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RESEARCH ARTICLE

Decreased force enhancement in skeletal muscle sarcomeres with a deletion in titin

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ABSTRACT

In the cross-bridge theory, contractile force is produced by cross-bridges that form between actin and myosin filaments. However, when a contracting muscle is stretched, its active force vastly exceeds the force that can be attributed to cross-bridges. This unexplained, enhanced force has been thought to originate in the giant protein titin, which becomes stiffer in actively compared with passively stretched sarcomeres by an unknown mechanism. We investigated this mechanism using a genetic mutation (*mdm*) with a small but crucial deletion in the titin protein. Myofibrils from normal and *mdm* mice were stretched from sarcomere lengths of 2.5 to 6.0 μm. Actively stretched myofibrils from normal mice were stiffer and generated more force than passively stretched myofibrils at all sarcomere lengths. No increase in stiffness and just a small increase in force were observed in actively compared with passively stretched *mdm* myofibrils. These results are in agreement with the idea that titin force enhancement stiffens and stabilizes the sarcomere during contraction and that this mechanism is lost with the *mdm* mutation.

KEY WORDS: Myofibrils, Eccentric contractions, Stiffness, Muscular dystrophy with myositis, Cross-bridges

INTRODUCTION

Movement involves the coordination of dynamic muscle contractions produced by proteins within sarcomeres. For nearly 60 years, contractile force has been understood as a Ca^{2+}-dependent cyclical formation of cross-bridges between thick (myosin) and thin (actin) filaments in the sarcomere (Huxley and Simmons, 1971) (Fig. 1). Titin, a viscoelastic spring in the sarcomere, was discovered decades after the cross-bridge model was developed (Maruyama, 1976). Thus, the cross-bridge theory does not account for this force-bearing protein in the sarcomere. Based on the cross-bridge theory, active, steady-state, isometric force must be proportional to the number of cross-bridges that can form (given by the sarcomere length for the plateau and descending limb regions of the force–length relationship), with maximal force produced at optimal filament overlap (Gordon et al., 1966). However, active muscle force greatly exceeds this predicted maximum force following stretching (Abbot and Aubert, 1952). Stretch-induced residual force enhancement has been observed at all structural levels, from entire muscles down to single sarcomere preparations (Leonard et al., 2010; Rassier, 2012). Therefore, an unidentified mechanism generates enhanced force in sarcomeres that persists in the isometric steady-state following active stretch. Previous investigations sought to describe this residual force enhancement within the framework of the cross-bridge theory. However, this property persists beyond filament overlap, where cross-bridges cannot contribute to force (Leonard and Herzog, 2010). Evidence suggests that titin becomes stiffer during muscle contraction, enhancing force during and following active stretch (Herzog and Leonard, 2002; Leonard and Herzog, 2010; Nishikawa et al., 2012; Powers et al., 2014).

A role for titin in active muscle force production has long been speculated (Edman et al., 1982; Noble, 1992) but remains controversial. Titin's function within the sarcomere was traditionally considered structural, but its dynamic nature has since been demonstrated with widespread structural, mechanical and signaling roles (Granzier and Labeit, 2004; Labeit et al., 2003; Leonard and Herzog, 2010; Nishikawa et al., 2012). Titin molecules (six per half thick filament) anchor the thin filaments at the Z-disc (Maruyama, 1976), running the full length of the thick filament to the M-line (Gregorio et al., 1999) to form a titin network that maintains the structural integrity of each sarcomere (Fig. 1). Structurally, titin's I-band consists of springs in series that resist sarcomere stretch. Upon stretch, the proximal immunoglobulin (Ig) domains elongate under low force, followed by stretching of the PEVK (Proline-Glutamate-Valine-Lysine) region, which contributes the majority of titin-based force (Fig. 1) (Granzier and Labeit, 2004). Located between the proximal Ig domain and the PEVK region, the N2A segment can also elongate during long sarcomere stretches and is a 'hot spot' for titin-based signaling (Granzier and Labeit, 2004). The distal Ig segment, connecting titin's I- and A-bands, is the most rigid of titin's springs. Transient increases in titin-based stiffness (15–20%) are observed when Ca^{2+} ions bind to the Ig and PEVK segments of titin (DuVall et al., 2013; Labeit et al., 2003). However, observations of up to 200% increase in titin stiffness in actively stretched rabbit and mouse sarcomeres provide evidence of an additional mechanism for modulation of titin-based force (Leonard and Herzog, 2010; Powers et al., 2014). This mechanistically elusive increase in titin-based stiffness in actively stretched sarcomeres is referred to as 'titin force enhancement' henceforth.

