

Effects of a titin mutation on negative work during stretch-shortening cycles in skeletal muscles.

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Summary Statement

Active muscles from mice with a small titin deletion exhibit large deficits in net negative work, suggesting that titin contributes to energy storage and dissipation during stretch-shortening cycles.

Abstract

Negative work occurs in muscles during braking movements such as downhill walking or landing after a jump. When performing negative work during stretch-shortening cycles, viscoelastic structures within muscles store energy during stretch, return a fraction of this energy during shortening, and dissipate the remaining energy as heat. Because tendons and extracellular matrix are relatively elastic rather than viscoelastic, energy is mainly dissipated by cross bridges and titin. Recent studies demonstrate that titin stiffness increases in active skeletal muscles, suggesting that titin contributions to negative work may have been underestimated in previous studies. The muscular dystrophy with myositis (*mdm*) mutation in mice results in a deletion in titin that leads to reduced titin stiffness in active muscle, providing an opportunity to investigate the contribution of titin to negative work in stretch-shortening cycles. Using the work loop technique, extensor digitorum longus and soleus muscles from *mdm* and wild type mice were stimulated during the stretch phase of stretch-shortening cycles to investigate negative work. The results demonstrate that, compared to wild type muscles, negative work is reduced in muscles from *mdm* mice. We suggest that changes in the viscoelastic properties of *mdm* titin reduce energy storage by muscles during stretch and energy dissipation during shortening. Maximum isometric stress is also reduced in muscles from *mdm* mice, possibly due to impaired transmission of cross bridge force, impaired cross bridge function, or both. Functionally, the reduction in negative work could lead to increased muscle damage during eccentric contractions that occur during braking movements.

Abbreviations. DF, degrees of freedom; EDL, extensor digitorum longus; L_0 , optimal muscle length; mDa, megaDaltons; *mdm*, muscular dystrophy with myositis; MM, muscle mass; RFD, rate of force development during stretch; P:A ratio, ratio of passive to active work; P_0 , maximum isometric stress.

Introduction

To meet the varying demands of locomotion, muscles function as motors, brakes, springs and struts (Dickinson et al., 2000). While most studies focus on muscles as motors that produce positive work for propulsion, negative work during braking is also important. By convention, positive work occurs during concentric contraction, whereas negative work occurs during eccentric contraction (James et al., 1995; James et al., 1996; Lindstedt, 2016). Negative work is functionally relevant during everyday movements that involve deceleration, such as walking downhill or down stairs, postural control (Lindstedt, 2016), or landing after a jump (Yeow et al., 2011). During these movements, negative work results when viscoelastic structures store kinetic energy during stretch and dissipate energy as heat during shortening – a useful mechanism for reducing velocity (Lindstedt, 2016). Lindstedt et al. (2001) estimated that a 70-kg person descending 500 m absorbs ~350 kJ of energy, enough to increase body temperature by 4-5°C. Defects in muscle braking during downhill walking could result in muscle damage if viscous and elastic properties fail to prevent over-stretch.

Previous studies have focused on identifying the structures in muscle-tendon units that store energy during stretch and dissipate energy during shortening, resulting in negative work. Tendons and collagen contribute little to net negative work because their forces are nearly the same during shortening as during stretch (Matson et al., 2012; Roberts, 2016). In contrast, cross bridges (De Winkel et al., 1995; Proske and Morgan, 1999) and titin (Bianco et al., 2007; Herzog et al., 2014; Minajeva et al., 2001) are viscoelastic structures that store energy during stretch and dissipate energy during shortening.

Historically, energy storage during stretch has been attributed to cross bridge properties (Huxley and Simmons, 1971; Lombardi and Piazzesi, 1990; Piazzesi and Lombardi, 1995). However, more recent studies suggest that cross bridges alone cannot account for energy storage during active stretch. In experiments that measured both heat and force during ramp stretches of single frog fibers, Linari et al. (2003) estimated that cross bridges account for only ~12% of the maximum energy stored during active stretch. Their cross bridge model, based on earlier models (Lombardi and Piazzesi, 1990; Piazzesi and Lombardi, 1995), included energy contributions from cross bridge elasticity (~2.2% of total energy storage; Linari et al., 2000) and redistribution of cross bridge states (~9.8% of total energy storage).

In a similar study, Pinniger et al. (2006) used a model based on Lynn and Taylor (1971) to estimate the contribution of cross bridges to increased force during stretch of rat fiber bundles. In contrast to Linari et al. (2003), their model assumed exponential strain dependence of cross bridge detachment rates. Pinniger et al. (2006) concluded that cross bridges could account for increasing muscle force from the onset of stretch through the 'P2 transition' (~18 nm/half-sarcomere), after which energy was stored in non-cross bridge components, likely titin.

Both Linari et al. (2003) and Pinniger et al. (2006) suggested that titin contributes significantly to energy storage during active stretch. At a size of ~3-4 mDa, titin is the largest known protein and spans the entire half sarcomere (Bang et al., 2001). Titin was first identified as a third filament in muscle sarcomeres by Maruyama (1976). The I-band region of titin contains two serially linked spring elements; tandem immunoglobulin (Ig) domains and the PEVK segment (Gautel and Goulding, 1996). Elongation of the I-band region of titin with sarcomere stretch (Linke et al., 1998b; Trombitás et al., 1998a) is thought to be a main source of passive tension in myofibrils (Horowitz, 1999; Horowitz et al., 1986; Linke et al., 1996; Maruyama, 1976), contributing up to 98% of passive force (Wang et al., 1993).

Although titin stiffness in passive muscle is too small to explain force enhancement during active stretch (Labeit et al., 2003; Linke et al., 1998b), recent studies in single myofibrils (Leonard and Herzog, 2010; Monroy et al., 2017; Powers et al., 2014; Powers et al., 2016) and intact muscles (Monroy et al., 2017) demonstrate that titin stiffness increases upon activation. It is therefore likely that earlier studies (Linari et al., 2003; Pinniger et al., 2006) underestimated titin's contribution to energy storage during stretch. Increased titin stiffness in active muscle is thought to explain residual force enhancement (Leonard and Herzog, 2010; Herzog, 2014; Herzog et al., 2016; Hessel et al., 2017; Lindstedt and Nishikawa, 2017; Nocella et al., 2014). Due to its length and viscoelastic properties (Bianco et al., 2007; Martonfalvi et al., 2014), titin is uniquely suited to store kinetic energy during stretch and also to dissipate energy as heat during shortening.

