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RESEARCH ARTICLE

Effects of a titin mutation on force enhancement and force depression in mouse soleus muscles

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ABSTRACT

The active isometric force produced by muscles varies with muscle length in accordance with the force–length relationship. Compared with isometric contractions at the same final length, force increases after active lengthening (force enhancement) and decreases after active shortening (force depression). In addition to cross-bridges, titin has been suggested to contribute to force enhancement and depression. Although titin is too compliant in passive muscles to contribute to active tension at short sarcomere lengths on the ascending limb and plateau of the force–length relationship, recent evidence suggests that activation increases titin stiffness. To test the hypothesis that titin plays a role in force enhancement and depression, we investigated isovelocity stretching and shortening in active and passive wild-type and *mdm* (muscular dystrophy with myositis) soleus muscles. Skeletal muscles from *mdm* mice have a small deletion in the N2A region of titin and show no increase in titin stiffness during active stretch. We found that: (1) force enhancement and depression were reduced in *mdm* soleus compared with wild-type muscles relative to passive force after stretch or shortening to the same final length; (2) force enhancement and force depression increased with amplitude of stretch across all activation levels in wild-type muscles; and (3) maximum shortening velocity of wild-type and *mdm* muscles estimated from isovelocity experiments was similar, although active stress was reduced in *mdm* compared with wild-type muscles. The results of this study suggest a role for titin in force enhancement and depression, which contribute importantly to muscle force during natural movements.

KEY WORDS: Skeletal muscle, Stretch, Isometric force, Sarcomere length, Locomotion, Muscular dystrophy with myositis

INTRODUCTION

Force enhancement is defined as an increase in force after stretch compared with the isometric force at the final length (Abbott and Aubert, 1952; Edman et al., 1982; Sugi and Tsuchiya, 1988). Likewise, a decrease in force after shortening compared with the isometric force at the final length is known as force depression (Abbott and Aubert, 1952; Edman, 1975; Edman et al., 1982; Sugi and Tsuchiya, 1988). In skeletal muscles, force enhancement (Abbott and Aubert, 1952) and force depression (Van Noten and Van Leemputte, 2011) are observed across a wide range of sarcomere lengths on both the ascending and descending limbs of the force–length relationship. Both phenomena increase with sarcomere length as well as with increasing magnitude of length changes.

Molecular mechanisms of force enhancement and force depression remain unexplained by the sliding-filament and cross-bridge theories of muscle contraction (Herzog, 2014; Meijer, 2002; Minozzo and Lira, 2013; Pinniger et al., 2006; Siebert et al., 2008). Although cross-bridge mechanisms have been suggested to account for force depression (Corr and Herzog, 2016) and force enhancement (Minozzo and Lira, 2013), other mechanisms have also been proposed. These include sarcomere length non-uniformity (Edman et al., 1993; Julian and Morgan, 1979) and elastic elements such as titin (Edman et al., 1993; Forcinito et al., 1998; Julian and Morgan, 1979; Nishikawa et al., 2012; Schappacher-Tilp et al., 2015). To date, there is no single accepted mechanism that explains force enhancement or force depression (Minozzo and Lira, 2013; Nishikawa et al., 2018). Yet, these muscle properties are important for locomotion because they allow animals to recover from perturbations instantaneously without requiring neural input (Daley and Biewener, 2011; Nishikawa et al., 2013; Seiberl et al., 2013).

Recent studies have suggested that the elastic titin protein may contribute to force enhancement (Leonard and Herzog, 2010; Nishikawa et al., 2012) and force depression (Forcinito et al., 1998; Nishikawa et al., 2012; Schappacher-Tilp et al., 2015). At up to 4.2 mDa (Warren et al., 2003), titin spans an entire half-sarcomere from M-line to Z-disk (Gregorio et al., 1999). Titin contributes to passive tension on the descending limb of the force–length relationship in myofibrils and muscle fibers (Linke et al., 1998a,b), as well as intact muscles (Brynnel et al., 2018). But, because of low force straightening of tandem Ig domains (Granzier and Labeit, 2004; Linke et al., 1998a), titin passive tension is too small to contribute to active tension on the ascending limb and plateau of the force–length relationship. However, new data suggest that N2A titin binds to actin upon activation of skeletal muscles, increasing titin stiffness and decreasing its equilibrium length (Dutta et al., 2018).