Titin force enhancement occurs in a force-dependent manner, decreasing in magnitude with reduced contractile force preceding stretch (Leonard and Herzog, 2010). Initiation of titin force enhancement requires strong cross-bridge formation (Herzog and Leonard, 2002; Joumaa et al., 2008; Powers et al., 2014), suggesting that the mechanisms underlying titin force enhancement and cross-bridge cycling are intimately connected in an active sarcomere. Distinct from Ca^{2+}-dependent cross-bridges, titin force enhancement persists following Ca^{2+} deactivation (Joumaa et al., 2008) and cannot be attributed solely to the effects of Ca^{2+} (Powers et al., 2014). Therefore, it appears that titin's spring properties are modulated in actively stretched sarcomeres by an unknown

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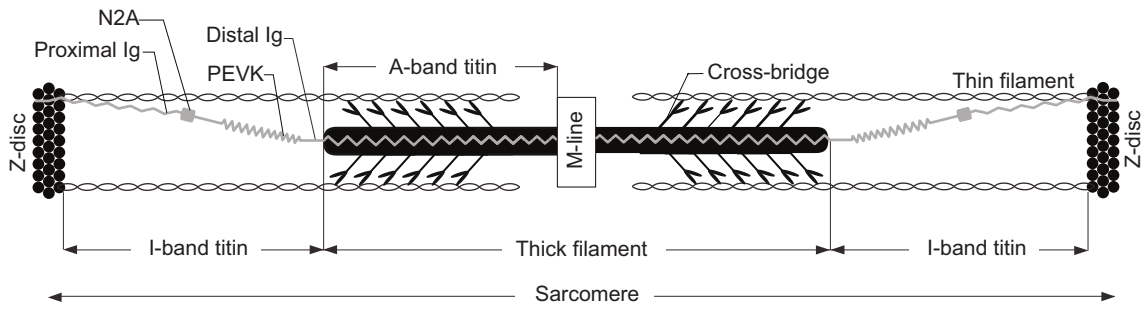

Fig. 1. Characterization of titin in the sarcomere. Three main proteins contribute to sarcomere force: the thick and thin filaments and titin (gray). Traditionally, force during contraction is explained by cross-bridges, which form between the thick and thin filaments, while the role of titin in sarcomere contraction is unknown. Titin is a structural and mechanical protein which extends the half sarcomere from the Z-disc to the M-line. The mechanical I-band region of titin consists of the proximal Ig, PEVK and N2A segments, which elongate to resist sarcomere stretch. The rigid distal Ig segment connects titin's mechanical I-band with its structural A-band, aligning the thick filament within the sarcomere. We propose a mechanism by which titin contributes to sarcomere force during stretch contractions.

mechanism that occurs in the presence of Ca^{2+} when cross-bridges cycle and contractile force is generated. It has been suggested that titin force enhancement could occur if titin binds to the thin filament to shorten and stiffen its available spring length during active sarcomere stretch (Herzog, 2014; Leonard and Herzog, 2010; Nishikawa et al., 2012; Powers et al., 2014). Conceptually, titin binding to the thin filaments provides a mechanism that can account for large, rapid and reversible force enhancement in actively stretched sarcomeres (Schappacher-Tilp et al., 2015). Therefore, if this hypothetical titin binding could be prevented, actively stretched sarcomeres would be more compliant (Fig. 2).

A spontaneous titin mutation presents the opportunity for this focused investigation of the mechanism underlying titin force enhancement. A titin mutation that occurs in muscular dystrophy with myositis (*mdm*) mice results in a predicted 83 amino acid deletion in the N2A and PEVK regions of the titin protein (Fig. 2) (Garvey et al., 2002). Muscles from *mdm* mice are actively more compliant (Monroy et al., 2012; Taylor-Burt et al., 2015), possibly owing to the deletion in titin's I-band region (Fig. 2). This suggests that modulation of titin stiffness in active sarcomeres by the proposed titin–thin filament interaction may be affected by the *mdm* mutation. We took this opportunity to investigate the mechanism of titin force enhancement by comparing the contractile properties of normal (WT) with mutant (*mdm*) myofibrils (composed of intact sarcomeres in series) during active and passive stretch to long sarcomere lengths beyond filament overlap. We hypothesized that titin force enhancement would be deficient if a critical region involved in titin–thin filament binding was deleted in the *mdm* mutation.

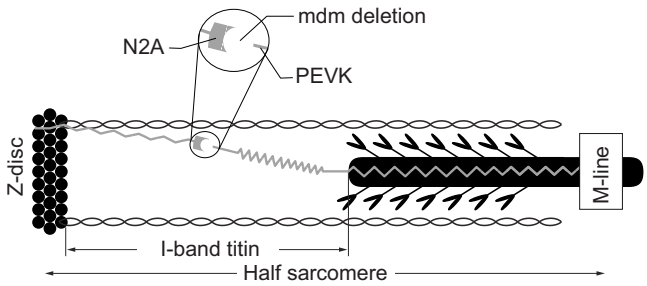

Fig. 2. Characterization of the *mdm* deletion in titin in the half sarcomere. In muscular dystrophy with myositis (*mdm*) a genetic mutation in the titin gene results in a predicted deletion of 83 amino acids from distal N2A and proximal PEVK in I-band titin (enlarged circled area highlights the region of the predicted deletion) with no measurable changes in thick and thin filament structure or function. This small deletion in the titin protein may affect titin's contribution to sarcomere force during stretch contractions.