Stretch-shortening cycles are often used to evaluate muscle function (Ahn, 2012; James et al., 1995; Josephson, 1985; Sawicki et al., 2015). Many such studies have focused on maximizing net positive work by stimulating muscles during the shortening phase of the cycle (Askew and Marsh, 1997; Askew and Marsh, 1998; James et al., 1995; Josephson, 1985). Though seldom studied, negative work is also important for safe and effective

locomotion (Lindstedt, 2016; Lindstedt et al., 2001). In the present study, we used the work loop technique (Josephson, 1985) to investigate negative work during stretch-shortening cycles. We stimulated muscles during lengthening, which provides a novel approach for quantifying negative work during cyclic movements.

The purpose of this study is to test the hypothesis that titin contributes to energy storage and dissipation during stretch-shortening cycles by comparing negative work loops in skeletal muscles from wild type and *mdm* (muscular dystrophy with myositis) mice. *Mdm* mice carry a 779 base pair deletion in the titin gene that leads to a predicted ~83 amino acid deletion in the N2A and proximal PEVK regions of titin in skeletal muscles (Garvey et al., 2002). Powers et al. (2016) reported that the increase in titin stiffness that normally occurs upon muscle activation in wild type myofibrils (Powers et al., 2014) is impaired in myofibrils from *mdm* psoas, perhaps due to deletion of amino acids critical for increasing titin stiffness in active muscle. Monroy et al. (2017) also found that intact soleus muscles from *mdm* mice were actively more compliant than wild-type muscles in rapid unloading tests. If titin contributes to energy storage and recovery during stretch-shortening cycles, then we predict that *mdm* muscles will store less energy during stretch and dissipate less energy during shortening, thereby reducing net negative work.

The effects of the *mdm* mutation may also vary among muscles due to differences in myosin isoforms (Kushmerick et al., 1992; Schiaffino and Reggiani, 2011), calcium cycling (Barclay, 2012), and/or titin isoform expression (Bang et al., 2001; Freiburg et al., 2000; Granzier et al., 2007; Prado et al., 2005). To test whether the effects of the *mdm* mutation vary among muscles, we quantified negative work in predominantly slow twitch soleus and predominantly fast twitch extensor digitorum longus (EDL) muscles (Kushmerick et al., 1992) from wild type and *mdm* mice.

Methods

Animal experiments were approved by the Institutional Animal Care and Use Committee of Northern Arizona University, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Animal surgery and experimental protocol

Heterozygous mice of the strain B6C3Fe a/a-Ttn *mdm* /J were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). A breeding colony was established to obtain wild type and homozygous recessive (*mdm*) mice. All mice had food and water *ad libitum*. Body mass was greater in wild type mice (20.81 ± 0.81 g) than in age-matched *mdm* mutants (6.14 ± 0.18 g; t-test, $p < 0.001$, age range 30-50 days old), as reported previously (Garvey et al., 2002; Taylor-Burt et al., 2015). Soleus ($n = 5$ *mdm* and $n = 8$ wild type) and EDL ($n = 7$ *mdm* and $n = 7$ wild type) muscles were prepared for *ex vivo* testing using standard procedures (Brooks and Faulkner, 1988; Hakim et al., 2013). EDL and soleus muscles of anesthetized mice were exposed surgically, and 4-0 silk sutures were tied to the distal and proximal ends at the muscle-tendon junction including as little tendon as possible without damaging the muscle fibers. The tendons were cut outside of the suture knots to extract the muscles. Wet muscle mass was smaller in *mdm* EDL and soleus compared to wild type (t-tests, $P > 0.05$, Table 1).

Extracted muscles were attached to a dual-mode muscle lever system (Aurora Scientific, Inc., Series 300B, Aurora, ON, Canada). Throughout all experiments, the muscles were bathed in a 21°C Krebs-Henseleit solution containing (in mM): NaCl (118); KCl (4.75); MgSO₄ (1.18); NaHCO₃ (24.8); KH₂PO₄ (1.18); CaCl₂ (2.54); glucose (10.0). The bath was aerated with a 95% O₂ : 5% CO₂ gas mixture. Each muscle was surrounded by two parallel platinum electrodes, which delivered 1 ms square pulses at supramaximal voltage (70 mV). The stimulation frequency was 200 Hz for the EDL and 130 Hz for the soleus (James et al., 1995). A custom LabVIEW (National Instruments Corp., Austin, Texas, USA) program was used to control the lever motor and record force, length and time at a sampling rate of 4 kHz. Muscle physiological cross-sectional area, measured using standard methods (Hakim et al., 2013), was used to calculate muscle stress (N/cm²). Maximum isometric stress (P_0) and optimal muscle length (L_0) were found by repeated tetanic stimulation at increasing muscle lengths, until a maximum value was identified. L_0 and P_0 were smaller in *mdm* EDL and soleus compared to wild type (t-tests, $P > 0.05$, Table 1).

The muscles were subjected to a work loop protocol similar to that described by James et al. (1995). Each work loop experiment began with muscles at L_0 . The muscles were first shortened, then lengthened, and finally shortened back to L_0 . Each muscle was moved through a 4 Hz sinusoidal length change between $L_0 \pm 5\%$ with a total strain amplitude of

10% L_0 . The stimulation duration was 40 ms, beginning 4 ms before the onset of lengthening and ending after $\sim 1/3$ of the total lengthening phase. To assess the contribution of passive structures (collagen, extracellular matrix, titin) to net negative work, the same work loop experiments described above were performed for each muscle with no stimulation.

