to bind calcium specifically. Further, we tested the existence of a titin-N2A/F-actin interaction using in vitro cosedimentation assays in the presence and absence of calcium, as well as transfection studies in C2C12 myoblastoma cells and C2C12-derived myotubes. The results identified distinct features in the 3-D structure of I81-I83 that may explain its ability to be specifically recognized by the binding partners of the N2A locus. However, we found no evidence of specific calcium-binding sites and also no evidence of a direct association of titin N2A and F-actin. Our findings highlight the unique scaffolding potential of N2A as signaling node, but do not bring support to its direct role in active force production through an association with F-actin.

### Materials and methods

#### Protein production

I81-I83 from human titin (residues 9582–9851; Uniprot accession no. Q8WZ42; to ease structural annotation, residue P9582 is taken as residue P1 when describing the crystal structure) was cloned into the vector pETM-11 (European Molecular Biology Laboratory collection) using NcoI and KpnI restriction sites. The pETM-11 vector adds a His6-tag and a tobacco etch virus cleavage site N-terminally to the inserted gene.

Protein samples were produced in *Escherichia coli* Rosetta (DE3; Merck Millipore) cultivated at 37°C in Luria–Bertani medium supplemented with kanamycin (100 µg/ml) and chloramphenicol (33 µg/ml). At an OD600 = 0.6, expression was induced with 0.5 mM isopropyl-β-d-thiogalactopyranoside, and cultures were grown further overnight at 18°C. Cells were harvested by centrifugation and lysed by sonication in buffer A (40 mM Tris-HCL, pH 8.0, 300 mM NaCl, 20 mM imidazole, and 1 mM dithiothreitol [DTT]) in the presence of an EDTA-free protease inhibitor cocktail (Roche Applied Science). Lysates were clarified by centrifugation (40,000 × g, 4°C, 40 min). I81-I83 was then isolated from the supernatant by Ni2+-affinity chromatography on a Histrap HP 5-ml column (GE Healthcare). Elution used a linear gradient of imidazole (20–500 mM). Next, the affinity tag was removed by overnight incubation with tobacco etch virus protease while in dialysis against buffer A. Tag and protease were removed using subtractive affinity chromatography, and the sample was further dialysed into buffer B (20 mM Tris-HCL, pH 8.0, 100 mM NaCl, and 1 mM DTT). The resultant sample was subjected to size exclusion chromatography on a Superdex S75 16/60 column (GE Healthcare) preequilibrated in buffer B. The purity of the sample obtained was assessed to be 99% by SDS-PAGE. The sample was flash-frozen in liquid nitrogen and stored at −80°C until further use.

Recombinant UN2A-I81 (residues 9472–9581; UniprotKB Q8WZ42) was produced as described above for I81-I83, except that the buffer system used was 25 mM HEPES, pH 7.5, with no DTT.

#### Crystal structure determination

I81-I83 was crystalized at 20°C on 96-well Intelliplates (Art Robbins Instruments) using sitting drops (400 nl total drop volume) containing equal volumes of protein (31 mg/ml) and reservoir solutions (25% [wt/vol] polyethylene glycol 3350, 100 mM bis-Tris, pH 5.5, and 200 mM lithium sulfate). For x-ray data collection, crystals were cryoprotected with mother liquor supplemented with 30% [vol/vol] ethylene glycol before vitrification in liquid nitrogen. X-ray diffraction data were collected at the Swiss Light Source synchrotron and processed using the XDS suite (Kabsch, 2010). Molecular replacement was conducted with Phaser (McCoy et al., 2007) using a version of the single Ig domain I81 (Protein Data Bank [PDB] accession no. 5JOE; Zhou et al., 2016) as search model, where BC and FG loops had been manually truncated. The search model corresponded to one twelfth (∼8%) of the crystallographic asymmetric unit. Initial automated model building was performed using the warpNtrace routine within ARP/wARP (Lamzin et al., 2012). Manual model building was performed in Coot (Emsley and Cowtan, 2004), and refinement used PHENIX (Adams et al., 2010). Refinement included noncrystallographic symmetry restraints and per-domain Translation/Libration/Screw (TLS) model of rigid-body harmonic displacements treatment. Model quality was assessed using MolProbity (Williams et al., 2018). Diffraction data and model statistics are given in Table 1. X-ray diffraction images have been deposited in Zenodo (Fleming, 2021). Structure factor amplitudes and model coordinates are deposited in the PDB under accession no. 7AHS.