MATERIALS AND METHODS

A single myofibril preparation was used for mechanical testing of *mdm* (muscular dystrophy with myositis, with a deletion in N2A–PEVK titin; Garvey et al., 2002) and WT mouse psoas sarcomeres (Fig. 2). For a thorough description of the methods employed in single myofibril experiments, see Powers et al. (2014). Briefly, experiments were conducted using an inverted light microscope (Axiovert 200M, Zeiss, Germany) equipped with a ×100 oil immersion objective (numerical aperture 1.3) and a ×2.5 Optovar (Immersol™, 518F, Zeiss). Force was calculated using custom-built nanofabricated silicon nitride nanolevers with a stiffness of 21 or 68 pN nm^{-1} (Fig. 3).

Mdm mice were acquired from a colony established at Northern Arizona University Animal Care Facility (Flagstaff, AZ, USA) using breeding pairs of B6C3Fe a-/a-*mdm* mice purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Homozygous recessive Ttn^{mdm} (*mdm*) mice were confirmed by genotyping (Lopez et al., 2008). The Institutional Animal Care and Use Committee at Northern Arizona University approved the use of these animals. Age matched C57-BL/6 mice from the University of Calgary were used as a healthy comparison. Ethics approval for the study was granted by the Life and Environmental Sciences Animal Ethics Committee of the University of Calgary.

Strips of mouse psoas muscle were extracted from euthanized normal (WT) and mutant (*mdm*) animals (male and female, 28–40 days old, mean±s.e.m. 33±3.4 days). Muscles were tied to wooden sticks to preserve the *in situ* length, placed in a rigor-glycerol solution (−20°C, pH 7.0) with protease inhibitors (Complete®, Roche Diagnostics, Montreal, QB, Canada) and stored at −20°C for 15–22 days (Leonard and Herzog, 2010). Muscle strips were homogenized in a rigor solution at 4°C and tested at 20°C (Leonard and Herzog, 2010). For experiments, chemically and mechanically isolated *mdm* and WT myofibrils were glued to one nanolever arm at one end and attached to a stiff glass needle (motor) at the other end (Fig. 3). Myofibril lengths were manually adjusted to an initial average sarcomere length of 2.5 µm. Average sarcomere length was determined by dividing the total length of the myofibril by the number of sarcomeres. Myofibrils were stretched by a piezoelectric motor controlled by custom-written LabView software at a rate of 0.1 µm per sarcomere per second to limit velocity-dependent increases in force. Displacement of the attached nanolever relative to the unattached reference arm

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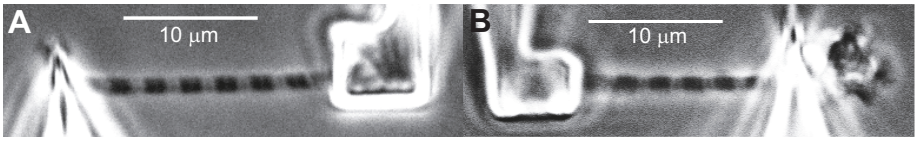

Fig. 3. Single myofibril experimental set-up. Single isolated myofibrils from normal (WT, A) and muscular dystrophy with myositis (*mdm*, B) mouse psoas. Myofibrils were mounted on one side to a motor to implement controlled length changes and on the other side to a nanolever (68 pN nm^{-1}) to calculate force for a given myofibril stretch. Myofibrils from WT and *mdm* mice were visually indistinguishable with no measurable difference in diameter (mean±s.e.m., WT: 1.27±0.05 μm, *N*=18; *mdm*: 1.28±0.06 μm, *N*=19) or calculated cross-sectional area (*t*-test, *P*=0.98). Thus, similar contractile force measurements in *mdm* and WT sarcomeres were expected.

was measured using a custom-written MATLAB program. Force was calculated from the measured displacement and lever stiffness and expressed in units of stress (nN μm^{-2}) by normalizing the calculated force to the cross-sectional area of each myofibril. Myosin and actin content in WT and *mdm* psoas was determined using 12% SDS-PAGE (Laemmli, 1970).

Individual myofibrils were suspended in a relaxing solution (pCa 8.0) for passive stretch. For active experiments, the bath was flushed with a Ca^{2+}-rich activation solution (pCa 3.5) prior to stretch. Myofibrils were stretched from an average initial sarcomere length of 2.5 μm to an average final length of 6.0 μm. At a sarcomere length of 4.0 μm, the mouse sarcomere is beyond myofilament overlap (Powers et al., 2014); thus, titin-based force within the intact sarcomere was considered at average sarcomere lengths exceeding 4.0 μm (Fig. 4).