A recent study further demonstrated that titin contributes not only to passive force but also to active force of skeletal muscle fibers at optimal length (Li et al., 2018). In this study, a transgenic mouse was developed in which a proteolytic cleavage site from tobacco etch virus was inserted into titin near the edge of the A-band. When titin was cleaved in fiber bundles from homozygous transgenic mice, both passive and active force of muscle fibers decreased by ∼50%. Titin stiffness also increases in response to activation (Leonard and Herzog, 2010; Powers et al., 2014). In myofibrils stretched beyond overlap of the thick and thin filaments, calcium activation increased titin-based force and stiffness (Leonard and Herzog, 2010; Powers et al., 2014). This increase in titin stiffness, termed ‘titin activation’, is impaired in muscles from muscular dystrophy with myositis (*mdm)* mice (Powers et al., 2016), with a

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Received 17 February 2019; Accepted 19 December 2019

Journal of Experimental Biology (2020) 223, jeb197038. doi:10.1242/jeb.197038

predicted 83 amino acid deletion in the N2A and PEVK regions of titin (Garvey et al., 2002; Powers et al., 2016).

Because titin activation is impaired in muscles from *mdm* mice, this mutation offers a unique opportunity to test the hypothesis that titin plays a role in force enhancement and force depression. Muscles and single fibers from *mdm* mice are passively stiffer and actively more compliant than wild-type muscles (Lopez et al., 2008; Monroy et al., 2017; Powers et al., 2017). Additionally, *mdm* fibers demonstrate reduced force enhancement when stretched beyond filament overlap (Powers et al., 2017), and both extensor digitorum longus and soleus muscles exhibit reduced mechanical energy storage in eccentric stretch and shortening cycles (Hessel and Nishikawa, 2017). These observations lead to the hypothesis that titin contributes to force enhancement and force depression during stretching and shortening of active muscle.

In this study, we compared force enhancement and force depression in intact soleus muscles from wild-type and *mdm* mice over a range of muscle lengths and activation levels using isovelocity stretch and shortening experiments (Sandercock and Heckman, 1997). We hypothesized that force enhancement and depression would be reduced in *mdm* soleus compared with wild-type soleus as a result of impaired titin activation of *mdm* muscles (Powers et al., 2016). Because force enhancement and force depression increase with higher levels of activation (Meijer, 2002), we measured force enhancement and force depression in maximally activated, submaximally activated and passive muscles. We hypothesized that force enhancement and depression would be higher in muscles stretched and shortened while maximally active compared with that following submaximal activation or after passive stretch or shortening. We also hypothesized that muscles from *mdm* mice would show no difference in force enhancement or force depression with activation level because of impaired titin activation (Leonard and Herzog, 2010; Powers et al., 2014).

MATERIALS AND METHODS

Animals

Heterozygous mice of the strain B6C3Fe a/a-Ttn^{mdm}/J were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and a breeding colony was established in the animal care facility at Northern Arizona University (NAU) to obtain wild-type and homozygous recessive mice (*mdm*). All mice were maintained in a temperature-controlled room with a 12 h:12 h light:dark cycle and were fed *ad libitum*. *mdm* mice could be identified by 21 days of age by a stiff gate and small body mass (Garvey et al., 2002). Heterozygous and wild-type littermates were identified using PCR of ear punches using standard primers (Lopez et al., 2008). Experiments were conducted on soleus muscles from juvenile mice of both sexes, including 8 wild-type mice (40±3.0 days old, 22.3±1.2 g body mass; means±s.e.m.) and 7 *mdm* mice (46±4.1 days old, 7.8±0.5 g body mass). The Institutional Animal Care and Use Committee at NAU approved the experimental protocol and use of animals.

Several previous studies have shown that *mdm* muscles and muscle fibers have normal sarcomere structure and normal active and passive tension at 24−30 days of age (Witt et al., 2004; Hessel et al., 2019). Central nuclei and fibrosis have been reported to increase with age as muscles degenerate and subsequently regenerate (Lopez et al., 2008; Heimann et al., 1996). To test whether progressive degeneration of *mdm* muscles might have affected residual force depression and enhancement, we conducted a correlation analysis of relationships among muscle age, muscle mass, maximum isometric stress, passive stress at optimal muscle length (L_0)+10%, force depression after shortening from L_0+10% to −10%, and force enhancement after stretch from L_0−10% to +10%.