Force and length data were analyzed using a custom MATLAB program (MathWorks Inc., Natick, MA, USA). To quantify the effect of stretch on active muscle force, we measured: 1) the rate of force development during active stretch (the change in force during the first 36 ms of lengthening divided by the time, normalized to stress per second, $\text{Ncm}^{-1}\text{s}^{-1}$); 2) the maximum stress during stretch (normalized to % P_0); and 3) the time to maximum stress (ms), measured from the onset of stimulation until the maximum stress was reached. Net negative work (J), a measure of the total energy dissipated, was calculated for each work loop as the area within the curve (James et al., 1995). By convention, negative work during a work loop occurs when forces are larger during stretch than during shortening (Josephson, 1985). Specific work (J/Kg) is equal to net work divided by muscle wet mass. Both active and passive specific work were measured for each muscle. To compare the relative contributions of passive and active net work, we calculated the ratio of passive work to active work (P:A ratio).

A 2-way factorial ANOVA was used to compare dependent variables (rate of force development during stretch, time to maximum stress during stretch, maximum stress during stretch, active work, passive work, P:A ratio, and P_0) between muscles and genotypes. The fixed factors were genotype (wild type, *mdm*), muscle (EDL, soleus), and the muscle x genotype interaction. A significant interaction would indicate that the soleus and EDL muscles are differentially affected by the *mdm* mutation. P-values < 0.05 were considered significant. The assumptions of normality and homogeneity in the residuals were tested using Shapiro-Wilk and Levene's tests, respectively. *Mdm* residuals were more variable than wild type for all dependent variables. All variables met the assumptions of normality and homoscedasticity after using best Box-Cox transformations. If an effect was significant, Tukey's pairwise comparisons and LS means plot analyses were used to compare group means. Data are presented as mean \pm standard error. Statistical analysis was conducted using JMP (JMP Pro 12.2, SAS Institute Inc., Cary, NC, USA).

Results

Work loops

Differences in work loop size and shape were observed between genotypes (wild type vs. *mdm*) and muscles (EDL and soleus). In wild type soleus muscles (Fig. 1A), stretch-shortening cycles were characterized by a rapid rise in stress during stretch that continued after the final stimulation pulse. Stress remained constant, or decreased slightly, until the onset of shortening. The maximum stress produced during stretch was larger than the maximum isometric stress (P_0) for wild type and *mdm* soleus. During shortening, stress decreased continuously and returned to passive levels before the end of shortening (Fig. 1A). Work loops from *mdm* soleus were smaller in area relative to wild type soleus (Fig. 1A). In passive stretch-shortening cycles, wild type and *mdm* soleus muscles exhibit typical hysteresis curves for passive muscle, with smaller stresses during shortening than during stretch (Fig. 1B). In *mdm* soleus, the passive stress often exceeded the active stress at the end of stretch (Figs. 1A, B).

During stretch-shortening cycles in wild type EDL, there was a rapid rise in stress above the maximum isometric level that reached a peak shortly after deactivation, and in most cases returned to passive levels before the end of lengthening (Fig. 2A). As for soleus, work loops from *mdm* EDL were smaller than for wild type EDL (Fig. 2A). In passive stretch-shortening cycles, wild type and *mdm* EDL muscles also exhibited typical hysteresis curves for passive muscles, with smaller forces during shortening than during stretch (Fig. 2B).

The area contained within a negative work loop quantifies the magnitude of the energy dissipated in each stretch-shortening cycle. The greater the area, the more energy dissipated. With few previously published papers on negative work in stretch-shortening cycles, we compared our measurements from wild type muscles to those of peak positive work reported by James et al. (1995), who performed 4 Hz stretch-shortening cycles using whole mouse EDL and soleus muscles over the same length range used in the present study. In that study, the stimulation pattern optimized positive work, which was approximately opposite to our stimulation pattern, with activation shortly before the end of lengthening and continuing for most of shortening. In James et al.'s (1995) study, the EDL produced 18.9 J/Kg of positive work, compared to 14.6 J/Kg negative work in this study (Fig. 3). The soleus produced 6.7 J/Kg of positive work (James et al., 1995); Fig. 2) compared to 15 J/Kg of

negative work in this study (Fig. 3). Given that we did not optimize the stimulation pattern to maximize negative work, and that we used a stimulation duration only 1/3 that of James et al. (1995), this comparison suggests that the EDL and soleus muscles are capable of producing substantial amounts of negative work relative to positive work in stretch-shortening cycles.

Differences between wild type and *mdm* muscles

The rate of force development during stretch was smaller ($F = 16.16$, $P = 0.0006$), active work was larger ($F = 48.16$, $P < 0.0001$), passive work was smaller ($F = 5.64$, $P = 0.03$), P:A ratio was larger ($F = 97.88$, $P < 0.0001$), and P_0 was smaller ($F = 46.03$, $P < 0.0001$) in muscles from *mdm* mice compared to wild type muscles (Table 2; Fig. 3). There was no difference between genotypes in maximum stress during stretch normalized to P_0 ($F = 0.46$, $P = 0.39$) or time to maximum stress ($F = 1.03$, $P = 0.46$). The lack of difference in time to maximum stress during stretch between genotypes for both soleus and EDL muscles suggests that deactivation kinetics, including calcium re-uptake (Calderón et al., 2014), are similar in wild type and *mdm* muscles.

Differences between soleus and EDL muscles

Compared to soleus muscles (Table 2; Fig. 3), EDL muscles had a larger rate of force development ($F = 24.10$, $P < 0.0001$), a shorter time to maximum stress ($F = 19.62$, $P = 0.0002$), and a larger P_0 ($F = 13.13$, $P = 0.001$) regardless of genotype. There were no differences between muscles in maximum stress during stretch ($F = 3.78$, $P = 0.065$), active work ($F = 0.28$, $P = 0.60$), passive work ($F = 0.29$, $P = 0.60$), or P:A ratio ($F = 2.29$, $P = 0.14$). As in previous studies (James et al., 1995), we found that the behavior of EDL and soleus muscles differed during stretch-shortening cycles. The predominantly fast twitch EDL exhibited a larger rate of force development, larger P_0 , and shorter time to maximum stress during stretch than predominantly slow twitch soleus muscles. These differences between muscles likely arise from differences in myosin isoforms and calcium handling (Barclay, 2012; Schiaffino and Reggiani, 2011) between fast and slow twitch fibers.