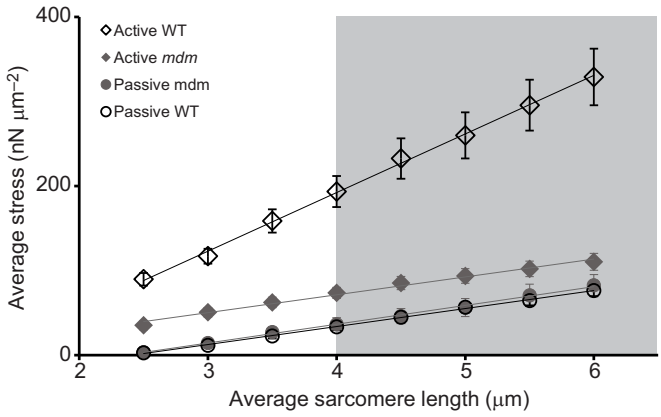

Fig. 4. Activated wild-type myofibrils generate more stress than *mdm* myofibrils. Average stress for a given average sarcomere length. Single psoas myofibrils from normal wild-type (WT, open symbols) and muscular dystrophy with myositis (*mdm*, filled symbols) mice were stretched from an average initial sarcomere length of 2.5 μm to an average final sarcomere length of 6.0 μm at a speed of 0.1 μm per sarcomere per second. Myofibrils were stretched actively (diamonds, following Ca^{2+} activation) or passively (circles, in an ATP relaxing solution). Thick and thin filament overlap is no longer present beyond 4.0 μm (indicated by the shaded region). Average stress of active WT myofibrils (*N*=7) was greater than that of *mdm* myofibrils (*N*=8) at all sarcomere lengths (*P*<0.01). Stress beyond filament overlap was much greater in actively stretched WT myofibrils than in actively stretched *mdm* myofibrils (*P*<0.01). There was no difference in passive stress between WT (*N*=9) and *mdm* (*N*=10) myofibrils (*P*>0.01). A repeated measures two-way analysis of variance and simple effect test were used to determine statistical differences between stresses by genotype and experimental condition. Comparison of individual slopes by group demonstrated that actively stretched WT myofibrils (69.3±9.5 nN μm^{-3}) are stiffer than actively stretched *mdm* myofibrils (21.1±7.5 nN μm^{-3}; *P*<0.01) and passively stretched *mdm* (22.2±11.1 nN μm^{-3}) and WT (21.9±5.9 nN μm^{-3}) myofibrils, while actively stretched *mdm* myofibrils do not differ in stiffness from passively stretched *mdm* and WT myofibrils (*P*>0.01). Kruskal–Wallis non-parametric analyses and Mann–Whitney *U* with Bonferroni corrections were used for slope comparisons.

A repeated measures two-way analysis of variance and simple effect test were used to determine statistical differences between stresses by genotype and experimental condition. To compare individual slopes by condition, Kruskal–Wallis non-parametric analyses and Mann–Whitney *U* with Bonferroni corrections were used. *t*-tests were used to compare independent groups. Significance was determined by an alpha level of 0.01.

RESULTS

Isolated myofibrils from *mdm* and WT were indistinguishable with no difference in diameter (Fig. 3; *P*=0.98; mean±s.e.m. *mdm*: 1.28±0.06 μm, *N*=19; WT: 1.27±0.05 μm, *N*=18) or contractile protein content, expressed as the ratio of myosin to actin content (myosin/actin) in *mdm* (1.39±0.09) and WT (1.41±0.09) psoas. Single isolated *mdm* and WT mouse psoas myofibrils were stretched actively (with Ca^{2+}) or passively (without Ca^{2+}) from an average sarcomere length of 2.5 μm to far beyond thick and thin filament overlap (6.0 μm) to observe titin-based stress in the absence of cross-bridges. Passive stress did not differ between *mdm* (*N*=10) and WT (*N*=9) myofibrils for the duration of stretch (*P*>0.01; Fig. 4). Active stress was lower in *mdm* (*N*=8) compared with *wt* myofibrils (*N*=7) at all sarcomere lengths (*P*<0.01; Fig. 4).

Individual slopes, indicative of titin stiffness at lengths beyond filament overlap, were compared by group and genotype for active WT (mean±s.d., 69.3±9.5 nN μm^{-3}, R^2=0.99, *N*=7), passive WT (21.9±5.9 nN μm^{-3}, R^2=0.99, *N*=9), active *mdm* (21.1±7.5 nN μm^{-3}, R^2=0.99, *N*=7) and passive *mdm* (22.2±11.1 nN μm^{-3}, R^2=0.99, *N*=10) myofibrils. Titin stiffness was three-times greater at the end of stretch (average sarcomere length, 6.0 μm) in active compared with passive WT myofibrils (Fig. 4). The slopes of active and passively stretched *mdm* myofibrils did not differ, indicating no change in titin stiffness from passive to activated *mdm* myofibrils (Fig. 4).

DISCUSSION

There was no difference in stress between passively stretched WT and *mdm* myofibrils, suggesting that the passive mechanical properties of *mdm* titin are not affected by the *mdm* deletion in titin (Fig. 4). Previous studies report no detectable difference in the molecular weight of *mdm* (predicted to be missing only 83 of over 30,000 amino acids) and WT titin (Huebsch et al., 2005). While we recognize that additional amino acids to those predicted based on the mutated titin gene may be deleted from *mdm* titin (Buck et al., 2014), the modest amino acid deletion in *mdm* titin (Fig. 2) does not alter titin's spring properties in a passive intact psoas sarcomere. Previous observations of increased passive stress in whole-muscle preparations from *mdm* mice (Huebsch et al., 2005; Lopez et al., 2008; Monroy et al., 2012; Taylor-Burt et al., 2015) are likely due to large endomysial and perimysial connective tissue deposits measured in *mdm* muscles (Lopez et al., 2008). With negligible connective tissue in the myofibril preparation, increased passive

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force was not observed in the present study and, thus, is unlikely to be the result of inherent changes to the passive stiffness of *mdm* titin.