#### Bioinformatic analysis of I81-I83 crystal structure

To identify possible calcium binding sites in I81-I83, chain A of the crystal structure was stripped from solvent atoms and analyzed using the IonCon (Hu et al., 2016) and Metsite (Sodhi et al., 2004) algorithms and the search restricted to calcium ions. For Metsite, false rate cutoffs of 1, 5, 10, and 20% were tested. Metsite uses an artificial neural network trained using all protein chains interacting with the specified metal ions from the PDB and clustering at a 25% sequence identity. The following classifying features were used to train the artificial neural network: sequence profile information, secondary structure, solvent accessibility, and distance matrices of site residues. Ioncon uses a support vector machine trained on sequence profiles, local structure properties (including solvent accessibility), and position- and segment-specific conservation scores of a nonredundant set of ion-binding proteins from the BioLiP database (Yang et al., 2013), which have a pairwise sequence identity <30%. Although there is some overlap between the input classifier types, and possibly training datasets, each approach uses its own machine learning technique and weightings and
Actin cosedimentation

Actin was prepared from rabbit back muscle (Spudich and Watt, 1971). G-actin was centrifuged at 100,000 g for 30 min, and the supernatant was polymerized in 50 mM NaCl, 20 mM MOPS, pH 7.0, and 5 mM MgCl₂. Titin I81-I83 and UN2A-I81 samples were centrifuged at 100,000 g for 30 min immediately before the assay. Titin UN2A-I81 or I81-I83 (20 µM) was added to actin (10 µM). Assay buffers were 50 mM NaCl, 20 mM MOPS, pH 7.0, and 5 mM MgCl₂, with either 2 mM CaCl₂ or 2 mM EGTA. Samples were incubated at 23°C for 1 h and centrifuged at 100,000 g for 30 min. Supernatant and pellet fractions were analyzed by SDS-PAGE with 12% acrylamide gels stained with Coomassie Brilliant Blue-G-250.

**Protein expression and localization in C2C12 cells**

For cellular studies, human titin fragments 180-UN2A-I81 (residues 9353–9671; UniprotKB accession no. Q8WZ42) and I81-I83 (residues 9582–9851; UniprotKB accession no. Q8WZ42) were amplified by PCR and subcloned in frame into mammalian expression vectors encoding fluorescent proteins N-terminally to the target insert, pEGFP-C1 (Clontech) or mCherry-C1. In the latter plasmid, the GFP encoded in pEGFP-C1 was replaced by mCherry. All constructs were confirmed by sequencing.

Mammalian expression constructs were then transfected into C2C12 cells (American Type Culture Collection) using Lipofectamine 2000 (Thermo Fisher Scientific) as previously described (Lange et al., 2012). Following 2 or 7 d of differentiation, cells were fixed with 4% paraformaldehyde in PBS for 5 min at room temperature. Cells were permeabilized using 1× PBS supplemented with 0.2% Triton X-100 for 5 min, and subsequently stained with a knockout-validated primary antibody against the sarcomeric protein obscurin (region covering IQ-Ig64; Blondelle et al., 2019) for 2 h at room temperature. Following three washes with 1× PBS for 5 min each, cells were stained for 1 h with a rabbit secondary antibody linked to either Alexa-488 or Alexa-594 (Jackson ImmunoResearch; 711-545-152 and 711-585-152) and Alexa-647 phalloidin (Thermo Fisher Scientific; A22287) dissolved in gold buffer (20 mM Tris-HCl, pH 7.5, 155 mM NaCl, 2 mM ethylene glycol tetraacetic acid, 2 mM MgCl₂, and 5% BSA). Alexa-647 phalloidin stained F-actin. After washing cells three times with 1× PBS, cells were mounted in fluorescent mounting medium (ProLong Gold antifade reagent; Invitrogen) and processed for imaging on a Leica SP5 confocal microscope in sequential scanning mode using a 63× oil-immersion objective and zoom rates between 1 and 4. Images were analyzed using ImageJ and the BioFormats Importer as well as Photoshop (Adobe).

**Results**

The crystal structure of I81-I83 reveals distinct molecular features

The I81-I83 tandem from titin has been proposed to be central to the calcium-dependent association of titin and F-actin at the N2A sarcomeric locus, with domain I83 suspected to be the key mediator of this interaction (Dutta et al., 2018; Nishikawa et al., 2019). As titin is a repetitive multi-Ig chain where component domains are highly homologous, we aimed to reveal the distinct molecular features of I81-I83 that could confer it the ability of mediating specific interactions and of coordinating calcium. For
this, we elucidated the 3-D structure of I81-I83 from human titin to 2.05 Å resolution using x-ray crystallography (Table 1). The crystal used in this study contained four molecular copies of I81-I83 in its asymmetric unit. These copies consistently revealed I81-I83 as a monomeric chain with an extended domain arrangement. Copies of individual domains were essentially identical across the four replicas (RMSD values: I81 = 0.68–0.98; I82 = 0.36–0.40; and I83 = 0.50–0.47). At the tandem level, the molecular copies were also highly similar, revealing only a minor deviation of 35° along the longitudinal molecular axis as a result of crystal packing. Overall, the four crystalline copies were in full agreement.