Genotype by muscle interactions

Soleus and EDL muscles differed in the effects of the *mdm* mutation. Active work ($F = 4.24$, $P = 0.05$; Table 2; Fig. 3A) and passive work ($F = 5.42$, $P = 0.03$; Table 2; Fig. 3B) were larger in *mdm* EDL than *mdm* soleus, and P_0 was larger in *mdm* EDL than in *mdm* soleus ($F = 5.44$, $P = 0.03$; Tables 1 and 2). No significant genotype x muscle interaction

(Table 2) was observed for P:A ratio ($F = 0.42$, $P = 0.52$; Fig. 3C), rate of force development during stretch ($F = 0.28$, $P = 0.60$; Fig. 3D), maximum stress during stretch ($F = 0.008$, $P = 0.93$; Fig. 3E), or time to maximum stress ($F = 0.012$, $P = 0.91$; Fig. 3F). These results suggest that the *mdm* mutation had a smaller effect on the EDL than on the soleus muscle.

Discussion

Differences in work loop characteristics between wild type and mdm muscles

Muscular dystrophy with myositis (*mdm*) is a recessive mutation that results in a 779 base pair deletion and a predicted ~83-amino acid deletion at the border between the N2A and PEVK regions of titin (Garvey et al., 2002). Previous studies demonstrate that *mdm* muscles exhibit increased passive tension compared to wild type muscles (Lopez et al., 2008; Monroy et al., 2017; Powers et al., 2017) due to an increase in collagen content (Powers et al., 2017). No increase in passive stress was observed in myofibrils from *mdm* psoas (Powers et al., 2016).

In contrast to wild type myofibrils (Powers et al., 2014), there is no increase in titin stiffness with calcium activation in *mdm* myofibrils (Powers et al., 2016). Failure of titin activation in intact *mdm* soleus is also observed during rapid unloading (Monroy et al., 2017). These studies demonstrate that skeletal muscles of *mdm* mice are actively more compliant than muscles from wild type mice (Lopez et al., 2008; Monroy et al., 2017; Taylor-Burt et al., 2015). In addition to titin-based active stiffness, maximum isometric stress is also reduced in *mdm* muscles compared to wild type muscles (see Table 1). This decrease in active stress has been observed in intact *mdm* soleus (Monroy et al., 2017; Taylor-Burt et al., 2015), in fiber bundles from *mdm* diaphragm (Lopez et al., 2008), and in single *mdm* psoas myofibrils (Powers et al., 2016). These observations suggest that titin or cross bridges, or both, may contribute to the deficit in negative work in *mdm* muscles.

When we performed stretch-shortening experiments in which muscles were stimulated while lengthening, we found that *mdm* EDL and soleus muscles exhibited a smaller rate of force development during active stretch, indicating that they store less energy during stretch than wild type muscles. We also found that *mdm* EDL and soleus muscles had a reduced ability to dissipate kinetic energy during shortening compared to wild type muscles, as indicated by their reduced negative work. Our results further showed that active work is significantly smaller relative to passive work (larger P:A ratio) in *mdm* muscles compared to wild type.

These findings are consistent with previous observations that the increase in titin stiffness that normally occurs upon activation is impaired in *mdm* muscles (Powers et al., 2016). Our results also demonstrate that passive work accounts for only a fraction of net negative work in both wild type and *mdm* muscles, as indicated by the small P:A ratios. For both *mdm* soleus and EDL, the observed increase in passive work was too small to compensate for the much larger reduction in negative work. Finally, the magnitude of the change was larger in soleus than EDL for active work, passive work, and P_0 , perhaps because passive stiffness is larger in *mdm* EDL compared to *mdm* soleus (see below; Freiburg et al., 2000). Furthermore, Heimann et al. (1996) noted that central nuclei, indicative of regeneration, appear earlier in soleus muscles from *mdm* mice than in other skeletal muscles. Many inherited myopathies exhibit phenotypic effects that vary among muscles, although the reasons for muscle-specific changes remain unknown (Ciciliot et al., 2013).

What structures are responsible for negative work in skeletal muscle?

The potential contribution of viscoelastic elements to negative work in a stretch-shortening cycle is governed not only by the amount of energy absorbed during stretch, but also by the amount of energy dissipated during shortening (Lindstedt, 2016). Negative work in stretch-shortening cycles requires that forces in viscoelastic structures are larger during lengthening than during shortening, due to energy dissipation. During stretch-shortening cycles, tendons forces are nearly equal during lengthening and shortening (Matson et al., 2012), suggesting that they do not contribute to net negative work during cyclic length changes (Holt et al., 2014). The same is true for most other collagen-based structures in muscle (Roberts, 2016). In contrast, both cross bridges (De Winkel et al., 1995; Proske and Morgan, 1999) and titin (Bianco et al., 2007; Mártonfalvi et al., 2014; Minajeva et al., 2001) are viscoelastic, storing energy during active stretch (Linari et al., 2003; Pinniger et al., 2006; Roots et al., 2007) and dissipating energy during shortening (Corr and Herzog, 2016; Schappacher-Tilp et al., 2015). Viscoelasticity of titin (Martonfalvi et al., 2014) and cross bridges (Proske and Morgan, 1999) likely involves a variety of complex mechanisms that are related but not limited to force enhancement and depression.

Cross bridges.- Lombardi and Piazzesi (1990) showed that a cross bridge model with three attached states and two detached states is compatible with experimental observations of increased force during stretch (Lombardi and Piazzesi, 1990), but required a 200-fold

increase in the reattachment rate of cross bridges forcibly detached from actin compared to unstrained cross bridges, an assumption for which there is no experimental support. Linari et al. (2003) used a modified cross bridge model (Piazzesi and Lombardi, 1995) to predict energy storage during stretch. Their model estimated that ~2.2% of the energy stored during stretch could be attributed to cross bridge strain, whereas up to 9.8% could be stored by redistributing cross bridges to higher energy states (Linari et al., 2003).

