Calcium increases titin N2A binding to F-actin and regulated thin filaments

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Mutations in titin are responsible for many cardiac and muscle diseases, yet the underlying mechanisms remain largely unexplained. Numerous studies have established roles for titin in muscle function, and Ca²⁺-dependent interactions between titin and actin have been suggested to play a role in muscle contraction. The present study used co-sedimentation assays, dynamic force spectroscopy (DFS), and in vitro motility (IVM) assays to determine whether the N2A region of titin, overlooked in previous studies, interacts with actin in the presence of Ca²⁺. Co-sedimentation demonstrated that N2A – F-actin binding increases with increasing protein and Ca²⁺ concentration, DFS demonstrated increased rupture forces and decreased k off in the presence of Ca²⁺, and IVM demonstrated a Ca²⁺-dependent reduction in motility of F-actin and reconstituted thin filaments in the presence of N2A. These results indicate that Ca²⁺ increases the strength and stability of N2A – actin interactions, supporting the hypothesis that titin plays a regulatory role in muscle contraction. The results further support a model in which N2A – actin binding in active muscle increases titin stiffness, and that impairment of this mechanism contributes to the phenotype in muscular dystrophy with myositis. Future studies are required to determine whether titin – actin binding occurs in skeletal muscle sarcomeres in vivo.

Titanopathies, inherited diseases caused by mutations in the titin (TTN) gene, are responsible for diverse cardiac and muscle diseases¹. In the heart, titin mutations are linked to numerous forms of cardiomyopathy²–⁵. In skeletal muscles, titin mutations are associated with a rapidly growing number of disorders ranging from early onset diseases characterized by muscle degeneration and early death to late onset diseases that affect a small set of muscles after years of apparently normal function¹. The underlying mechanisms that produce degenerative phenotypes in titinopathies remain largely unexplained¹.

Since the discovery of titin⁶, many studies have investigated its interactions with other sarcomeric proteins using several different techniques. These studies revealed a variety of interactions in the M-line and Z-disk which highlight the important role of titin as a sarcomeric scaffold for myofibrillogenesis⁷. In the Z-disc, titin Z repeats interact with F-actin, alpha-actinin, and other proteins including telethonin. In the M-line, titin FnIII domains bind to the tail portion of myosin, as well as to MyBP-C and myomesin. Numerous studies have established roles for titin in myofibrillar assembly⁸, myofibrillar passive tension⁹, and hypertrophic signaling¹⁰. In addition to these established roles for titin, Ca²⁺-dependent interactions between titin and actin have long been suggested to play a role in muscle contraction. Experimental evidence supports the existence of Ca²⁺-dependent interactions between I-band titin and actin. Using in vitro motility and binding assays, Kellermayer and Granzier¹¹ found that a T2 fragment of titin (comprising the N2-line to M-line) from rabbit longissimus dorsi interacts with actin filaments and reconstituted thin filaments at pCa < 6.0. Although such interactions have been suggested to increase titin stiffness in active skeletal muscle¹²–¹⁴, this model has been slow to gain wide-spread acceptance¹⁵.

Despite significant efforts, subsequent attempts to discover Ca²⁺-dependent interactions between I-band titin and actin have been largely unsuccessful. Several studies investigated interactions between PEVK titin and actin using constructs based on human soleus (GenBank X90569) and human cardiac titin (GenBank

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concentration was determined using Bradford assays. Purified protein was stored at −20 °C in 50% glycerol.

ble protein was removed by centrifugation and N2A protein was loaded onto a HIS-Trap column (GE Healthcare) in inclusion body using a French press at 20,000 PSI with a 5 min hold before collection of lysate. Remaining insoluble proteins were inhibited by S100A1 when Ca²⁺ was present. Only one study examined interactions between N2A titin (X90569, residues 5510–5507, Ig80-IS-Ig81-Ig82) and actin. Using co-sedimentation and immunofluorescence microscopy, this study found no interactions between titin and F-actin or reconstituted thin filaments outside the Z-disc. Notably, however, the terminal Ig83 domain was not included in this construct.

Analysis of previous studies that investigated interactions between titin constructs and actin demonstrates that no previous studies included a sequence of 115 amino acids (residues 5508–5617 from X90569) at the N2A-PEVK intersection in titin, spanning most of Ig83 and the first 30 amino acids of the proximal PEVK region. Therefore, the goal of the present study was to test the hypothesis that amino acid residues in the N2A region of titin interact with actin filaments in the presence of Ca²⁺. We hypothesized that this previously unexamined region of N2A titin could be responsible for the Ca²⁺-dependent interactions with actin and reconstituted thin filaments observed previously by Kellermayer & Granzier. Additional motivation came from the observations that: 1) an 83 amino acid deletion at the N2A-PEVK intersection in muscular dystrophy with myositis (mdm) mice leads to early muscle degeneration and death; and 2) the mdm mutation prevents the increase in titin stiffness that normally occurs upon activation of wild type muscles, perhaps by disrupting titin-actin interactions. In the present study, we used co-sedimentation, in vitro motility assays (IVM), and dynamic force spectroscopy (DFS) to characterize interactions between an N2A titin construct (Ig80-IS-Ig81-Ig82-Ig83) and F-actin or reconstituted thin filaments in the presence and absence and Ca²⁺.