Under the hypothesis that *mdm* muscles become increasingly abnormal with age, we would expect active stress to decrease with an age-related increase in disruption of myofibrillar structure or cross-striations. Likewise, we would expect passive stress to increase with age-related fibrosis or other effects on cytoskeletal structures that might increase passive tension. If degeneration, fibrosis or weakness of *mdm* muscles contributed to reduced force enhancement or depression, then we would expect muscle mass and maximum isometric stress to decrease with age, passive stress to increase with age, and force enhancement and depression to decrease with age and to increase with muscle mass and maximum isometric force.

Muscle preparation

Soleus muscles were extracted from wild-type and *mdm* mice killed with an isoflurane overdose followed by cervical dislocation. Using 4-0 silk suture, the muscles were tied off securely at the muscle–tendon junction to minimize the contribution of extramuscular connective tissue to the experiments. Muscles were immersed in a mammalian Krebs–Ringer solution bath (in mmol l^{-1}: 137 NaCl, 5 KCl, 1 NaH_2PO_4, 24 $NaHCO_3$, 2 $CaCl_2$, 1 $MgSO_4$ and 11 dextrose, pH 7.4; buffered with 95% O_2 and 5% CO_2) maintained at 25°C, a temperature at which wild-type mammalian muscles produce 90% of the maximum isometric force they would produce at 37°C (James et al., 2015). Wild-type and *mdm* muscles are affected similarly by temperature (data not shown).

The distal ends of the soleus muscles were attached to an inflexible hook and the proximal ends were attached to a dual servomotor force lever (Aurora Scientific, Inc., Series 300B, Aurora, ON, Canada) to measure muscle force and length. Initial muscle length was measured with digital calipers, and was used to calibrate the lever and measure lengths throughout the experiment. Muscles were stimulated using two platinum electrodes connected to a Grass S48 stimulator placed parallel to the muscle in the bath. L_0 was determined by adjusting muscle length until maximum isometric force (F_0) was obtained during maximal tetanic stimulation (usually 80 V, 75 Hz). After testing, the Achilles tendon and tibial tendinous origin were removed, and the soleus muscle was dabbed dry and weighed. The physiological cross-sectional area of the muscle was determined by multiplying the muscle mass by the cosine of the pennation angle (8.5 deg; Burkholder et al., 1994) and then dividing by the product of muscle fiber length (muscle length×0.80 in both genotypes; Monroy et al., 2017) and mammalian skeletal muscle density (1.06 g cm^{-3}; Sacks and Roy, 1982).

Data collection

A series of isovelocity (isokinetic) tests was used to measure force enhancement and force depression in wild-type and *mdm* soleus muscles. Muscles were stretched from −10% to 10%, −8% to 8%, −6% to 6%, −4% to 4%, and −2% to 2% of L_0, and then shortened from 2% to −2%, 4% to −4%, 6% to −6%, 8% to −8%, and 10% to −10% of L_0 to obtain a range of normalized velocities from 0.2 to 1.0 muscle lengths s^{-1} (ML s^{-1}). An isovelocity experiment consisted of stretching or shortening a muscle at a constant velocity while measuring force in passive and maximally or submaximally activated muscles (Herzog and Leonard, 1997; Sandercock and Heckman, 1997). In each active isovelocity lengthening or shortening trial, muscles were activated for a total of 1450 ms. Length was changed between 700 and 900 ms after the onset of

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stimulation on the plateau of isometric tension. Muscles were held at the final length for 550 ms, and were then deactivated and returned to their original length. All experiments were performed in this order with a minimum of 3 min rest between trials. To ensure that any damage to muscles due to stretching or shortening was minimal, maximum isometric force at L_0 was measured before and after testing. Data were excluded from analysis if the force decreased by more than 12% from F_0 measured before the experiments were conducted.

To minimize the number of tests per muscle, reduce fatigue and ensure that as many muscles as possible completed all of the tests, we used the steady-state isometric stress 700 ms after the onset of stimulation at each initial length to calculate residual force enhancement and depression (Fig. 1), as well as to measure the passive and active force–length relationships for wild-type and *mdm* soleus muscles. To confirm that isometric stress had reached a plateau after 700 ms, we calculated the average of the first derivative of stress during the final 100 ms of each isometric contraction. We found that the average of the first derivative was not significantly different from zero (−0.00028 to 0.00075 N cm^{-2} in wild-type and −0.00009 to 0.000005 N cm^{-2} in *mdm* muscles), showing that the isometric force had reached a steady state.