While these models demonstrate the potential for cross bridges to contribute to enhanced force during stretch, Linari et al.'s (2003) model and other observations suggest that cross bridges alone cannot explain the increase in muscle force during active stretch. Pinniger et al. (2006) found that, during ramp stretches of 5% L_0 , ~40% of the total energy storage remained unexplained by the strain of cross bridges or thick and thin filaments. Pinniger et al. (2006) also suggested that myosin heads can store elastic energy during stretch only up to ~18 nm/half-sarcomere (~1.5% L_0 , the P2 transition; Lombardi and Piazzesi, 1990), at which point they detach from the thin filaments. However, energy storage and force enhancement by muscles continue beyond the P2 transition, not only *in vitro* (Linari et al., 2003) but also during natural movements such as locomotion in mice (James et al., 1995). Therefore, it is likely that a more extensible elastic element, such as titin, also contributes to increased muscle force during stretch (Roots et al., 2007).

Titin. - Because titin is extensible (Linke et al., 1996; Linke et al., 1998b; Trombitás et al., 1998b) and viscoelastic (Herzog et al., 2014; Mártonfalvi et al., 2014), and because its stiffness increases upon muscle activation (Leonard and Herzog, 2010; Powers et al., 2016), titin is a likely candidate for energy storage during active muscle stretch, contributing to energy storage during eccentric contractions as suggested in previous studies (Linari et al., 2003; Pinniger et al., 2006). Mártonfalvi et al. (2014) stretched and shortened single titin molecules using high-resolution optical tweezers. During stretch-shortening cycles at constant velocity, they found that the force of single titin molecules increased substantially during stretch, and decreased quickly during shortening, producing significant negative work. Similar observations have been reported for single myofibrils as well (Herzog et al., 2014). In contrast to cross bridges, the I-band region of titin can extend up to several hundred nanometers during stretch (Linke et al., 1998a; Linke et al., 1998b), and therefore can potentially store energy during stretches to lengths beyond the P2 transition ($> 1.5 \% L_0$).

Both Linari et al. (2003) and Pinniger et al. (2006) concluded that, in addition to cross bridges, titin is likely a major contributor to energy storage during active stretch of skeletal muscles. Linari et al. (2003) estimated the contribution of titin using a model of passive tension based on force-extension measurements from single titin molecules (Kellermayer et al., 2001) and Morgan's (Morgan, 1990; Morgan, 1994) theory of sarcomere inhomogeneity, in which a small fraction (4.5%) of highly extended sarcomeres contributes to energy storage. This model estimated that passive titin could contribute up to 16.3 % of the total energy stored during stretch (Linari et al., 2003). In contrast, Pinniger et al. (2006) assumed that all energy beyond the P2 transition was stored in non-cross bridge structures, presumably titin. Thus, titin could account for an increased force during stretch up to 36.5% of P_0 , or ~40% of total energy storage.

Our understanding of titin in active muscles has advanced since Linari et al. (2003) and Pinniger et al. (2006) estimated titin energy storage during stretch. Recent experiments demonstrate that titin stiffness increases in active muscle (Leonard and Herzog, 2010; Powers et al., 2014; Powers et al., 2016). Leonard and Herzog (2010) stretched passive and active myofibrils beyond filament overlap, where cross bridges can no longer contribute to myofibrillar stiffness. They found that titin stiffness increases upon calcium activation. In similar experiments, Powers et al. (2014) also found that titin stiffness increases upon calcium activation of wild type myofibrils, but not in *mdm* myofibrils (Powers et al., 2016). Titin stiffness appears to increase by a factor of ~3-4 in active compared to passive muscle (Leonard and Herzog, 2010; Powers et al., 2016; Monroy et al., 2017). It has been suggested that calcium-dependent titin-actin interactions may contribute to increased titin stiffness in active muscle (Herzog et al., 2016; Nishikawa, 2016; Schappacher-Tilp et al., 2015). The mechanism(s) responsible for the increase in titin stiffness during activation appear to be impaired in *mdm* muscles (Powers et al., 2016), and are currently under investigation (Hessel et al., 2017; Nishikawa, 2016).

Decreased active stiffness of titin in *mdm* muscles (Powers et al., 2016) likely contributes to the decrease in rate of force development and active work during stretch – shortening cycles observed in this study. While the contribution of titin has likely been underestimated in previous studies (Linari et al., 2003; Pinniger et al., 2006), it is difficult to estimate the potential contribution of titin viscoelasticity to muscle force during active stretch. Although increased titin stiffness in active muscle is thought to account for residual force enhancement that persists after stretch (Herzog et al. 2016), this purely elastic titin-

based force is too small to account for the larger increase in force that occurs during stretch. During stretch, viscous forces from cross bridges and titin may also contribute to force enhancement. Yet, stretching rates are typically too slow in single molecule and myofibril experiments (<250 nm/s; (Kellermayer et al., 2001; Leonard and Herzog, 2010; Linke et al., 1998b; Mártonfalvi et al., 2014; Powers et al., 2016) to allow estimation of viscous forces.

Bianco et al. (2007) estimated viscous forces that result from PEVK-actin interactions by stretching single PEVK constructs over a wide range of stretch rates from 250 - 5000 nm/s. We used data from Bianco et al. (2007) stretching titin PPAK constructs at 1250 nm/s (compared to 960 nm/s in the present study and up to ~1700 nm/s in Linari et al., 2003) to estimate the expected energy storage due to viscous forces in titin during active stretch. Our analysis assumes that only PEVK titin contributes to force during stretch of active muscle due to calcium-dependent interactions between titin and thin filaments (Leonard and Herzog, 2010; Nishikawa et al., 2012; Powers et al., 2014; Schappacher-Tilp et al., 2015). At a stretch rate of 1250 nm/s, Bianco et al. (2007) estimated a viscous force of ~32 pN per titin (see their Fig. 4) which, following Linari et al.'s (2003) calculations, could account for up to 23.8% of the total energy absorbed during stretch of active muscles, in contrast to Linari et al.'s (2003) original estimate that passive titin could account for ~ 16.3% of the total energy. These calculations likely underestimate the viscous forces in PEVK titin during stretch of active muscle due to the likelihood that PEVK binds to each actin filament at multiple sites (Bianco et al., 2007).