Methods

Expression and Purification of N2A construct. An N2A construct (Fig. 1) containing an N-terminal HIS-tag and two N-terminal cysteine residues was synthesized and subcloned into a pET 100/D plasmid (GeneArt, Biomatik, Regensburg, Germany). Expression was performed in BL21 (DE3) cells using auto-inducing media and overnight incubation at 30 °C with shaking at 300 rpm. Expressed N2A protein was found in the inclusion body. Induced cells were harvested and re-suspended in PBS with lysozyme, DNase, MgCl₂, Triton X-100 and protease inhibitors. Re-suspended cells were freeze-thawed and sonicated. The inclusion body was harvested by centrifugation and the process was repeated three times, including 0.375 M urea in the third wash to remove non-specifically associated proteins. Following centrifugation, the pellet was re-suspended in chromatography buffer (20 mM Tris, 150 mM NaCl, 25 mM imidazole, 0.1% Tween-20). N2A protein was released from the inclusion body using a French press at 20,000 PSI with a 5 min hold before collection of lysate. Remaining insoluble protein was removed by centrifugation and N2A protein was loaded onto a HIS-Trap column (GE Healthcare) and eluted using 150 mM imidazole. Purity was assessed using Coomassie stained SDS-PAGE gels and protein concentration was determined using Bradford assays. Purified protein was stored at −20 °C in 50% glycerol.

Actin Co-Sedimentation Assay. The N2A construct and F-actin were diluted in 25X reaction buffer (RB: 2.5 mM ATP, 5 mM DTT, 2.5 mM CaCl₂, 25 mM MgCl₂, 2.5 M NaCl and 50 mM Tris, pH 8) to a final concentration of 1X and 2 µM F-actin was mixed with three concentrations of N2A (2, 3, or 5 µM) in the presence of Ca²⁺ (pCa = 4). The assays were repeated in the absence of Ca²⁺ (pCa = 10) as a negative control for N2A binding. Ca²⁺-free samples were created by adding 1 mM EGTA (final concentration). Co-sedimentation assays were repeated three times for each combination of [Ca²⁺] and [N2A] for a total of 18 samples. Samples were incubated for 1 hr at 4 °C and F-actin was sedimented by centrifugation at 30,000 g for 30–45 min. Pelleted F-actin was washed with 1X RB to remove non-specifically bound protein and pelleted F-actin was solubilized using 8 M urea + 1 mM β-ME for >2.5 hours. Sample volumes were equalized using 1X RB and samples were separated on a 10% SDS-PAGE gel. Densitometry was performed using the rolling-ball algorithm on ImageJ. Two-way analysis of variance (ANOVA) was used to determine significance of effects of [Ca²⁺], [N2A], and their interaction on titin-actin binding as estimated from gel densitometry (α = 0.05).

Figure 1. (a) Schematic of experimental setup showing N2A construct (lg domains in red) attached to the AFM tip interacting with F-actin paracrystals on the surface. (b) Schematic of the N2A clone used in these studies. The position of the HIS-tag (HHHHHH) and cysteine residues (CC) are shown at the N-terminus. Numbers at the domain boundaries indicate the amino acid sequence of the clone based on annotation of NP_035782 in the NCBI database.
Titin attachment to AFM tip. N2A constructs were attached to AFM tips (Fig. 1) using standard procedures for thiol-maleimide crosslinking\textsuperscript{26,27}. Silicon nitride AFM tips (MSNL, Bruker Inc., \(k = 30–40\) pN/nm) were amino (APTES) functionalized following a previously published protocol\textsuperscript{28}. Briefly, tips were treated with 30 µL of APTES and 10 µL triethylamine-TEA solutions inside a desiccator for 2 hours. After amino-functionalization, tips were treated with N-\(\gamma\)-maleimidobutyryl-oxysuccinimide ester before attachment. AFM tips were incubated overnight with thiol de-protected N2A constructs (7 nM) in PBS buffer (1X, pH 7.4) inside a humid chamber at 4°C. Cross-linking was achieved through covalent-bond formation between the sulfhydryl groups present in the two N-terminal cysteines on the N2A-construct and the maleimide functionalized AFM tip.