First, we examined the structure of the individual domains I81, I82, and I83 to assign them to established Ig fold subclasses in titin. Titin Ig domains have been classified into “N-conserved” and “N-variable” types according to the features of the loop cluster at the N-terminal side of their fold (Marino et al., 2005). In brief, N-conserved domains have a lengthened Ig fold characterized by the presence of (i) a PPxX motif at the N-terminal β-strand A; (ii) a lengthened FG β-hairpin housing a conserved NxxG motif; and (iii) an extended BC loop containing proline residues that often form a PPh (h, hydrophobic residue) or PxP motif, where at least one proline adopts a cis conformation. β-strand A, BC loop, and FG β-hairpin cluster tightly at the N-terminal side of the Ig fold, with prolines in β-strand A and BC loop stacking together and the asparagine residue in the NxxG sequence of the FG β-turn forming a crucial hydrogen bond to the BC loop that secures the mutual packing. N-variable Ig domains lack these features and are shorter in length, defined by short BC and FG elements and a β-strand A that is variable in sequence. Canonical representatives of the N-conserved subclass are the Ig domains of the differentially spliced, skeletal I-band tandem (e.g., I65-I70; von Castelmur et al., 2008), while typical members of the N-variable subclass form the constitutive I-band tandem (e.g., I9-I11; Bogomolovas et al., 2016). A structural and sequence comparison of I81, I82, and I83 with classified titin domains of known 3-D structure revealed that I82 is a typical member of the N-variable titin subclass, while I81 and I83 display distinct features in that their BC loops and FG β-turns are mismatched (Fig. 1, A and B). I81 has a long BC loop but a short FG β-hairpin (Zhou et al., 2016), while contrarily I83 has a short BC loop but an extended FG β-hairpin. The different length of these loops in I81 and I83 causes them to no longer pack against each other. This releases the constraints in sequence composition. In I81 the BC loop is atypically long, even one residue longer than the typical N-conserved Ig type. In I83, the FG β-turn now contains an atypical LEPR motif (instead of the canonical NxxG motif), and the short BC loop lacks prolines (Fig. 1 B). In the position of β-strand A, which normally is an integral component of the ABDE β-sheet, both I82 and I83 possess instead a β-bulge that protrudes from the surface of the domain. Whether this feature is also important to allow for recognition of specific protein partners bears further investigation.

To complete the analysis of Ig domain components of titin N2A, we analyzed the sequence of I80 and performed its homology modeling using I-TASSER (Yang and Zhang, 2015) and Phyre-2 (Kelley et al., 2015). Based on sequence features and supported by 3-D models, we concluded that I80 displays canonical features of the N-conserved subclass (Fig. 1, A and B). This is the class of the majority of I-band Igs. Unlike I81 and I83, it does not show novel features.

In summary, our findings reveal that titin N2A is composed of heterogeneous Ig domains, where I80 and I82 belong to N-conserved and N-variable types, respectively, and I81 and I83 present unique, individual loop structures (Fig. 1 B).

### 81-I83 display a unique tandem architecture

In titin, Ig domains are linked in series connected by short linker sequences, thereby forming domain tandems. Available crystal structures of titin Ig tandems (reviewed in Zacharchenko et al., 2015) have revealed that these normally adopt an extended chain conformation, where Ig-Ig interfaces show few direct contacts. Interestingly, 3-D structures also show that the tandems display local rotational order along their longitudinal chain axes. Such rotational order is largely a steric consequence of the BC loop and FG β-hairpin features of the individual ig in the tandem, as such loops form the Ig-Ig interfaces (Fig. 2 A). The skeletal I-band tandem composed of N-conserved Ig domains with elongated BC and FG features shows a characteristic up-down arrangement, where individual Igs are rotated 180° with respect to each other along the chain (Fig. 2, B and C; von Castelmur et al., 2008). On the other hand, the constitutive I-band tandem, which is composed of short N-variable Ig domains, shows slight domain rotations of 45–68° angles (Fig. 2, B and C; Bogomolovas et al., 2016). The N2A tandem I81-I83 shows individual Igs arranged at a 90° angle with respect to one another, which results from the hybrid loop features of its component domains (Fig. 2 C). The interfaces between these domains in N2A are restrained (Fig. 2 A). The I181-2I82 interface shows hydrogen bonds from the domains to the linker (I181-K15 to linker E90 and I182-S115 to linker V116) as well as a hydrophobic patch (I181-L14, I181-S189, and I182-V116) and the hydrophobic patch will energetically prevent the rotation of the interface, while the hydrogen bonds stabilize it. The interface I182-I83 is also restrained with a direct salt bridge (I82-E104 to I83-R258), among other stabilizing hydrogen bonds (I82-R258 to linker P179). Interestingly, this reveals that the N2A tandem not only displays individualized features at the local domain level but also has a unique overall 3-D chain topography that is distinct from that of other Ig tandems in titin’s I-band.