The foregoing considerations suggest that cross bridges alone cannot account for energy storage during stretch of active muscle and that the contribution of titin to energy storage has likely been underestimated in previous studies. Nevertheless, it is also likely that cross bridge forces are reduced in *mdm* muscles, either via impaired force transmission or altered cross bridge kinetics. As in previous studies (Lopez et al., 2008; Monroy et al., 2017; Powers et al., 2016), we found that maximum isometric stress is reduced in *mdm* muscles. We also found that the maximum stress during the stretch phase of active work loops in *mdm* muscles decreased in proportion to the reduction in P_0 , suggesting that cross bridges also contribute to the reduction of negative work in *mdm* muscles. Although no direct evidence suggests that cross bridge function *per se* is impaired in *mdm* muscles, some fibers have weak striation patterns at 30-50 days of age, which could contribute to reduced isometric stress (Powers et al., 2017). The actin and myosin content of single *mdm* myofibrils appears normal (Powers et al., 2016), and gene expression studies show little or no change in expression of

thick or thin filament proteins, or proteins involved in calcium cycling (Witt et al., 2004). Nonetheless, an analysis of cross bridge kinetics in *mdm* muscles, for example by measuring ATP hydrolysis rates (Barclay, 2012), remains to be conducted.

While further work is required to test the hypothesis that cross bridge function *per se* is impaired in muscles from *mdm* mice, it is likely that the reduced active stiffness of titin impairs transmission of cross bridge forces in muscle sarcomeres (Lindstedt and Nishikawa, 2017; Nishikawa, 2016). This hypothesis is consistent with the results of Horowitz et al. (1986), who demonstrated that selectively degrading titin with low doses of ionizing radiation resulted in decreased tension in single muscle fibers. Furthermore, Higuchi (1996) found that short exposure of skinned fibers to trypsin also reduced isometric force. In agreement with Higuchi (1996), Leonard & Herzog (2010) found that degradation of titin using mild trypsin digestion completely eliminated both passive and active force in single myofibrils stretched beyond overlap of the thick and thin filaments. Taken together, these studies suggest a role for titin in transmission of cross-bridge forces from A-band to Z-line.

Studies of other inherited muscle diseases also suggest a role for titin in force transmission. Patients with Ehlers-Danlos Syndrome express a stiffer isoform of titin in skeletal muscle (Ottenheijm et al., 2012). By increasing submaximal force production, the increased stiffness of titin partly compensates for muscle weakness due to increased compliance of the extracellular matrix. The markedly higher titin-based stiffness likely increases calcium sensitivity of force production. Based on this line of reasoning, it seems possible that the larger passive stiffness of *mdm* EDL compared to soleus muscles (Freiburg et al., 2000) could contribute to the relatively smaller effect of the *mdm* mutation on EDL compared to soleus observed in this study.

Comparison between *in vitro* and *in vivo* muscle work

It is interesting to consider how the deficit in negative work in *mdm* muscles might affect locomotor biomechanics in the mouse. Pace et al. (2017) compared limb morphology and walking kinematics between *mdm* and wild type mice. They found that differences in limb proportions (i.e., relative thigh, shank, metatarsal and toe lengths) between wild type and *mdm* mice were small, and these subtle differences in limb proportions did not account for larger changes in walking kinematics. *Mdm* mice display relatively large decreases in duty

factor (from 0.69 in wild type to 0.62 in *mdm*) and ankle range of motion (from $\sim 67^\circ - 110^\circ$ in wild type to $\sim 67^\circ - 80^\circ$ in *mdm*) during walking (Pace et al., 2017).

Compared to wild type mice, *mdm* mice exhibit a nearly complete absence of ankle dorsiflexion at the start of the stance phase (Pace et al., 2017), despite the fact that the ankle range of motion is normal during manipulation of the joint. During walking, energy absorbed by the posterior muscles of the shank is subsequently recovered to amplify work and power during plantarflexion (Lipfert et al., 2014; Roberts and Azizi, 2011). Therefore, reduced dorsiflexion likely limits the speed and efficiency of walking in *mdm* mice. Pace et al. (2017) concluded that an increase in passive stiffness of *mdm* muscles (Monroy et al., 2017) and other compensatory mechanism may lead to reduced ankle range of motion to protect the shank muscles from damage. Our results support this interpretation. The deficits in energy storage and dissipation in muscles from *mdm* mice could increase muscle lengthening during locomotion, potentially leading to muscle damage.

In contrast to level walking, muscles absorb energy when actively lengthened and subsequently dissipate energy as heat, functioning like shock-absorbers (Lindstedt et al., 2001). In *mdm* muscles, both energy storage and shock-absorbing functions of muscles are impaired. In contrast to our experimental design, in which length changes are imposed on muscles by a lever, muscles *in vivo* must produce enough force during active stretch to prevent lengthening, or excessive lengthening will destabilize movement and result in muscle damage. Our results predict that, in comparison to wild-type mice, *mdm* mice should experience particular difficulty in controlling their limb movements and velocity during downhill walking. A biomechanical analysis of *mdm* mice walking down a treadmill could provide additional insights into the consequences of reduced negative work on gait kinematics and stability.

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Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1: Morphological and physiological characteristics of wild type and *mdm* muscles. MM = muscle mass, L_0 = optimal muscle length, P_0 = maximal isometric stress. * indicates significant difference between wild type and *mdm* muscles (t-tests, $P < 0.05$).