Polymerization and deposition of F-actin on lipid bilayer surface. To obtain small unilamellar lipid vesicles, a 1:1 mix of dried 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and 1,2-dipalmitoyl-sn-glycero-3-ethylphosphocholine was hydrated in buffer (10 mM Tris, 100 mM NaCl, 3 mM CaCl\(_2\), pH 7.4) at 1 mM final lipid concentration, followed by sonication for 10 min. A charged lipid bilayer was formed on a freshly cleaved mica surface by incubating at 55°C for 20 min in a hydrated environment\textsuperscript{29}. On the day of each experiment, globular actin (Cytoskeleton, Inc.) was polymerized to F-actin in buffer (5 mM Tris, 1 mM ATP, 0.5 mM CaCl\(_2\), 0.5 mM DTT, pH 8.0). F-actin was attached to the lipid bilayer surface by incubating for 30 min and stability was verified using AFM imaging (Fig. 2). F-actin polymerized using this approach was stable for ~3 hours, consistent with previously published results\textsuperscript{29}, avoiding use of non-physiological stabilizing factors, such as phallloidin, that may affect molecular interactions.

Single molecule force spectroscopy (SMFS) and dynamic force spectroscopy (DFS). SMFS and DFS experiments enable direct measurement of molecular interaction forces between N2A constructs and F-actin. All SMFS and DFS experiments were performed using an Asylum atomic force microscope (MFP3D-infinity, Oxford Instruments, Santa Barbara, CA, USA) and silicon nitride cantilevers with stiffness of ~20–30 pN/nm. To verify the structure of N2A constructs, SMFS was performed by pulling the N2A constructs adsorbed on a mica surface in PBS (1X, pH 7.4) and unfolding behavior was observed. SMFS experiments were conducted in probing buffer (1X PBS, 1 mM ATP) in the presence (pCa = 4.0) or absence of Ca\(^{2+}\). Measurements in the absence of Ca\(^{2+}\) were performed by adding 700 µM EGTA (Sigma Aldrich) to the probing buffer to eliminate free Ca\(^{2+}\). Measurements of Ca\(^{2+}\) were performed by adding 700 µM EGTA (Sigma Aldrich) to the probing buffer to eliminate free Ca\(^{2+}\). During experiments, the AFM tip was brought in contact with the surface until a triggering force of 100 pN was applied and held for 0.5 s, allowing the N2A construct to interact with F-actin on the surface\textsuperscript{26}. The tip was then retracted by 300 nm at constant, physiologically relevant velocities between 300–2000 nm/sec\textsuperscript{17}. Probing was performed using multiple approach-retract cycles of the AFM tip over various locations on the lipid bilayer surface.

For each pulling velocity, ~100 force curves showing N2A-actin interactions, identified by polymer-like extension of the N2A constructs, were collected as described previously\textsuperscript{26,27}. Each force curve was fit with the extensible worm-like chain (WLC) model to estimate contour length (L\(_c\)), persistence length (L\(_p\)), and rupture force (F), with L\(_c\) and L\(_p\) as free parameters. Because the insertion sequence allows the construct to behave like a tether, the loading rate could not be determined directly from the experimental data and the apparent loading rate (ALR) for each curve was instead calculated using Eq. 1\textsuperscript{30}:

\[
\frac{1}{r} = \frac{1}{k_v v} \left( 1 + \frac{k_p L_c}{4} \frac{k_b T}{L_p F^3} \right)
\]

where \(r\) is ALR, \(k_v\) is the spring constant of the cantilever, \(v\) is the pulling speed, \(L_c\) is the contour length, \(k_b\) is the Boltzmann constant, \(T\) is room temperature (298 K), \(L_p\) is the persistence length, and \(F\) is the rupture force.
Rupture force values for each experiment were measured, and histograms of rupture force distributions for N2A - F-actin interactions in the absence and presence of Ca²⁺ were plotted. For a rupture force distribution at a given apparent loading rate, the most probable rupture force was calculated by fitting the distribution with a probability density function (PDF)\(^{36,27,30-33}\):

\[
p(F) = \frac{k_{\text{off}}}{k_{\text{off}} + k_{\text{on}}} \left( \frac{F}{k_{\text{off}} T} \right)^{\frac{x_B}{k_{\text{off}} T}} \exp\left( -\frac{F}{k_{\text{off}} T} \right)
\]

where \(k_{\text{off}}\) is the off-rate constant and \(x_B\) is the distance of the transition state from the bound state. While \(k_{\text{off}}\) and \(x_B\) can theoretically be fit using Eq. 2, a more accurate fit is gained by measuring rupture forces at multiple loading rates. The relationship between rupture force and apparent loading rate is given by Eq. 3\(^{34}\):

\[
F = \frac{k_{\text{off}}}{k_{\text{off}} + k_{\text{on}}} \ln \left( \frac{F}{k_{\text{off}} T} \right)
\]

Plotting values of rupture forces corresponding to maxima of PDF distributions against the logarithm of mean apparent loading rate allowed estimation of \(k_{\text{off}}\) and \(x_B\) from linear fits. The most probable rupture forces in the presence and absence of Ca²⁺ were compared using two-tailed T-tests (\(\alpha = 0.05\)), and effects of ALR and [Ca²⁺] on \(k_{\text{off}}\) were tested using covariance (ANCOVA, \(\alpha = 0.05\)), with [Ca²⁺] as the main effect and ALR as the covariable.