### No specific calcium binding sites can be identified in I81-I83

The crystal structure of I81-I83, which includes its solvent molecules (Table 1), did not reveal any divalent ions according to experimental electron density values. Thus, in order to identify possible calcium binding sites in I81-I83, the topography of its electrostatic surface potential was investigated using bioinformatic tools. For identified negatively charged surface loci, the geometry of surface amino acids was inspected to evaluate their capability to coordinate divalent metals. For the specific coordination of a calcium ion by a protein, the protein surface must offer a negatively charged pocket with a minimum of four planar coordinating atoms. Six coordinating atoms are more commonly observed and eight coordinating atoms are seen in high-affinity calcium binding sites in proteins (Katz et al., 1996). Common calcium-coordinating residues are the side chains of residues Asp and Glu, with Asn, Gln, Ser, and Thr possible but less frequently observed (Hu et al., 2016; Katz et al.,...
Additionally, main chain oxygens and water molecules can contribute to the calcium ion’s coordination (Katz et al., 1996). An examination of the crystal structure of I81-I83 showed that, despite the existence of negatively charged surface regions (red areas, Fig. 3), no surface residue clusters satisfy those requirements, even when considering alternative side-chain rotamer rearrangements. To further confirm the lack of calcium sites, the structure was submitted to two independent structure-based metal binding predictor servers: IonCom (Hu et al., 2016) and Metsite (Sodhi et al., 2004). No calcium bindings sites were found by these servers. We therefore concluded that I81-I83 as here observed is unlikely to bind calcium specifically and with high affinity.

**Titin N2A components do not colocalize with F-actin in C2C12 cells**

To investigate if the particular characteristics of I81-I83 mediate an interaction with F-actin, we studied the localization of the titin N2A constructs I80-UN2A-I81 and I81-I83 in transfected C2C12 muscle cells. For this, I80-UN2A-I81 or I81-I83 fused C-terminally to the fluorescent proteins GFP or mCherry was transfected into C2C12 myoblasts and actin-stained with Alexa-647 phalloidin to investigate the respective subcellular localizations. In cells differentiated for 2 d, I80-UN2A-I81 and I81-I83 both displayed a diffuse localization (Fig. 4 A), with I81-I83 also showing nuclear enrichment and forming a few cytoplasmic puncta (Fig. 4 A, right). Neither titin fragment displayed an association with actin filaments. However, F-actin during early differentiation of C2C12 myoblasts may still incorporate the nonmuscle β-actin isotype, and may not be decorated with sarcomeric proteins seen in the thin filaments of mature muscle fibers. Hence, to account for actin isotype or the need of associated proteins, we investigated the subcellular localization of these titin fragments in C2C12 cells that had been differentiated for 7 d (Fig. 4 B). The differentiated myotubes displayed a typical cross-striated pattern that here was revealed by staining of obscurin and F-actin. Obscurin is positively correlated with progressive myoblast differentiation, and its cross-striated distribution is seen during the assembly of the myofibrillar contractile apparatus (Borisov et al., 2008). However, also in the 7 d...
differentiated cells, I80-UN2A-I81 and I81-83 continued to display a diffuse cytoplasmic localization and did not form striations colocalized with actin that could be indicative of an interaction. Thus, no evidence could be obtained here for the association of the investigated titin N2A region with thin filaments.

**Titin N2A components do not cosediment with F-actin in the presence or absence of calcium**

To further study the lack of titin/F-actin association observed in C2C12 cells, we performed in vitro cosedimentation assays where titin UN2A-I81 and I81-183 components were incubated with F-actin in the presence and absence of calcium (Fig. 5). Here, both UN2A-I81 and I81-183 constructs were produced as recombinant samples in soluble form. Previous data on UN2A-I81 (Zhou et al., 2016) and the crystal structure of I81-183 in this study demonstrate that the samples are monodisperse, stable, and structurally integral. Structural integrity and solubility are important considerations for this assay, as poorly folded proteins are prone to nonspecific interactions and aggregation, resulting in false positives.

In this assay, soluble protein samples that do not interact with actin remain in the supernatant fraction upon high-speed centrifugation (100,000 g), while the large actin filaments sediment. Upon binding to F-actin, proteins that would otherwise remain in the soluble fraction after centrifugation will cosediment and appear in the pellet. In our control assays, we observed that single titin samples (in the absence of actin) show mild sedimentation behavior (Fig. 5). However, neither construct increases its basal level of sedimentation in the presence of F-actin, regardless of the presence or absence of calcium. Our cosedimentation data indicate that these titin-N2A segments do not associate with F-actin in vitro, thereby confirming the results in C2C12 cells.

**Discussion**

Titin N2A is a signaling hub that selectively recruits protein factors in the sarcomeric I-band. Proteins known to associate directly with N2A are MARPs, SMYD2, and P94/calpain3 (Hayashi et al., 2008; Miller et al., 2003; Lun et al., 2014; Voelkel...
HSP90 (Voelkel et al., 2013) are inert. The leading hypothesis is that to achieve binding specificity, domain components of titin are highly conserved, and their fold presents only small local differences, mostly in loop regions. Thus, the question remains of how Ig domains in I-band signaling loci have undergone (1) individualization as to display distinct local features in their fold. This fold diversification is often accompanied by (2) the development of multi-domain binding sites, where domains are packed serially along the titin chain with a distinct relative orientation that leads to a unique rotational architecture of the titin chain at that locus (Zacharchenko et al., 2015). These strategies are illustrated by the 3-D structures of the dual Ig-tandem Z1Z2 that binds telethonin in the Z-disc (Zou et al., 2006) and the A168-A170 tandem that recruits MuRF1 to M-line titin (Mrosek et al., 2007). The unique features of I81-I83, with its heterogeneous Ig components and distinct rotational chain order, further support this hypothesis. These findings confirm that the chain context of titin domains and the influence of loops and linkers on the chain architecture need to be considered to understand titin function in health and disease. For example, the analysis of inter-domain motions and features has been shown to be important when assessing the damage potential of single nucleotide variants (Bogomolovas et al., 2016; Fleming et al., 2020), with the recently developed Domain Interface Score a useful parameter for assessing the damage potential of variants in linker regions (Fleming et al., 2020).