The mechanism of titin force enhancement is of primary interest in this study. We observed a 3- to 4-fold increase in titin stiffness in actively compared with passively stretched WT mouse sarcomeres (4.0–6.0 µm), which was not observed in *mdm* sarcomeres (Fig. 4). This observation suggests that the mechanism of titin force enhancement is inhibited by the *mdm* mutation. In a passively stretched sarcomere, proximal Ig domains elongate, bearing little force until recruitment of the PEVK segment, which contributes the majority of titin-based force in the sarcomere (Granzier and Labeit, 2004). PEVK (Labeit et al., 2003) and Ig domains (DuVall et al., 2013) become measurably stiffer when Ca^{2+} ions are released from the sarcoplasmic reticulum during activation. Additional observations demonstrate Ca^{2+}-dependent PEVK interactions with the thin filament (Kellermayer and Granzier, 1996) as well as interactions between tropomyosin, actin filaments and titin (Raynaud et al., 2004). The results from this and previous studies demonstrate a distinct modulation of titin stiffness when passive sarcomeres are activated and cross-bridges are allowed to cycle and generate force (Leonard and Herzog, 2010; Powers et al., 2014). The mechanism of titin force enhancement stiffens titin and, consequently, stiffens the actively stretched sarcomere as well. It was initially suggested that titin force enhancement may occur when titin binds to the thin filament, becoming shorter and stiffer in actively stretched sarcomeres (Leonard and Herzog, 2010). A binding location was later predicted at N2A, limiting titin's extendible spring length to include only its stiffest PEVK and distal Ig segments (Nishikawa et al., 2012). A mathematical model predicting N2A binding to the thin filament underestimated experimental values of titin force enhancement (Powers et al., 2014; Schappacher-Tilp et al., 2015). Thus, PEVK must become stiffer (beyond the effects of Ca^{2+} and beyond the effects of N2A binding) in an actively stretched sarcomere to explain experimental observations of titin force enhancement. Nishikawa et al. (2012) previously suggested that PEVK may become stiffer when it is wound around the thin filament with translation and rotation of cross-bridges (Nishikawa et al., 2012). It is also possible that PEVK binds directly to the thin filament at one or many sites along its length to become stiffer (Schappacher-Tilp et al., 2015) or to a different protein in the sarcomere such as tropomyosin (Raynaud et al., 2004). Whether by binding or winding or an alternative mechanism, we acknowledge that there are many ways in which titin may become stiffer in an activated sarcomere. Nevertheless, these and previous findings in *mdm* suggest that PEVK and N2A play a critical role in the mechanism of titin force enhancement.

Some considerations are worth noting regarding the effect of the *mdm* deletion on the structure and function of the titin protein. Protein conformation is dependent on the interactions of the individual amino acids within a protein. Even a small amino acid deletion can lead to modifications in protein conformation that subsequently alter its function (Akhter et al., 2014). In healthy muscle, length-specific titin isoforms that vary in stiffness are routinely generated by selective amino acid deletion during post-translational processing (Granzier and Labeit, 2004). Local conditions can also initiate post-translational processing of titin to generate length-specific titin isoforms that suit the altered mechanical needs of the muscle (Granzier and Labeit, 2004). While the *mdm* mutation is predicted to remove 74 amino acids from distal N2A and 9 amino acids from proximal PEVK (Fig. 2), the effect of the *mdm* mutation on post-translational processing is unknown. Subtle deletions in titin have previously been shown to elicit variant splicing during post-translational processing, resulting in additional, unpredicted deletion of amino acids along the length of the protein (Buck et al., 2014). Therefore, we acknowledge the possibility that additional amino acids to those in N2A and PEVK may be missing from *mdm* titin with further consequences to the function of the protein. In the absence of a comprehensive amino acid sequence of *mdm* titin, and with no measurable change in the molecular weight of *mdm* titin (Garvey et al., 2002; Huebsch et al., 2005), we assume a reasonably accurate prediction primarily affecting the N2A and PEVK segments of *mdm* titin (Fig. 2). However, whether the amino acids deleted in *mdm* titin directly or indirectly affect the mechanism of titin force enhancement is not clear. Determining the amino acid sequence of the mutant titin is an important focus of future investigation as the amino acids deleted in *mdm* may prove critical to the function of titin in an active sarcomere.

Considering the mechanism of titin force enhancement, one must acknowledge that *mdm* sarcomeres generated considerably less active stress than *wt* myofibrils within and beyond filament overlap (Fig. 4). With no increase in *mdm* stiffness during active stretch, we speculate that the titin force enhancement mechanism does not occur in *mdm* sarcomeres and that contractile dysfunction plays a role in the loss of titin force enhancement in *mdm*. Titin force enhancement is abolished with chemical inhibition of cross-bridges (Powers et al., 2014) and the magnitude of titin force enhancement has been shown to be dependent on contractile force (Leonard et al., 2010). These previous observations along with the present observation that active force and titin force enhancement are both affected in *mdm* sarcomeres leads us to the intriguing question: is there an inherent dysfunction in cross-bridge function that eliminates titin force enhancement in *mdm* or does the *mdm* deletion in titin alter the active mechanics of the sarcomeres?