**In-Vitro Actin Motility (IVM) Assay.** IVM assays measure the velocity of actin filaments on a substrate of myosin\(^{30}\) and have been used previously to investigate interactions between titin fragments and actin or reconstituted thin filaments\(^{31,36,37}\). Addition of actin binding proteins induces a frictional load on the filaments\(^{39}\), resulting in reduced velocity. In this study, chicken skeletal myosin in myosin buffer (0.3 M KCl, 25 nM BES, 5 mM EDTA, 4 mM MgCl₂, and 0.1 M DTT) was mixed with F-actin in actin buffer (55 mM KCl, 25 nM BES, 5 mM EDTA, 4 mM MgCl₂, and 0.1 M DTT) and 1 mM ATP. Samples were centrifuged at 30,000 g for 25 minutes to remove inactive myosin heads. Flow cells were prepared by treating sterilized cover glass with 1% nitrocellulose and attaching the cover glass to microscope slides using double-sided tape.

Myosin (235 nM) and N2A (475 nM) or BSA (475 nM) were incubated for 1 min in flow cells to allow proteins to bind to the nitrocellulose. Addition of BSA produced controls with the same ratio of myosin to protein in the presence and absence of N2A. Flow cells were washed with BSA (15 µM) in actin buffer to block unoccupied binding sites on nitrocellulose. Non-functional myosin heads were removed by adding 1 µM actin for 2 min and washing with actin buffer plus 1 mM ATP, followed by actin buffer. TRITC-labeled porcine cardiac actin (5 nM) in actin motility buffer (40 mM KCl, 25 mM BES, 5 mM EDTA, 4 mM MgCl₂, and 0.1 M DTT) and 1 mM ATP was centrifuged at 30,000 g for 25 minutes to remove inactive myosin heads. Flow cells were prepared by treating sterilized cover glass with 1% nitrocellulose and attaching the cover glass to microscope slides using double-sided tape.

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Motions of fluorescently labeled F-actin was determined at pCa = 10.0, 8.0, 6.0 and 4.0 (n = 116–342 filaments per group, total = 1616 filaments), and repeated using reconstituted thin filaments at pCa = 10.0, 8.0, 7.5, 7.0, 6.5, 6.0, 5.0 and 4.0 (n = 116–408 filaments per group, total = 3441 filaments). Reconstituted thin filaments were generated by incubating F-actin with native rabbit skeletal muscle tropomyosin (600 nM) and recombinantly expressed α-tropomyosin (600 nM) in actin buffer. Two-way ANOVA was used to determine significance of effects of treatment (N2A vs. BSA), [Ca²⁺], and their interaction on motility of F-actin and reconstituted thin filaments (\(\alpha = 0.05\)). ANOVA was performed using the average filament velocity including stalled filaments. The data were fit to the Hill cooperativity equation using non-linear regression. Shapiro-Wilk tests (\(\alpha = 0.05\)) verified the assumption that the data were distributed normally (\(p = 0.8747\) for BSA control and \(p = 0.7055\) for N2A). V_max, pCa_90 and the Hill coefficient were determined for each condition (BSA vs. N2A).

**Results**

We used three approaches to test the hypotheses that N2A titin binds to F-actin, and that Ca²⁺ increases the strength of this interaction. First, we used co-sedimentation assays to estimate the dissociation constant between N2A and actin under equilibrium conditions. Next, we used dynamic force spectroscopy (DFS) to directly measure the rupture forces and binding stability associated with dynamic interactions between single N2A and F-actin molecules. Finally, we used *in vitro* motility (IVM) assays to measure the effect of titin constructs on velocity of actin filaments and reconstituted thin filaments in the presence of myosin.

**N2A structural validation.** The structure of N2A constructs was verified by obtaining force-extension curves using SMFS. When the AFM tip was retracted from the lipid bilayer surface, saw-tooth patterns corresponding to unfolding of each of the four Ig domains (Ig80, Ig81, Ig82 and Ig83) were observed (Fig. 3). The observed saw-tooth patterns exhibited Lc ~ 50 nm for the fully extended 117 amino acid insertion sequence (IS) and Lc ~ 30 nm for the Ig domains, verifying that the N2A constructs had the expected composition and structure.