A prominent interacting partner proposed for N2A is F-actin (Dutta et al., 2018). This interaction is not expected to play a direct role in signaling, but to be of relevance for the mechanical regulation of the sarcomere (Dutta et al., 2018; Nishikawa et al., 2019, 2020). The hypothesized interaction of F-actin and titin N2A is thought to be enhanced by calcium (Dutta et al., 2018). Recent work showed that domain I83 has a notably reduced fold stability compared with I81 and I82, but that its stability increases in the presence of calcium (Kelly et al., 2020). This points to the possibility of I83 hosting a calcium-binding site, which could mediate a calcium-dependent association with actin. The elucidation of the crystal structure of I81-I83 in this work reveals the fold boundaries for these Ig domains, and sets the C terminus of I83 at residue T9851. By comparison, the N2A and I83 samples used in previous studies (Dutta et al., 2018; Kelly et al., 2020) were C-terminally truncated, missing three terminal residues in I83. The truncation compromised β-strand G that packs against β-strand A’, sealing together the N and C termini of the domain fold. Truncations of just two to four terminal residues in titin Ig domains are known to result in notable domain destabilization, as exemplified by the truncations of MS (Politou et al., 1994; Pfuhl et al., 1997) and I10 (Bogomolovas et al., 2016). It is thus possible that the instability of I83 previously reported might originate from the truncation of its β-strand G, even more so as I83 does not contain well-formed β-strands A and A’ capping the β-strand G region. Furthermore, based on an analysis of the crystal structure, we found no molecular evidence for the existence of a specific calcium-binding site in I81-I83. This suggests that calcium effects on these Ig domains might be unspecific and result from the predominantly negatively charged surface of the domains, especially I83, which has the lowest pI value (pI = 5.05). This value is similar to that of Ig domain I27 (pI = 4.94), which is well characterized structurally and does not host specific metal-binding sites, but is stabilized by unspecific calcium interactions (DuVall et al., 2013).
Beyond the domain level, it cannot be ruled out, however, that a large-scale chain rearrangement could cause the creation of a cryptic metal binding site between domains, as observed in a highly infrequent conformation captured in the crystal structure of the Z-line titin tandem Z1Z2, where the domains fold onto themselves, forming a tight V shape stabilized by a metal ion sandwiched in between (Marino et al., 2006). Future studies will be required to explore the putative existence of such complex scenario.

In this work, we attempted to validate the N2A/F-actin interaction using both recombinant soluble samples in vitro and transfected C2C12 myoblasts. Under our experimental conditions, we did not observe an interaction between titin-N2A components and F-actin, independently of the presence or absence of calcium. The data show that I83 is not a critical mediator of actin binding. Our results agree with findings from an early study that assessed the competitive binding of the I79-I80-UN2A-I81-I82 segment to actin in the sarcomere by monitoring myofibril stiffness (Linke et al., 1997). However, our results did not concur with those of a recent study (Dutta et al., 2018) that used recombinant I80-UN2A-I81-I82-I83 extracted from insoluble inclusion bodies in cosedimentation studies. Inclusion body extraction is a difficult method to obtain soluble protein free of fold defects and without a tendency to aggregate unspecifically (Humer and Spadiut, 2018). In the actin cosedimentation assay reported (Dutta et al., 2018), the pelleting behavior of the isolated titin sample was not tested as a comparative control; therefore, the tendency of that sample to pellet and to associate unspecifically cannot be evaluated. Alternatively, a lack of cosedimentation in our work might result from the shorter length of our constructs or the absence of domain I80. However, the presence of I80, which has canonical structural features and lacks individualized elements, did not induce actin colocalization in C2C12-derived myotubes. Thus, this domain per se does not appear deterministic of actin binding. Future investigations will be required to clarify the existence of a potential titin-N2A/F-actin interaction in the sarcomere.

In conclusion, our data reveal distinct features in titin N2A components that might underpin their selective role in signaling. However, the findings do not bring up evidence to support its proposed mediation in active force production through an association with actin.