Mdm sarcomeres generated lower active force within the region of filament overlap than normal WT sarcomeres. Gel analyses confirmed that myosin/actin were not differentially degraded in *mdm*. Thus, at the same sarcomere length and presumably similar overlap of thick and thin filaments, *mdm* myofibrils should generate comparable forces to WT myofibrils during active stretch unless there is inherent contractile protein malfunction. While it is reasonable to suspect that actin and myosin may have been affected by secondary effects of the *mdm* mutation, there is currently no evidence to suggest that structural changes to the contractile proteins may render *mdm* sarcomeres functionally deficient. *Mdm* sarcomeres do not differ in size or striation and appear physically unaltered (Fig. 3). Nevertheless, decreased active force has been observed in various preparations in *mdm* (Lopez et al., 2008; Monroy et al., 2012; Taylor-Burt et al., 2015) and down to the basic contractile unit of the muscle. This suggests that active *mdm* sarcomeres are deficient in their ability to generate force. Characterization of the *mdm* mutation by Lopez et al. (2008) showed an increase in the slow isoform of myosin heavy chain with *mdm* disease progression. However, whether (and how) a switch in myosin heavy chain isoforms results in contractile dysfunction in *mdm* is unclear. The N2A protein signaling complex is also disturbed in *mdm*, affecting various signaling pathways and the levels of numerous signaling molecules in the *mdm* sarcomere (Witt et al., 2004). However, how perturbed signaling may contribute to the contractile dysfunction observed in *mdm* remains speculative.

Our current hypothesis is that when the cross-bridges cycle and generate force, titin binds to the thin filament, shortening its extendable spring length to become stiffer in actively stretched sarcomeres (Herzog and Leonard, 2002; Leonard and Herzog,

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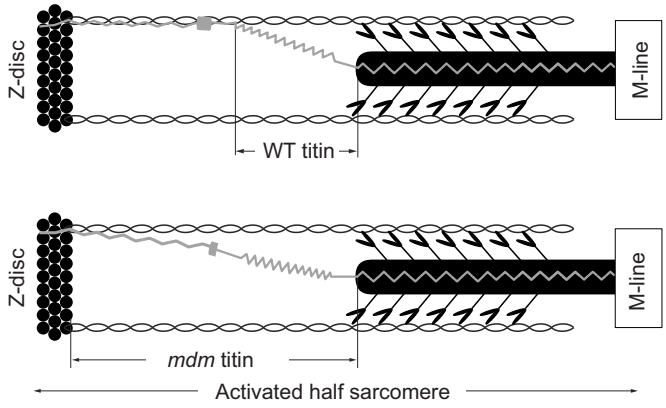

Fig. 5. Proposed mechanism: *mdm* titin is longer and more compliant than WT titin in activated half sarcomeres. We propose that one or many sites within I-band titin bind to the thin filament in an activated (WT) half sarcomere (top), resulting in a shorter and stiffer titin spring (WT titin) and half sarcomere. The shortened WT titin is crucial to the structural integrity of a contracting sarcomere and contributes to force following active sarcomere stretch. In the *mdm* half sarcomere (bottom), a truncated N2A and proximal PEVK segment prevents proposed titin–thin filament binding. This results in a longer and more compliant titin spring (*mdm* titin) and *mdm* half sarcomere with reduced structural integrity and compromised active force production.

2010; Nishikawa et al., 2012; Powers et al., 2014). How cross-bridge cycling alters the environment in the sarcomere to allow for titin binding is not understood. We previously demonstrated that movement of the regulatory protein troponin C does not initiate titin force enhancement (Powers et al., 2014). However, it is possible that movement of (or interaction with) the regulatory protein tropomyosin exposes potential titin binding site(s) on the thin filaments. We acknowledge the possibility that titin force enhancement may occur by a number of mechanisms. Nevertheless, with more than 300% titin force enhancement in WT myofibrils compared with 30% in *mdm* myofibrils at 6.0 µm, we demonstrate that following the *mdm* mutation in the titin gene, the titin force enhancement mechanism is lost.