**N2A-actin Co-sedimentation.** In co-sedimentation assays, performed to estimate the dissociation constant for the interaction between the N2A construct and actin filaments, varying concentrations of N2A protein...
from 2–5 µM were incubated with F-actin at either pCa = 10 or pCa = 4. ANOVA demonstrated significant effects of [N2A] (p < 0.0001), [Ca2+] (p = 0.0003), and their interaction (p = 0.0125) on N2A – F-actin binding as estimated by densitometry. The amount of bound N2A protein increased as a function of N2A concentration (between 2–5 µM) and a fit of the data to estimate K_d also showed that K_d increases with Ca2+ concentration (K_d ~ 7 µM at pCa = 10 compared to K_d ~ 17 µM at pCa = 4; Fig. 4). While the small number of data points does not allow a definitive estimate of K_d, the analysis shows that Ca2+ significantly increases binding of N2A to F-actin.

Single molecule force spectroscopy (SMFS). Direct measurements of single molecule interactions between N2A constructs and F-actin were made using SMFS. retracting the AFM tip from the surface applied a mechanical force to the N2A - F-actin complex, producing a non-linear, polymer-like extension due to stretching of the N2A construct (Fig. 5). The construct detached from F-actin at rupture forces ranging from ~30–200 pN. During retraction of the AFM probe, extension of IS and Ig domains (Fig. 5) was well approximated by the extensible WLC model (extension length/contour length > 0.8), enabling determination of rupture force (F), persistence length (L_p), and contour length (L_c). L_p was 0.3 ± 0.15 nm in both the presence and absence of calcium, which is consistent with the persistence length value of an extended polymer reported elsewhere. In control experiments performed in the presence and absence of Ca2+, when N2A constructs were probed against a charged lipid bilayer surface without F-actin, no polymer-like extension was observed and measured force values were in the range of instrument noise (10–15 pN). Similar results were obtained when a bare AFM tip with no N2A construct was probed against the actin functionalized lipid bilayer surface. These results confirm the specificity of the experimental approach in detecting N2A - F-actin interactions.

Rupture events corresponding to dissociation of N2A - F-actin complexes were measured in the presence (pCa = 4) and absence of Ca2+ (Fig. 6a,b). The yield of interaction events was ~5% in both cases. In the absence of Ca2+ (Fig. 6c), the maximum of the PDF was 70 ± 40 pN, while in the presence of Ca2+ (Fig. 6d), the maximum was 100 ± 40 pN (two tailed t-test, p < 0.001). This increase in rupture force demonstrates that Ca2+ significantly increases the strength of N2A - F-actin interactions.

The stability of N2A - F-actin interactions in the presence (pCa = 4) and absence of Ca2+ was further characterized using dynamic force spectroscopy (DFS) by measuring the rupture force at various loading rates and using
the Bell-Evans model\textsuperscript{38} to estimate $k_{\text{off}}$ and $x_\beta$ using Eq. 3. Because the N2A construct itself can stretch during loading due to the presence of the partially unstructured IS\textsuperscript{39,40}, the apparent loading rate in Eq. 1 was calculated using $L_c$ and $L_p$ values for the stretched length of the construct that included the extended IS and Ig domains\textsuperscript{26,31}. Both data sets were fit well using Eq. 3 ($R^2 = 0.9$ for no calcium; $R^2 = 0.8$ for pCa 4.0; $P < 0.0001$; Fig. 7), suggesting that the dissociation process undergoes a single barrier\textsuperscript{38}. For the interaction between N2A and F-actin, the model yielded $k_{\text{off}} = 15.6 \pm 2.7 \text{s}^{-1}$ and $x_\beta = 0.2 \pm 0.01 \text{nm}$ in the absence of Ca$^{2+}$, and $k_{\text{off}} = 4.7 \pm 2.9 \text{s}^{-1}$ and $x_\beta = 0.2 \pm 0.03 \text{nm}$ at pCa = 4. Thus, $k_{\text{off}}$ decreased significantly in the presence (circles) vs. absence (diamonds) of Ca$^{2+}$ (ANCOVA, $p < 0.0001$), indicating that Ca$^{2+}$ increases the lifetime of N2A - F-actin interactions. The slope was not significantly different in the presence vs. absence of Ca$^{2+}$ (ANCOVA, $p = 0.7118$).

**Figure 5.** Force-extension curve (black) showing interaction between a single N2A construct and F-actin and fit to worm-like chain (WLC) model (red).