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References

Adams, P.D., P.V. Afonine, G. Bunkóczi, V.B. Chen, I.W. Davis, N. Echols, J.J. Headd, L.-W. Hung, G.J. Kapral, R.W. Grosse-Kunstleve, et al. 2010. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66:212-218. https://doi.org/10.1107/S0907444909052925

Adams, M., J.R. Fleming, E. Riehle, T. Zhou, T. Zacharchenko, M. Markovic, and O. Mayans. 2019. Scalable, Non-denaturing Purification of Phosphoproteins Using Ga3+-IMAC: N2A and MIM2 Titin Components as Study case. Protein. J. 38:181-189. https://doi.org/10.1007/s10930-019-09815-w

Bang, M.L., T. Centner, F. Fornoff, A.J. Geach, M. Gotthardt, M. McNabb, C.C. Marino, M., P. Zou, D. Svergun, P. Garcia, C. Edlich, B. Simon, M. Wilmanns, et al. 2014. Single-Molecule Force Spectroscopy on Titin: N2A domain. Acta Crystallogr. D Biol. Crystallogr. 66:125-132. https://doi.org/10.1107/S0907444913044774

Bartunik, A.O., M.O. Raeker, and M.W. Russell. 2008. Developmental expression and differential cellular localization of obscurin and obscurin-associated kinase in cardiac muscle cells. J. Cell. Biochem. 103:1621-1635. https://doi.org/10.1002/jcb.21155

Cazorla, O., A. Freiburg, M. Helmes, T. Centner, M. McNabb, Y. Wu, K. Taguchi, M. Yanagida, S. Hirner, et al. 2008. Multiple molecular interactions implicate the connectin/titin N2A region as a modulating scaffold for p94/calpain 3 activity in skeletal muscle. J. Biol. Chem. 283:14801-14814. https://doi.org/10.1074/jbc.M708262200

Cazarl, A., J. Leventhal, and C.C. Nishikawa. 2018. Overview of the muscle cytoskeleton. Compr. Physiol. 7:891-944. https://doi.org/10.1002/cphy.c160033

Hesse, A.L., S.L. Lindstedt, and K.C. Nishikawa. 2017. Physiological Mechanisms of Isometric Contraction and Its Applications: A Role for the Giant Titin Protein. Front. Physiol. 8:70. https://doi.org/10.3389/fphys.2017.00070

Hu, X., Q. Dong, J. Yang, and Y. Zhang. 2016. Recognizing metal and acid radical ion-binding sites by integrating ab initio modeling with template-based transfers. Bioinformatics. 32:3260-3269. https://doi.org/10.1093/bioinformatics/btw396

Huebsch, K.A., E. Kudryashova, C.M. Wooley, R.B. Sher, K.L. Seburn, M.J. Spencer, and G.A. Cox. 2005. Mdm muscular dystrophy: interactions with calpain 3 and a novel functional role for titin’s N2A domain. Hum. Mol. Genet. 14:2801-2811. https://doi.org/10.1038/hmg/dd1311

Humer, D., and O. Spadlut. 2018. Wanted: more monitoring and control during inclusion body processing. World J. Microbiol. Biotechnol. 34:158. https://doi.org/10.1007/s11274-018-32952-8

Kabsch, W. 2010. Software XDS for image rotation, recognition and crystal symmetry assignment. Acta Crystallogr. D Biol. Crystallogr. 66:125-132. https://doi.org/10.1107/S0907444913044774

Katz, A.K., J.P. Glusker, S.A. Beebe, and C.W. Bock. 1996. Calcium ion coordination: A comparison with that of beryllium, magnesium, and zinc. J. Am. Chem. Soc. 118:5752-5763. https://doi.org/10.1021/ja95343i

Kellermayer, M.Z.S., and H.L. Granzier. 1996. Calcium-dependent inhibition of in vitro thin-filament motility by native titin. FEBS Lett. 380:281-286. https://doi.org/10.1016/0014-5793(96)00555-5

Kelley, L.A., S. Mezulis, C.M. Yates, M.N. Wass, and M.J.E. Sternberg. 2005. The Phyre2 web portal for protein modeling, prediction and analysis. Nat. Protoc. 10:845–858. https://doi.org/10.1038/nprot.2015.053

Kelly, C.M., S. Manukian, E. Kim, and M.J. Gage. 2020. Differences in stability and calcium sensitivity of the Ig domains in titin’s N2A region. Protein Sci. 29:1160-1171. https://doi.org/10.1002/pro.3848

Labeit, D., K. Watanabe, C. Witt, H. Fujita, Y. Wu, S. Lahmers, T. Funck, S. Labeit, and H. Granzier. 2003. Calcium-dependent molecular spring elements in the giant protein titin. Proc. Natl. Acad. Sci. USA:100:13716-13721. https://doi.org/10.1073/pnas.2335652100

Lamain, V.S., A. Perrakis, and K.S. Wilson. 2012. ARP/wARP – automated model building and refinement. In International Tables for Crystallography, C.P. Brock, T. Hahn, W. W contaletsche, U. Müller, U. Shmueli, E. Prince, A. Authier, V. Kopsky, D.B. Litvin, E. Arnold, et al. Editors. Hoboken, New Jersey. https://doi.org/10.1002/j.1097-0282.2006.000862

Lange, S., L. Perera, P. Teh, and J. Chen. 2012. Obscurin and CRD6 regulate cullin-dependent small ankyrin-1 (sAnk1) protein turnover. Mol. Biol. Cell. 23:2490-2504. https://doi.org/10.1091/mbc.E12-01-0052

Lanzicher, T., T. Zhou, C. Saripalli, V. Keschrumrus, J.E. Smith III, O. Mayans, V. Sbaizero, H. Granzier. 2020. Multiple molecular interactions implicate the connectin/titin N2A region as a modulating scaffold for p94/calpain 3 activity in skeletal muscle. J. Biol. Chem. 283:14801-14814. https://doi.org/10.1074/jbc.M708262200