Although various observations indicate a mechanism by which titin becomes up to four times stiffer in an actively stretched sarcomere (Leonard and Herzog, 2010; Powers et al., 2014), there is currently no direct evidence that modulation of titin force is elicited by titin binding to the thin filament. Since early investigations of residual force enhancement, the 'recruitment of a passive element' has been suggested as an explanation for the observations of increased force following active stretch (Edman et al., 1978). Subsequent studies have explored the possible recruitment of titin, the primary passive element in the sarcomere. Still, the details of titin recruitment remain vague and subject to speculation. With previous research demonstrating some affinity of titin for actin (Bianco et al., 2007; Kellermayer and Granzier, 1996) and the ability to reproduce experimental observations of sarcomere force enhancement by modeling titin–thin filament binding (Powers et al., 2014; Schappacher-Tilp et al., 2015), we remain in support of the idea that titin binds to the thin filament in activated skeletal muscle sarcomeres (Fig. 5), offering a rapid and reversible increase in stiffness. How this mechanism occurs and how it influences whole-muscle function and, subsequently, animal locomotion, are important topics for future investigations. In the case of the *mdm* mouse, movement is severely compromised, with animals exhibiting an abnormal gait (Huebsch et al., 2005; Taylor-Burt et al., 2015) while actively stretched sarcomeres are three times more compliant than *wt* sarcomeres (Fig. 4). Whether the compliance of actively stretched *mdm* sarcomeres directly contributes to deficiencies in *mdm* mouse locomotion cannot be deduced from the results of the present study. The ability to scale mechanical observations from subcellular, to whole-muscle and *in vivo* observations, where force enhancement is also present (Herzog and Leonard, 2002), is an important consideration for the design of future experiments investigating titin force enhancement. Whether titin is primarily (or partially) responsible for *in vivo* residual force enhancement observations remains an interesting consideration.

While titin has traditionally been viewed as a contributor strictly to passively stretched sarcomeres, it is now evident that its mechanical role extends to actively stretched sarcomeres. A major stiffening of titin has been observed in actively stretched intact sarcomeres of rabbit (Leonard and Herzog, 2010) and mouse (Powers et al., 2014). In this study, we have used a titin mutation model (*mdm*) to investigate how an amino acid deletion affects the elusive mechanism of titin force enhancement in skeletal muscle sarcomeres. The results suggest that the mechanism of titin force enhancement does not occur in *mdm* sarcomeres. Actively stretched *mdm* sarcomeres generate much lower force than healthy WT controls, suggesting that contractile force and titin force enhancement are intimately connected. Decreased active force in *mdm* has been observed previously in whole muscle (Monroy et al., 2012; Taylor-Burt et al., 2015) and fiber bundles (Lopez et al., 2008), and now in myofibrils, introducing the possibility that titin plays a critical role in active force that translates across the muscle hierarchy. Decreased active stress prior to stretch (2.5 µm) suggests that titin force enhancement is initiated immediately upon activation and is coupled to the sarcomere's ability to generate contractile force.

Titin can no longer be viewed as a passive structural spring in the sarcomere, but should be considered a dynamic adaptable spring, adjusting its stiffness to suit the temporary and extended mechanical demands of the sarcomere. However titin force enhancement may occur, this mechanism stiffens the structural network of the sarcomere for enduring the high forces of cross-bridges and stretch during dynamic locomotion. Titin-based force enhancement is essentially absent in *mdm* sarcomeres where amino acids in N2A and PEVK titin are deleted, indicating these specific regions along titin are paramount in increasing titin stiffness in an active sarcomere. With this spontaneous titin mutation (*mdm*), nature has provided us with a stepping-stone to explore the long-elusive mechanism of residual force enhancement and to further establish the role of titin in an active sarcomere. It is time to reconsider traditional views of skeletal muscle contraction as an exclusively cross-bridge-based mechanism and embrace the role of titin in the active sarcomere.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.P. carried out data collection, analysis, interpretation and paper preparation; K.N. carried out conceptual design, interpretation and revision of the paper; V.J.

performed data collection, analysis and revision of the paper; W.H. carried out conceptual and experimental design, interpretation and final revision.

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References

Abbot, B. C. and Aubert, X. M. (1952). The force exerted by active striated muscle during and after change of length. *J. Physiol.* **117**, 77-86.

Akhter, S., Bueltmann, K., Huang, X. and Jin, J.-P. (2014). Restrictive cardiomyopathy mutations demonstrate functions of the C-terminal end-segment of troponin I. *Arch. Biochem. Biophys.* **552-553**, 3-10.

Bianco, P., Nagy, A., Kengyel, A., Szatmári, D., Mártonfalvi, Z., Huber, T. and Kellermayer, M. S. Z. (2007). Interaction forces between F-actin and titin PEVK domain measured with optical tweezers. *Biophys. J.* **93**, 2102-2109.

Buck, D., Smith, J. E., III, Chung, C. S., Ono, Y., Sorimachi, H., Labeit, S. and Granzier, H. L. (2014). Removal of immunoglobulin-like domains from titin's spring segment alters titin splicing in mouse skeletal muscle and causes myopathy. *J. Gen. Physiol.* **143**, 215-230.

DuVall, M. M., Gifford, J. L., Amrein, M. and Herzog, W. (2013). Altered mechanical properties of titin immunoglobulin domain 27 in the presence of calcium. *Eur. Biophys. J.* **42**, 301-307.

Edman, K. A., Elzinga, G. and Noble, M. I. (1978). Enhancement of mechanical performance by stretch during tetanic contractions of vertebrate skeletal muscle fibres. *J. Physiol.* **281**, 139-155.

Edman, K. A., Elzinga, G. and Noble, M. I. (1982). Residual force enhancement after stretch of contracting frog single muscle fibers. *J. Gen. Physiol.* **80**, 769-784.

Garvey, S. M., Rajan, C., Lerner, A. P., Frankel, W. N. and Cox, G. A. (2002). The muscular dystrophy with myositis (mdm) mouse mutation disrupts a skeletal muscle-specific domain of titin. *Genomics* **79**, 146-149.