**Figure 6.** Ca$^{2+}$ increases rupture forces of interacting N2A and F-actin molecules. Overlay of force-extension curves in the (a) absence and (b) presence of Ca$^{2+}$. Histograms of N2A - F-actin rupture forces and most probable rupture force at a loading rate of 8000 pN/s in the (c) absence of Ca$^{2+}$ (70 ± 40 pN) and (d) presence of Ca$^{2+}$ (100 ± 40 pN).
In-Vitro Motility Assays (IVM). Co-sedimentation and force spectroscopy experiments demonstrate the ability of N2A to bind to F-actin or thin filaments. IVM was used to explore how the N2A constructs affect sliding velocity of F-actin and reconstituted thin filaments on a lawn of myosin. ANOVA showed that the velocity of F-actin decreased significantly in the presence of N2A vs. BSA (p < 0.0001) and also decreased (p < 0.0001) with increasing [Ca²⁺] (Fig. 8a). There was no difference in slope between N2A vs. BSA (p = 0.1268).

Similar experiments were conducted using regulated thin filaments, in which troponin and tropomyosin were present (Fig. 8b). The data were fit to the Hill cooperativity equation to determine Vₘₐₓ, pCa₅₀ and the Hill coefficient. Non-linear regression models were statistically significant for both BSA and N2A treatments (p ≤ 0.0007). Vₘₐₓ, pCa₅₀ and the Hill coefficients also explained a significant proportion of the variance in both groups (all p ≤ 0.0281). The pCa₅₀ was unchanged in the presence of N2A (BSA control = 6.70, N2A = 6.76). The N2A-containing sample showed an increase in cooperativity based on the Hill coefficients (BSA control = 0.88, N2A = 1.564). Vₘₐₓ was ~1 µm/sec slower in the presence of N2A (3.13) compared to BSA control (4.09). There was little movement of reconstituted filaments at pCa 8 or 10, consistent with myosin binding sites being inaccessible due to the presence of troponin. ANOVA demonstrated significant effects of N2A vs. BSA (p < 0.0001), [Ca²⁺] (p < 0.0001), and their interaction (p < 0.0001) on the velocity of regulated filaments. At pCa 4 and 5, there was a significant decrease in filament velocity in the presence of the N2A construct (Tukey’s HSD, p < 0.05).

Discussion
Although many previous studies have investigated titin – actin interactions in muscle, only Kellermayer and Granzier found Ca²⁺-dependent interactions between actin and the T2 fragment of titin, which extends from the N2-line to the M-line of muscle sarcomeres. Using in vitro motility assays, they demonstrated that T2 reduced the velocity of F-actin and reconstituted thin filaments moving on a lawn of heavy meromyosin in the

**Figure 7.** Dependence of N2A - F-actin rupture force on apparent loading rate (semi-log, in the absence (diamonds, k_{off} = 15.6 ± 2.7 s⁻¹, x₀ = 0.2 ± 0.01 nm) and presence (pCa = 4) of Ca²⁺ (circles, k_{off} = 4.7 ± 2.9 s⁻¹, x₀ = 0.2 ± 0.03 nm). Error bars represent standard error of the mean.

**Figure 8.** In vitro motility of F-actin and reconstituted thin filaments. (A) The N2A construct (triangles) decreases motility of F-actin in the presence of Ca²⁺ compared to BSA controls (circles). (B) In vitro motility of reconstituted thin filaments decreases in the presence of N2A at pCa = 4.0 and 5.0.
presence and absence of Ca\(^{2+}\). They further showed that Ca\(^{2+}\) (pCa < 6.0) further decreased actin motility. Titin–actin binding was confirmed using an *in vitro* immunofluorescence binding assay.

Since 1996, no studies have succeeded in replicating the results of Kellermayer & Granzier\(^{11}\) by showing Ca\(^{2+}\)-dependent interactions between titin and actin. Various *in vitro* experiments demonstrated Ca\(^{2+}\)-independent interactions between F-actin or reconstituted thin filaments and PEVK constructs based on skeletal muscle titin (GenBank X90568\(^{17,19}\)). Furthermore, interactions between cardiac PEVK constructs (GenBank X90568) and actin are inhibited by the Ca\(^{2+}\)-binding protein S100A1 in the presence of Ca\(^{2+}\) 20. However, all previous investigations excluded a critical region of 115 amino acids in the N2A-PEVK border region of skeletal muscle titin, which notably includes 53 of the 83 amino acids deleted in *mdm*\(^{22}\). The results of the present study demonstrate Ca\(^{2+}\)-dependent interactions between N2A titin constructs (Ig80–IS-Ig81-Ig82-Ig83) and F-actin or reconstituted thin filaments at pCa < 6.0, similar to the results of Kellermayer and Granzier\(^{11}\).