LeWinter, M.M., and H. Granzier. 2010. Cardiac titin: a multifunctional giant. Circulation. 121:2137-2145. https://doi.org/10.1161/CIRCULATIONAHA.109.860171

Linke, W.A., M. Ivmeyem, S. Labeit, H. Hinsen, J.C. Ruegg, and M. Gautel. 1997. Actin-titin interaction in cardiac myofibrils: probing a physiological role. Biochem. J. 326:905–919. https://doi.org/10.1042/0006-2957(97)01781-2

Lun, A.S., J. Chen, and S. Lange. 2014. Probing muscle ankryin-repeat protein (MARp) structure and function. Anat. Rec. (Hoboken). 297:1619-1625. https://doi.org/10.1002/ar.22968

Lee, E.J., J. Nedrud, P. Schemmel, M. Gotthardt, T.C. Irving, and H.L. Labeit. 2013. Calcium sensitivity and myofilament lattice structure in titin N2B KO mice. Arch. Biochem. Biophys. 535:76–83. https://doi.org/10.1016/j.abb.2012.12.004

LeWinter, M.M., and H. Granzier. 2010. Cardiac titin: a multifunctional giant. Circulation. 121:2137-2145. https://doi.org/10.1161/CIRCULATIONAHA.109.860171

Linke, W.A., M. Ivmeyem, S. Labeit, H. Hinsen, J.C. Ruegg, and M. Gautel. 1997. Actin-titin interaction in cardiac myofibrils: probing a physiological role. Biochem. J. 326:905–919. https://doi.org/10.1042/0006-2957(97)01781-2

Lun, A.S., J. Chen, and S. Lange. 2014. Probing muscle ankryin-repeat protein (MARp) structure and function. Anat. Rec. (Hoboken). 297:1619-1625. https://doi.org/10.1002/ar.22968

Marino, M., D.I. Svergun, L. Krepplak, P.V. Konarev, B. Maco, D. Labeit, and O. Mayans. 2005. Poly-Ig tandems from I-band titin share extended molecular arrangements irrespective of the distinct features of their modular constituents J. Muscle Res. Cell Motil. 26:355–365.

Marino, M., P. Zou, D. Svergun, P. Garcia, E. Edlich, B. Simon, M. Wilmanns, C. Mühle-Goll, and O. Mayans. 2006. The Ig doublet ZIIZ: a model system for the hybrid analysis of conformational dynamics in Ig tandems from titin. Structure. 14:1437-1447. https://doi.org/10.1016/j.str.2006.07.009
McCoy, A. J., R. W. Grosse-Kunstleve, P. D. Adams, M. D. Winn, L. C. Storoni, and R. J. Read. 2007. Phaser crystallographic software. J. Appl. Cryst. 40: 658–674. https://doi.org/10.1107/S0021889807012206

Miller, M. K., M.-L. Bang, C. C. Witt, D. Labeit, C. Trombitas, K. Watanabe, H. Granzier, A. S. McElhinny, C. C. Gregorio, and S. Leibet. 2003. The muscle ankyrin repeat proteins: CARP, ankrd2/Arp and DARP as a family of titin filament-based stress response molecules. J. Mol. Biol. 333:951–964. https://doi.org/10.1016/j.jmb.2003.09.012

Mroshek, M., D. Leibet, S. Witt, H. Heerklotz, E. von Castelmir, S. Leibet, and O. Mayans. 2007. Molecular determinants for the recruitment of the ubiquitin-ligase MuRF-1 onto M-line titin. FASEB J. 21:1383–1392. https://doi.org/10.1096/fj.06-7644com

Nishikawa, K., S. Dutta, M. DuVall, B. Nelson, M. J. Gage, and J. A. Monroy. 2010. Chirality, J. S. Bryson, L. J. McGuffin, J. J. Ward, L. Wernisch, and D. T. Jones. 2004. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle.

Nishikawa, K., S. L. Lindstedt, A. Hessel, and D. Mishra. 2019. N2A titin: Signaling hub and mechanical switch in skeletal muscle. Int. J. Mol. Sci. 21:3974. https://doi.org/10.3390/ijms21133974

Petersen, E. F., T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, and T. E. Ferrin. 2004. UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25:1605–1612. https://doi.org/10.1002/jcc.20084

Pfuhl, M., S. Improta, A. S. Politou, and A. Pastore. 1997. When a module is also a domain: the role of the N terminus in the stability and the dynamics of immunoglobulin domains from titin. J. Mol. Biol. 265:242–256. https://doi.org/10.1006/jmbi.1996.0728

Politou, A. S., M. Gault, M. Pfuhl, S. Leibet, and A. Pastore. 1994. Immunoglobulin-type domains of titin: same fold, different stability? Biochemistry. 33:4730–4737. https://doi.org/10.1021/bi00181a004