Isovelocity tests at each length were performed at three activation levels: maximal, submaximal and passive. Maximal force (F_0) was typically obtained using 80 V and 75 Hz stimulation. Submaximal activation (average 80% of F_0) was achieved by reducing both voltage (typically 34–45 V), which activates fewer muscle fibers, and frequency (typically 45 Hz), which produces unfused submaximal force. Custom-designed software (LabVIEW 7.1, National Instruments, Austin, TX, USA) controlled servomotor parameters and recorded muscle force and length from the lever system at 4000 Hz.

Data analysis

Residual force enhancement and depression were calculated 1440 ms after the onset of activation by subtracting the total steady-state isometric force at the final length (measured 700 ms after the onset of activation, see Fig. 1). To account for the high

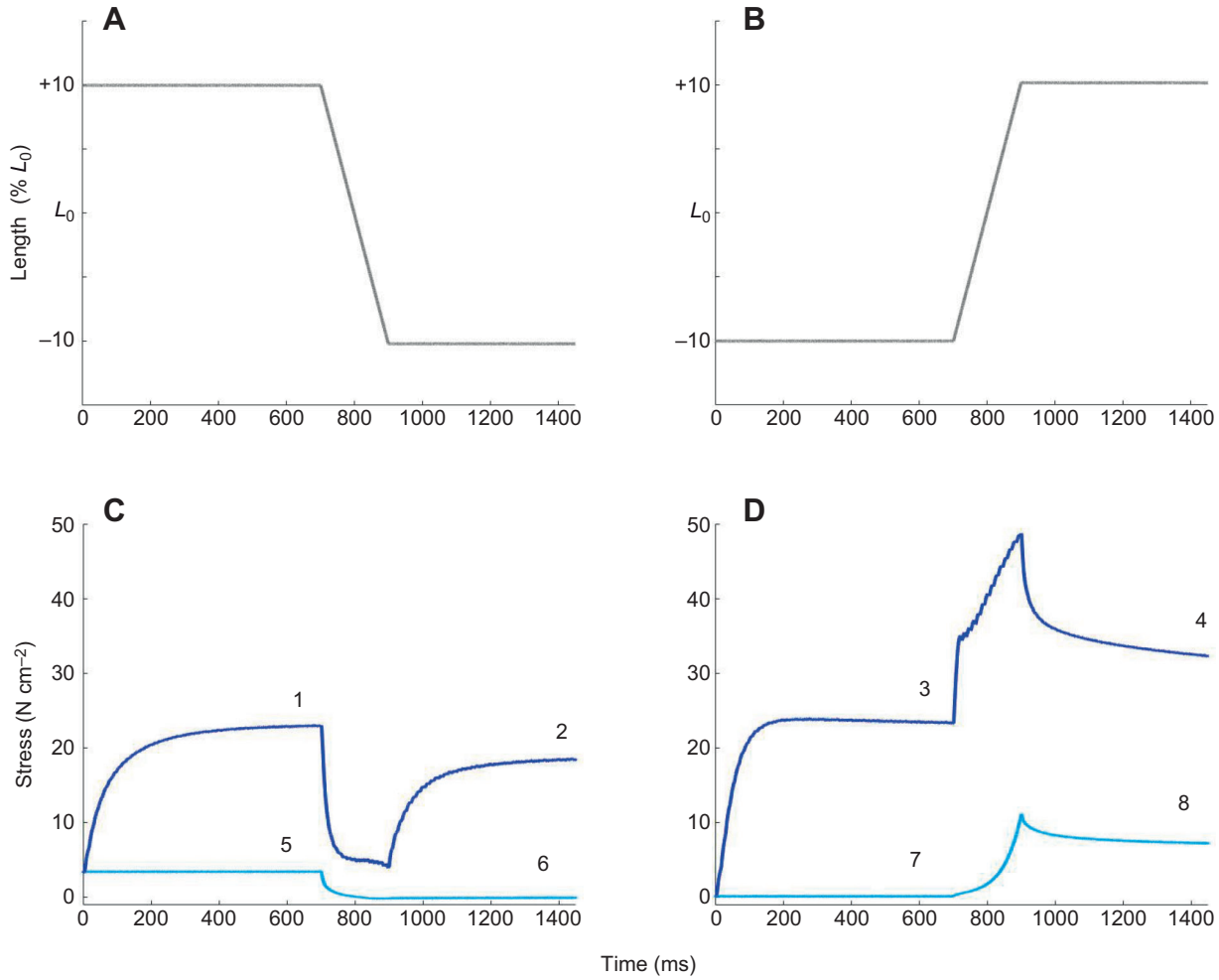

Fig. 1. Methods for measuring residual force enhancement and depression and accounting for contributions of passive stress. (A) Isovelocity shortening from +10% to −10% optimal muscle length (L_0) and (B) stretch from −10% to +10% L_0 in isolated whole mouse soleus muscles. Muscles were shortened or lengthened at constant velocity for 200 ms starting 700 ms after the onset of stimulation (time=0). (C,D) Stress of a representative wild-type soleus muscle during passive (light blue) and active (dark blue) isovelocity shortening (C) and lengthening (D). Residual force depression was calculated by subtracting the total isometric force at the final length (measured at point 3) from the total stress after shortening (point 2). To account for the contribution of passive tension, the change in passive stress (point 7−point 6) was further subtracted from residual force depression. Residual force enhancement was calculated by subtracting the total isometric force at the final length (measured at point 4) from the isometric stress at the final length (point 1). To account for the contribution of passive tension, the change in passive stress (point 8−point 5) was further subtracted from residual force enhancement.

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passive force in muscle from *mdm* mice, we also calculated force enhancement and depression after further subtracting the change in passive force (i.e. the passive force after shortening or lengthening – the passive steady-state isometric force at the stretched or shortened length) from the residual force enhancement or depression (see Fig. 1).