	Soleus		EDL	
	<i>Mdm</i>	Wild type	<i>Mdm</i>	Wild type
MM (mg)	$1.66 \pm 0.58^*$	6.10 ± 0.54	$2.39 \pm 0.35^*$	8.33 ± 0.83
L_0 (mm)	$5.57 \pm 0.18^*$	9.31 ± 0.09	$5.21 \pm 0.10^*$	8.99 ± 0.17
P_0 (N/cm ²)	$3.66 \pm 0.89^*$	16.71 ± 0.90	$11.31 \pm 2.01^*$	20.23 ± 2.90

Table 2: Results of 2 -way ANOVA for work loop characteristics. Results are presented as (F-statistic, P-value). * = $P < 0.05$. For each ANOVA, DF = 1, 23.

	Genotype		Muscle		Genotype*Muscle	
	F	P	F	P	F	P
RFD	16.16	0.0006*	24.10	<0.0001*	0.28	0.60
Time to Max Stress	2.64	0.46	19.62	0.0002*	0.012	0.91
Max Stress (% P_0)	0.78	0.39	3.78	0.065	0.008	0.93
Passive Work	5.64	0.03*	0.29	0.60	5.42	0.03*
Active Work	48.16	< 0.0001*	0.28	0.60	4.24	0.05*
P:A Ratio	97.88	<0.0001*	2.29	0.14	0.42	0.52
P_0	46.03	<0.0001*	13.14	0.001*	5.44	0.03*

Figures

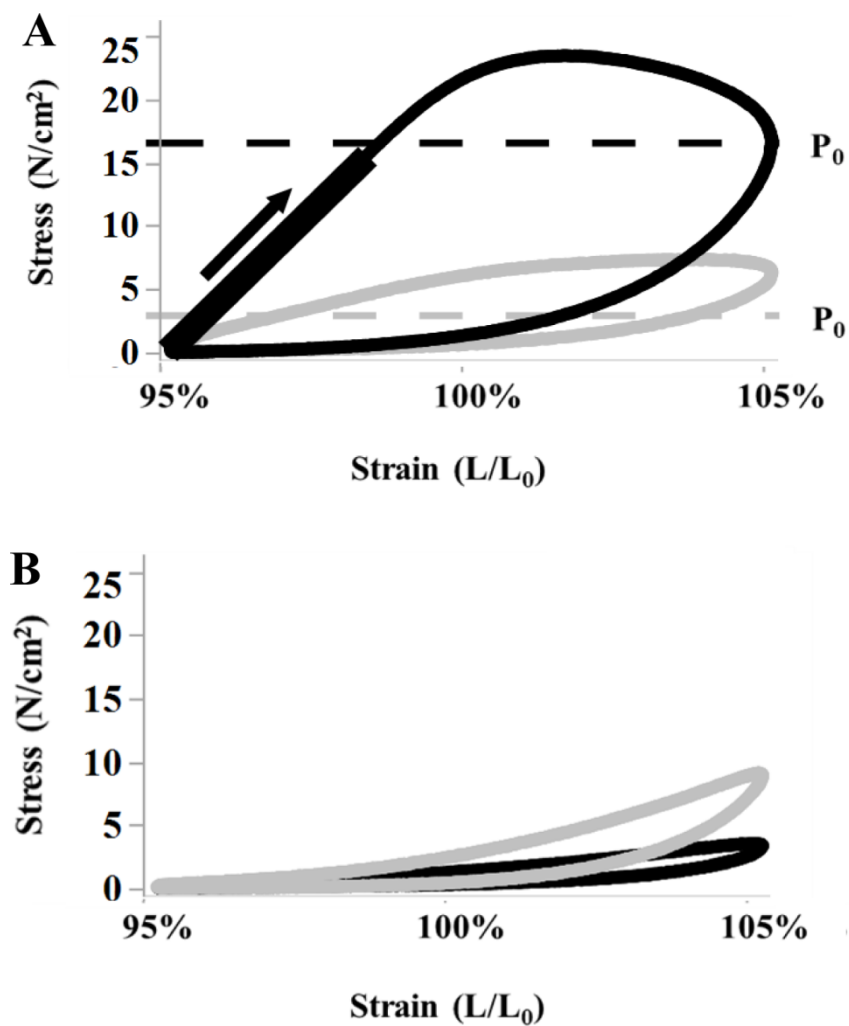


Figure 1: Representative work loops for active (A) and passive (B) soleus muscles.

Negative work is larger in wild type muscles (black) than *mdm* muscles (gray). Horizontal dashed lines indicate P_0 ; colors match genotype. The direction of the work loop (black arrow) and stimulation phase (black bar) are shown for the wild type soleus only, but are the same for all active work loops. Passive work loops (B) were smaller for both wild type and *mdm* soleus compared to active muscles (A).