**Co-sedimentation and *in vitro* motility.** Co-sedimentation experiments demonstrate that Ca\(^{2+}\) decreases the dissociation constant for the N2A – F-actin interaction. The small number of data points limit drawing conclusions about how the affinity changes as a function of Ca\(^{2+}\) beyond the demonstration that bound N2A increases with higher Ca\(^{2+}\) concentration (see Fig. 4), as also found by Kellermayer & Granzier\(^{11}\). Furthermore, the motility of both polymerized actin filaments (see Fig. 8a) and reconstituted thin filaments (see Fig. 8b) decreases in the presence of N2A in a Ca\(^{2+}\)-dependent manner. These results support previous findings\(^{11}\) that the titin T2 fragment, which includes N2A and PEVK regions, reduced the velocity of actin filaments at pCa < 6. Previous studies observed that actin-binding proteins reduce the sliding velocity of actin on myosin, presumably because binding applies a frictional load to actin filaments, resulting in slower velocities\(^{21}\). Taken together, these studies suggest that N2A titin, specifically the Ig80–IS-Ig81-Ig82-Ig83 region, is responsible for binding of titin to actin in the presence and absence of Ca\(^{2+}\), and that titin–actin interactions are stronger when Ca\(^{2+}\) is present, as observed previously for the T2 fragment of titin\(^{11}\).

These results imply that N2A-actin binding is likely mediated by Ca\(^{2+}\) stabilization of actin binding sites in Ig domains. It is possible that Ca\(^{2+}\)-dependent binding is mediated through Ig83. This model is consistent with the observed loss of function observed in *mdm* mice\(^{23}\), which have a partially deleted Ig83 domain\(^{22}\). This also would explain why titin-actin interactions were observed in our studies but were absent in Linke et al.\(^{21}\) as the constructs used in that study did not include Ig83. While stable binding might require additional Ig domains beyond Ig83, the existing data suggest that this region likely contains amino acids required for binding.

**Single molecule dynamic force spectroscopy.** While co-sedimentation measures equilibrium binding, DFS experiments provide a direct measure of molecular interaction forces and off-rates between the proteins. As Kellermayer and Granzier\(^{11}\) observed for the T2 fragment, DFS also demonstrated that the N2A construct binds to F-actin in the presence and absence of Ca\(^{2+}\). Higher rupture forces in the presence of Ca\(^{2+}\) (see Fig. 6) indicate that the N2A-actin interaction is stabilized by Ca\(^{2+}\). The wide distribution of rupture forces in the presence and absence of Ca\(^{2+}\) observed in the present study implies a variety of molecular interactions. The most likely cause of this wide force distribution is random variation in the interaction due to stochastic thermal fluctuations and variable loading directions\(^{22,23}\). Other possible explanations include multiple N2A – F-actin interactions\(^{44}\) or multiple F-actin binding sites within each N2A molecule\(^{22}\), but both are unlikely. It is unlikely that the wide distribution is due to multiple N2A molecules interacting with F-actin because only force curves demonstrating single interaction events were included in the analysis. The force distribution in the absence of Ca\(^{2+}\) exhibits what may be a small second peak at approximately double the force of the primary peak, which could be caused by rare simultaneous rupture events. Because these events appear to be rare and at much higher forces, they are unlikely to contribute to the width of the distribution. Also, the first peak of the PDF (see Eq. 2) fit to the rupture force histogram should correspond to a single interaction\(^{45}\). That this force was significantly greater than the force observed with no actin present (15–20 pN) indicates that the measurement corresponds to interactions with actin rather than the lipid surface. Alternatively, if N2A constructs have multiple F-actin binding sites, then the rupture force histogram should exhibit multiple peaks corresponding to the interactions at each of these sites. Multiple peaks are not apparent in the rupture force distributions beyond the small second peak already noted (see Fig. 6c,d) making the presence of multiple binding sites unlikely, although the possibility that multiple peaks might emerge with a larger number of force curves cannot be definitively ruled out. The N2A-F-actin rupture length in both the presence and absence of Ca\(^{2+}\) showed wide variability, but some rupture length clustering was observed in the more pronounced rupture events in the presence of Ca\(^{2+}\). This result seems to point toward a specific actin binding location within the N2A construct, but more experiments involving individual Ig domains are required to confirm this possibility.

The value of \(k_{\text{off}}\) for N2A – F-actin interactions was significantly lower in the presence of Ca\(^{2+}\), suggesting that Ca\(^{2+}\) increases not only rupture forces but also the stability of the interaction. When plotted as a function of the log of the ALR, the linear fit of rupture forces between N2A constructs and F-actin in the presence and absence of Ca\(^{2+}\) also suggests a one-dimensional dissociation energy landscape. For comparison, at comparable loading rates, the stability of the N2A – F-actin interaction measured here in the presence of Ca\(^{2+}\) (\(k_{\text{off}} \approx 4.7 \text{ sec}^{-1}\)) is an order of magnitude lower than the stability of Ca\(^{2+}\)-independent skeletal PEVK interactions with F-actin (\(k_{\text{off}} \approx 0.4 \text{ sec}^{-1}\))\(^{37}\). This difference could be due to several factors, including different actin binding sites on N2A and PEVK, as well as reduced entropic flexibility of actin filaments when stabilized by heavy meromyosin and phal- lidin, as in their study. The higher conformational flexibility of our N2A construct combined with the dynamic nature of F-actin paracrystals formed on a lipid surface could reduce the stability of the N2A – F-actin interaction, as higher protein configurational entropy can reduce mechanical stability\(^{46}\).