To determine the force–velocity relationship at maximal and submaximal activation, the average velocity of stretch or shortening was calculated between 750 and 850 ms after the onset of stimulation. The corresponding force was measured at 800 ms when the muscle length was equal to L_0. The force–velocity curves in the shortening domain were fitted to the Hill equation $(v+b)(F+a)=b(F_0+a)$; where v represents the prescribed velocity of shortening and F_0 represents the force at L_0 (Hill, 1938). Least squares regression analysis (MATLAB, MathWorks, Natick, MA, USA) was used to estimate parameters a (force constant) and b (velocity constant). For maximal and submaximal activation, maximum shortening velocity (V_{max}) was estimated using the formula $V_{max}=(b\times F_0)/a$ (Hill, 1938).

Statistical analysis

All data are reported as means±s.e.m. unless otherwise noted. Power analysis was performed using G*Power (Heinrich Heine University, Dusseldorf, Germany). Based on an average effect size of 1.5 (range 0.97–1.83), α=0.05 and β=0.80, the minimum sample size was calculated to be 7 animals per group. All statistical tests were performed using JMP Pro 14 (SAS Institute, Inc., Cary, NC, USA). *t*-tests (α=0.05) were used to compare age, body mass, and optimal length of wild-type and *mdm* soleus muscles. Active and passive force–length relationships were analyzed using two-way analysis of variance (ANOVA) with genotype and length as the main effects. Because residuals from the ANOVA demonstrated that 6 of 20 datasets had unequal variance between genotypes and 6 of 40 datasets deviated from normality, we used the best Box–Cox transformation and also analyzed the data using non-parametric Wilcoxon tests.

Differences between genotypes in stress after stretch and shortening were compared using ANCOVA with activation (maximal, submaximal and passive) and genotype (wild-type, *mdm*) as main effects and amplitude (20%, 16%, 12%, 8% and 4%) as the continuous covariate. To account for the high passive stress of *mdm* muscles (see Figs 2C and 4C), we performed the analysis on residual force enhancement and depression before and after subtracting the change in passive stress (see Fig. 1). Individual animals nested within genotype were treated as a random factor. A full-factorial analysis was performed on force enhancement and depression. *Post hoc* differences among means were evaluated using Tukey's honestly significant difference (HSD) tests.