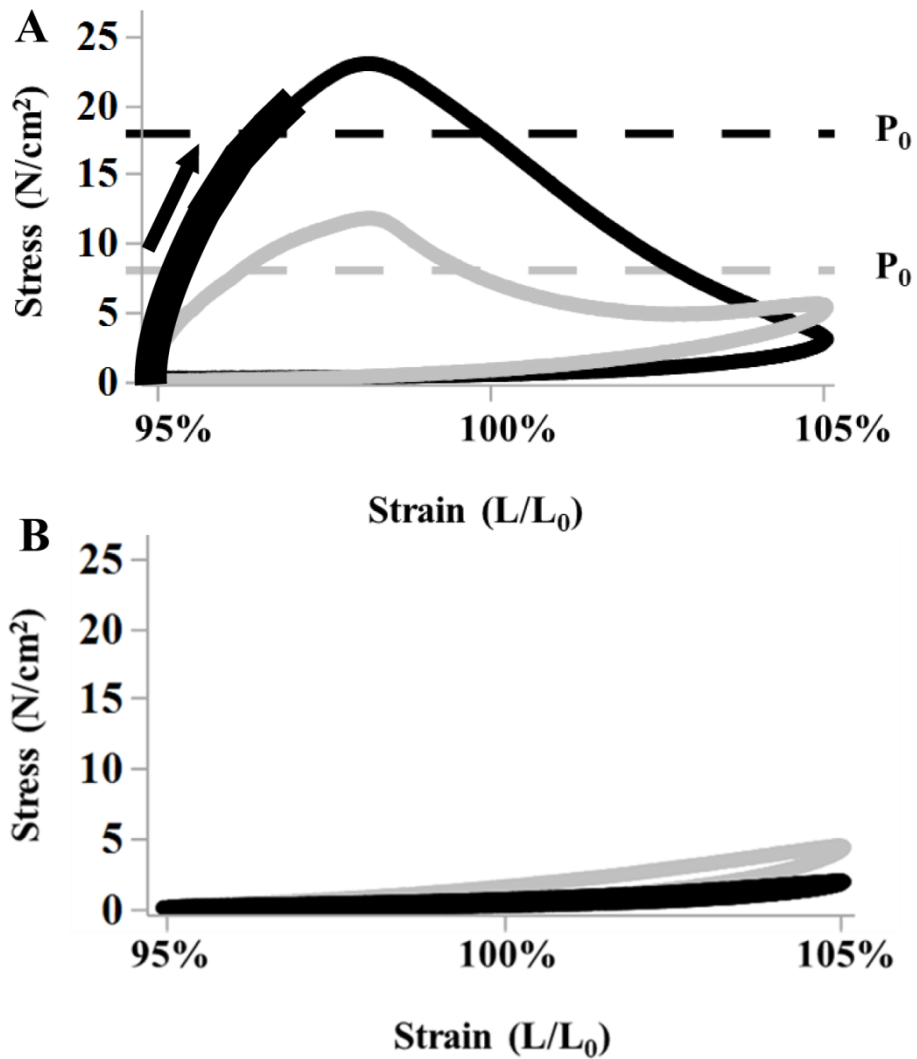


Figure 2: Representative work loops for active (A) and passive (B) EDL muscles. Negative work is larger in wild type muscles (black) than *mdm* muscles (gray). Horizontal dashed lines indicate P_0 ; colors match genotype. Stimulation pattern as for Fig. 1. Passive work loops (B) were smaller for both wild type and *mdm* EDL compared to active muscles (A).

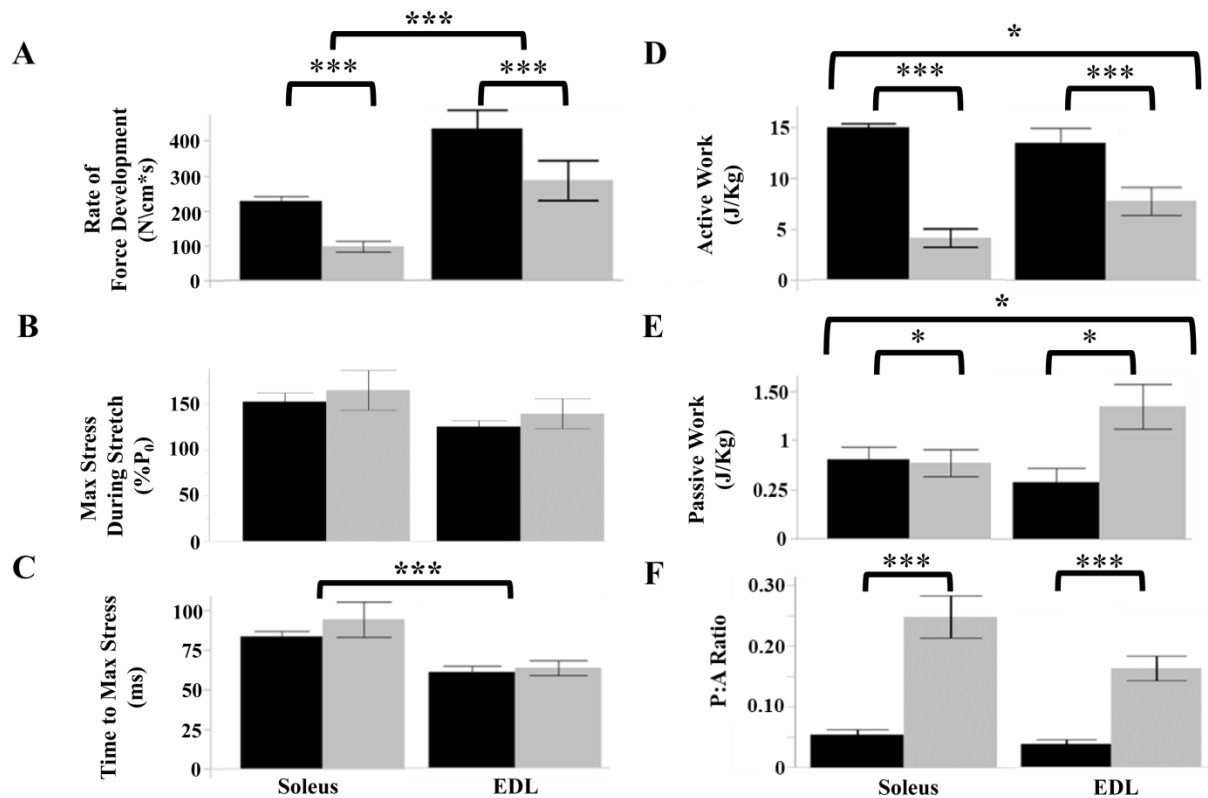


Figure 3: Characteristics of work loops from wild type (black) and *mdm* (gray) soleus and EDL muscles. (A) Active work was greater in wild type than in *mdm* muscles, and was also greater in *mdm* EDL than in *mdm* soleus. (B) Passive work was greater in *mdm* than in wild type muscles, and was greater in *mdm* EDL than in *mdm* soleus. (C) P:A ratio was greater in *mdm* than in wild type muscles. (D) Rate of force development was greater in wild type than *mdm*, and greater in EDL than soleus. (E) Maximum stress during stretch showed no significant effects of genotype, muscle, or genotype x muscle interaction. (F) Time to maximum stress was greater in soleus than in EDL. Asterisks denote significance levels * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. Small bracket denotes significant differences between genotypes. Medium bracket denotes significant differences between muscles. Large bracket denotes significant genotype x muscle interaction. Error bars are mean \pm s.e.m.