Gordon, A. M., Huxley, A. F. and Julian, F. J. (1966). The variation in isometric tension with sarcomere length in vertebrate muscle fibres. *J. Physiol.* **184**, 170-192.

Granzier, H. L. and Labeit, S. (2004). The giant protein titin: a major player in myocardial mechanics, signaling, and disease. *Circ. Res.* **94**, 284-295.

Gregorio, C. C., Granzier, H., Sorimachi, H. and Labeit, S. (1999). Muscle assembly: a titanic achievement? *Curr. Opin. Cell Biol.* **11**, 18-25.

Herzog, W. (2014). Mechanisms of enhanced force production in lengthening (eccentric) muscle contractions. *J. Appl. Physiol.* **116**, 1407-1417.

Herzog, W. and Leonard, T. R. (2002). Force enhancement following stretching of skeletal muscle: a new mechanism. *J. Exp. Biol.* **205**, 1275-1283.

Huebsch, K. A., Kudryashova, E., Wooley, C. M., Sher, R. B., Seburn, K. L., Spencer, M. J. and Cox, G. A. (2005). Mdm muscular dystrophy: interactions with calpain 3 and a novel functional role for titin's N2A domain. *Hum. Mol. Genet.* **14**, 2801-2811.

Huxley, A. F. and Simmons, R. M. (1971). Proposed mechanism of force generation in striated muscle. *Nature* **233**, 533-538.

Joumaa, V., Rassier, D. E., Leonard, T. R. and Herzog, W. (2008). The origin of passive force enhancement in skeletal muscle. *Am. J. Physiol. Cell Physiol.* **294**, C74-C78.

Kellermayer, M. S. and Granzier, H. L. (1996). Calcium-dependent inhibition of in vitro thin-filament motility by native titin. *FEBS Lett.* **380**, 281-286.

Labeit, D., Watanabe, K., Witt, C., Fujita, H., Wu, Y., Lahmers, S., Funck, T., Labeit, S. and Granzier, H. (2003). Calcium-dependent molecular spring elements in the giant protein titin. *Proc. Natl. Acad. Sci. USA* **100**, 13716-13721.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Leonard, T. R. and Herzog, W. (2010). Regulation of muscle force in the absence of actin-myosin-based cross-bridge interaction. *Am. J. Physiol. Cell Physiol.* **299**, C14-C20.

Leonard, T. R., DuVall, M. and Herzog, W. (2010). Force enhancement following stretch in a single sarcomere. *Am. J. Physiol. Cell Physiol.* **299**, C1398-C1401.

Lopez, M. A., Pardo, P. S., Cox, G. A. and Boriek, A. M. (2008). Early mechanical dysfunction of the diaphragm in the muscular dystrophy with myositis (Ttnmdm) model. *Am. J. Physiol. Cell Physiol.* **295**, C1092-C1102.

Maruyama, K. (1976). Connectin, an elastic protein from myofibrils. *J. Biochem.* **80**, 405-407.

Monroy, J. A., Powers, K. L., Gilmore, L. A., Uyeno, T. A., Lindstedt, S. L. and Nishikawa, K. C. (2012). What is the role of titin in active muscle? *Exerc. Sport Sci. Rev.* **40**, 73-78.

Nishikawa, K. C., Monroy, J. A., Uyeno, T. E., Yeo, S. H., Pai, D. K. and Lindstedt, S. L. (2012). Is titin a 'winding filament'? A new twist on muscle contraction. *Proc. R. Soc. B Biol. Sci.* **279**, 981-990.

Noble, M. I. (1992). Enhancement of mechanical performance of striated muscle by stretch during contraction. *Exp. Physiol.* **77**, 539-552.

Powers, K., Schappacher-Tilp, G., Jinha, A., Leonard, T., Nishikawa, K. and Herzog, W. (2014). Titin force is enhanced in actively stretched skeletal muscle. *J. Exp. Biol.* **217**, 3629-3636.

Rassier, D. E. (2012). Residual force enhancement in skeletal muscles: one sarcomere after the other. *J. Muscle Res. Cell Motil.* **33**, 155-165.

Raynaud, F., Astier, C. and Benyamin, Y. (2004). Evidence for a direct but sequential binding of titin to tropomyosin and actin filaments. *Biochim. Biophys. Acta* **1700**, 171-178.

Schappacher-Tilp, G., Leonard, T., Desch, G. and Herzog, W. (2015). A Novel three-filament model of force generation in eccentric contraction of skeletal muscles. *PLoS ONE* **10**, e0117634.

Taylor-Burt, K. R., Monroy, J., Pace, C., Lindstedt, S. and Nishikawa, K. C. (2015). Shiver me titin! Elucidating titin's role in shivering thermogenesis. *J. Exp. Biol.* **218**, 694-702.

Witt, C. C., Ono, Y., Puschmann, E., McNabb, M., Wu, Y., Gotthardt, M., Witt, S. H., Haak, M., Labeit, D., Gregorio, C. C. et al. (2004). Induction and myofibrillar 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.