Powers, K., K. Nishikawa, V. Joumara, and W. Herzog. 2016. Decreased force enhancement in skeletal muscle sarcomeres with a deletion in titin. J. Exp. Biol. 219:131–1316. https://doi.org/10.1242/jeb.153202

Sodhi, J. S., K. Bryson, L. J. McGuillin, J. J. Ward, L. Wernisch, and D. T. Jones. 2004. Predicting metal-binding site residues in low-resolution structural models. J. Mol. Biol. 342:307–320. https://doi.org/10.1016/j.jmb.2004.07.019

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin–troponin complex with actin and the proteolytic fragments of myosin. J. Biol. Chem. 246:4866–4871. https://doi.org/10.1016/S0021-9258(18)62016-2

Tahir, U., J. A. Monroy, N. A. Rice, and K. C. Nishikawa. 2020. Effects of a titin mutation on force enhancement and force depression in mouse soleus muscles. J. Exp. Biol. 223:jeb197038. https://doi.org/10.1242/jeb.197038

Tiffany, H., K. Sonkar, and M. J. Gage. 2017. The insertion sequence of the N2A region of titin exists in an extended structure with helical characteristics. Biochim. Biophys. Acta. Proteins Proteomics. 1865:1–10. https://doi.org/10.1016/j.bbapap.2016.10.003

Trombitas, K., M. Greaser, S. Leibet, J. P. Jin, M. Kellermayer, M. Helmes, and H. Granzier. 1998. Titin extensibility in situ: entropic elasticity of permanently folded and permanently unfolded molecular segments. J. Cell Biol. 140:853–859. https://doi.org/10.1083/jcb.140.4.853

Voelkel, T., C. Andresen, A. Unger, S. Just, W. Rottbauer, and W. A. Linke. 2013. Lysine methyltransferase Smyd2 regulates Hsp90-mediated protection of the sarcomeric titin springs and cardiac function. Biochim. Biophys. Acta. 1833:812–822. https://doi.org/10.1016/j.bbamcr.2012.09.012

von Castelmir, E., M. Marino, D. J. Svergun, L. Kreplak, Z. Uçurtum-Fotiadis, P. V. Konarev, A. Urzhumtsev, D. Leibet, S. Leibet, and O. Mayans. 2008. A regular pattern of Ig super-motifs defines segmental flexibility as the elastic mechanism of the titin chain. Proc. Natl. Acad. Sci. USA. 105:1186–1191. https://doi.org/10.1073/pnas.0707163105

Williams, C. J., J. J. Heald, N. W. Moriarty, M. G. Prisant, L. L. Videau, L. N. Deis, V. Verma, D. A. Keedy, B. J. Hintze, V. B. Chen, et al. 2018. MolProbity: More and better reference data for improved all-atom structure validation. Protein Sci. 27:293–315. https://doi.org/10.1002/pro.3330

Witt, C. C., Y. Ono, E. Puschmann, M. McNabb, Y. Wu, M. Gotthardt, S. H. Witt, M. Haak, D. Leibet, C. C. Gregorio, et al. 2004. Induction and myofilibrillar targeting of CARP, and suppression of the Nkx2.5 pathway in the MDM mouse with impaired titin-based signaling. J. Mol. Biol. 336:145–154. https://doi.org/10.1016/j.jmb.2003.12.021

Yamaski, R., M. Berri, Y. Wu, K. Trombitas, M. McNabb, M. S. Z. Keller- mayer, C. Witt, D. Leibet, S. Leibet, M. Greaser, and H. Granzier. 2001. Titin–actin interaction in mouse myocardium: passive tension modulation and its regulation by calcium/S100A1. Biochem. Biophys. J. 81:2297–2313. https://doi.org/10.1021/bi050876-6

Yang, J., and Y. Zhang. 2015. i-TASSER server: new development for protein structure and function predictions. Nucleic Acids Res. 43(W1): W174–W181. https://doi.org/10.1093/nar/gkv342

Yang, J. A. Roy, and Y. Zhang. 2013. BioLIP: a semi-manually curated database for biologically relevant ligand-protein interactions. Nucleic Acids Res. 41(Database issue, D1): D1095–D1103. https://doi.org/10.1093/nar/gks966

Zacharchenko, T., E. von Castelmir, D. J. Rigden, and O. Mayans. 2015. Structural advances on titin: towards an atomic understanding of multi-domain functions in myofilament mechanics and scaffolding. Biochem. Soc. Trans. 43:850–855. https://doi.org/10.1042/BST20150084

Zhou, T., J. R. Fleming, B. Franke, J. Bogomolovas, I. Barsukov, D. J. Rigden, S. Leibet, and O. Mayans. 2016. CARP interacts with titin at a unique helical N2A sequence and at the domain Ig1 to form a structured complex. FEBS Lett. 590:3098–3110. https://doi.org/10.1016/j.febslet.2016.04.037

Zou, P., N. Finotsis, S. Lange, Y.-H. Song, A. Popov, I. Mavridis, O. M. Mayans, M. Gault, and M. Wilmanns. 2006. Palindromic assembly of the giant muscle protein titin in the sarcomeric Z-disc. Nature. 439:229–233. https://doi.org/10.1038/nature04343