Although single molecule force measurements can be difficult to interpret, a number of factors support the N2A – F-actin interactions observed in this study. Because F-actin and N2A constructs are polymers, their spatial
arrangement is likely to influence how they interact under physiological conditions. In this experiment, the pulling direction of N2A constructs attached to the AFM tip was orthogonal to the axis of the F-actin filaments. Although this geometric arrangement may not be identical to how native N2A might interact with thin filaments under physiological conditions, covalent attachment of the N2A constructs to the AFM tip via N-terminal cysteines frees the entire N2A molecule to interact with F-actin. Additionally, because the loading direction of N2A constructs was perpendicular to actin filaments, the measurement was not affected by inter-connecting actin bonds being pulled at the same time, as would be the case if the pulling direction was parallel to the filament axis. Our simple experimental approach also requires no additional stabilizing factors, such as phalloidin, which might affect any measured interactions.

Control experiments demonstrated no interactions between N2A constructs and the charged lipid bilayer surface, and it is unlikely that measured forces could represent rupture of lipid bilayers or F-actin filaments. Puncturing a lipid bilayer using an AFM probe requires forces in the range of nN, much larger than forces measured in the present study (~30–200 pN). Mechanically breaking actin filaments or paracrystals of actin filaments requires forces ~600 pN, also much larger than those measured in this study.

Role of N2A-F-actin interaction in muscle sarcomeres. Based on their observation that titin T2 fragments decreased in vitro motility of actin and thin filaments in the presence of heavy meromyosin (HMM), Kellermayer and Granzier hypothesized that, in addition to generation of passive tension, titin might play a motility of actin and thin filaments in the presence of heavy meromyosin (HMM), in vitro experiments decreased titin stiffness by preventing elongation of proximal Ig domains during active stretch. Recent reports using specific cleavage of titin in a transgenic mouse with tobacco etch virus protease inserted into the distal Ig region, suggest that titin bears considerable force during isometric contraction. Titin cleavage was observed to reduce isometric contractile force by 50% in homozygous muscle. Such large titin forces cannot occur without an increase in titin stiffness compared to passive muscle, in which straightening of proximal Ig domains exhibits very low effective stiffness. Recent experiments in which single myofibrils were stretched beyond overlap of the thick and thin filaments (where cross-bridges per se no longer contribute to force production), showed that titin stiffness increased by a factor of ~4 compared to passive myofibrils at the same length. Further studies demonstrated that this increase in titin stiffness fails to occur in psoas muscles from mdm mice, with a 779 bp deletion corresponding to 83 amino acids in the distal Ig83 and proximal PEVK regions. Taken together, these results are consistent with a model in which binding of N2A titin to actin/thin filaments in active muscle increases titin stiffness by preventing elongation of proximal Ig domains at low force, as occurs during passive stretch, and that this mechanism for Ca2+-dependent increase in titin stiffness is likely impaired by the mdm mutation.

Future studies are required to test whether titin-actin interactions as reported here in vitro limit extension of proximal Ig domains during active stretch of skeletal muscle sarcomeres. Granzier suggested that labeling of myofibrils with anti-titin antibodies could be used to directly observe extension of titin segments in situ during passive and active stretch. A recent study used the F146 antibody, which binds in the distal PEVK segment near the edge of the A-band, to investigate segmental titin elongation. They found evidence for an effect of Ca2+ but no evidence for actin interactions on extension of proximal and distal segments. It is important to point out, however, that the F146 antibody binds far from the N2A region and the proximal segment therefore includes most of the PEVK segment, making it relatively insensitive to changes in elongation of the N2A region. A more definitive test of whether N2A titin binds to actin in skeletal muscle sarcomeres requires use of antibodies that bind closer to the N2A region itself or between N2A and the Z-line.

Data Availability
The datasets generated during the current study are available from the corresponding author on reasonable request.

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Acknowledgements
This work was supported the W. M. Keck Foundation and the Technology Research Initiative Fund of Northern Arizona University. We thank Stan Lindstedt for helpful comments on an earlier draft of the manuscript.

Author Contributions
S.D. collected and analyzed DFS data and prepared Figs 1–3 and 5–6. C.T. collected and analyzed cosedimentation and IVM data. S.S. and J.M. assisted with design and execution of IVM experiments. MG analyzed cosedimentation and IVM data and prepared Figs 4 and 8. K.N. performed all statistical analyses and guided the project. K.N., B.N., S.D. and M.G. wrote the main manuscript. All authors reviewed the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-32952-8.

Competing Interests: The authors declare no competing interests.

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