Analysis of the residuals from ANCOVA on force enhancement and depression demonstrated that 15 of 50 datasets had unequal variance between genotypes, and 29 of 100 datasets deviated from normality. We therefore used the best Box–Cox transformation. However, even after transformation, 22 of 100 datasets deviated from normality and 14 of 50 datasets demonstrated unequal variance between genotypes. Because the assumptions of normality and equal variance were violated, we also examined the data using non-parametric Wilcoxon tests for one-way comparisons or Steel–Dwass tests for non-parametric ANOVA. Both tests gave similar results to the less conservative parametric tests (Sokal and Rohlf, 1994). An alpha level of 0.05 was used to determine statistical significance and a Bonferroni correction was used for non-parametric analyses when more than one analysis was performed using the same dataset. All data and analyses reported in the paper are available from the Dryad digital repository (doi:10.5061/dryad.3vm2818).

RESULTS

Contractile properties of *mdm* versus wild-type muscles

mdm mice weighed less than wild-type mice (wild-type 22.3±1.2 g; *mdm* 8.1±0.47 g; *t*-test, $P<0.001$) although age at testing was not significantly different between genotypes (wild-type 41.6±3.0 days; *mdm* 46.3±4.4 days; *t*-test, $P=0.35$). Because of their larger size, optimal muscle length (L_0) for wild-type mice soleus (10.9±0.5 mm) was longer than that for *mdm* mice (8.37±0.52 mm; *t*-test, $P=0.004$). Active isometric stress at L_0 was lower in *mdm* (7.0±1.9 N cm^{-2}) than in wild-type muscles (21.0±1.97 N cm^{-2}; *t*-test, $P=0.0002$). Passive stress at L_0 was not significantly different between wild-type (0.91±0.09 N cm^{-2}) and *mdm* soleus (2.0±0.61 N cm^{-2}; *t*-test, $P=0.11$). However, passive stress increased significantly faster in *mdm* soleus than in wild-type soleus at lengths above optimal (Fig. 3A; ANOVA, genotype×length, $P=0.0002$). Active stress was higher in wild-type than in *mdm* soleus at all lengths tested (Fig. 3B; ANOVA, genotype, $P<0.0001$). The genotype×length interaction was not significant (ANOVA, $P=0.0684$), demonstrating that although *mdm* muscles generated less active force than wild-type muscles, the active force–length relationship was similar (Fig. 3B). The results of non-parametric tests were similar to those of parametric tests (see Fig. 3).

In contrast to expectations based on age-related degeneration of *mdm* muscles, correlation analysis (Table 1) showed that neither muscle mass nor passive stress at L_0 +10% was significantly related to age in wild-type or *mdm* muscles (all $P>0.05$). Our previous studies showed that *mdm* mice and their muscles are small, not because they degenerate over time, but rather because growth stops at an early age (Pace et al., 2017). In contrast to expectations based on age-related muscle degeneration, we also found that maximum isometric stress increased significantly with age (Table 1) in our sample of *mdm* muscles ($n=7$, $P=0.0055$) but not in wild-type muscles ($n=8$, $P=0.2859$). This might be expected if only the strongest muscles completed the protocol with <12% decrease in active stress. In both wild-type and *mdm* muscles, both maximum isometric stress and passive stress at L_0+10% were negatively correlated with residual force depression (all $P<0.05$) and positively correlated with residual force enhancement (all $P<0.05$).

At maximal activation, parameters of the force–velocity relationship were similar between wild-type and *mdm* soleus

Table 1. Correlation coefficients among muscle age, muscle mass, maximum isometric stress, passive stress at L_0+10%, residual force depression (RFD) at L_0–10% and residual force enhancement (RFE) at L_0+10%

	Wild-type					
mdm	Age	Mass	Active stress	Passive stress	RFD	RFE
Age	–	0.64	0.43	0.41	–0.59	0.47
Mass	–0.05	–	–0.13	0.35	–0.24	0.08
Active stress	0.90**	–0.06	–	0.55	–0.76*	0.87**
Passive stress	0.63	–0.33	0.77*	–	–0.79*	0.84**
RFD	–0.62	0.31	–0.81*	–0.98***	–	–0.80*
RFE	0.75	–0.09	0.78*	0.92**	–0.89**	–

Correlation coefficients for age (days), mass (g), active stress, passive stress L_0+10%, RFD L_0–10% and RFE L_0+10% are shown. Values for wild-type muscles are shown above the diagonal and those for *mdm* muscles are shown below the diagonal, shaded gray. *$P<0.05$, **$P<0.01$, ***$P<0.0001$.

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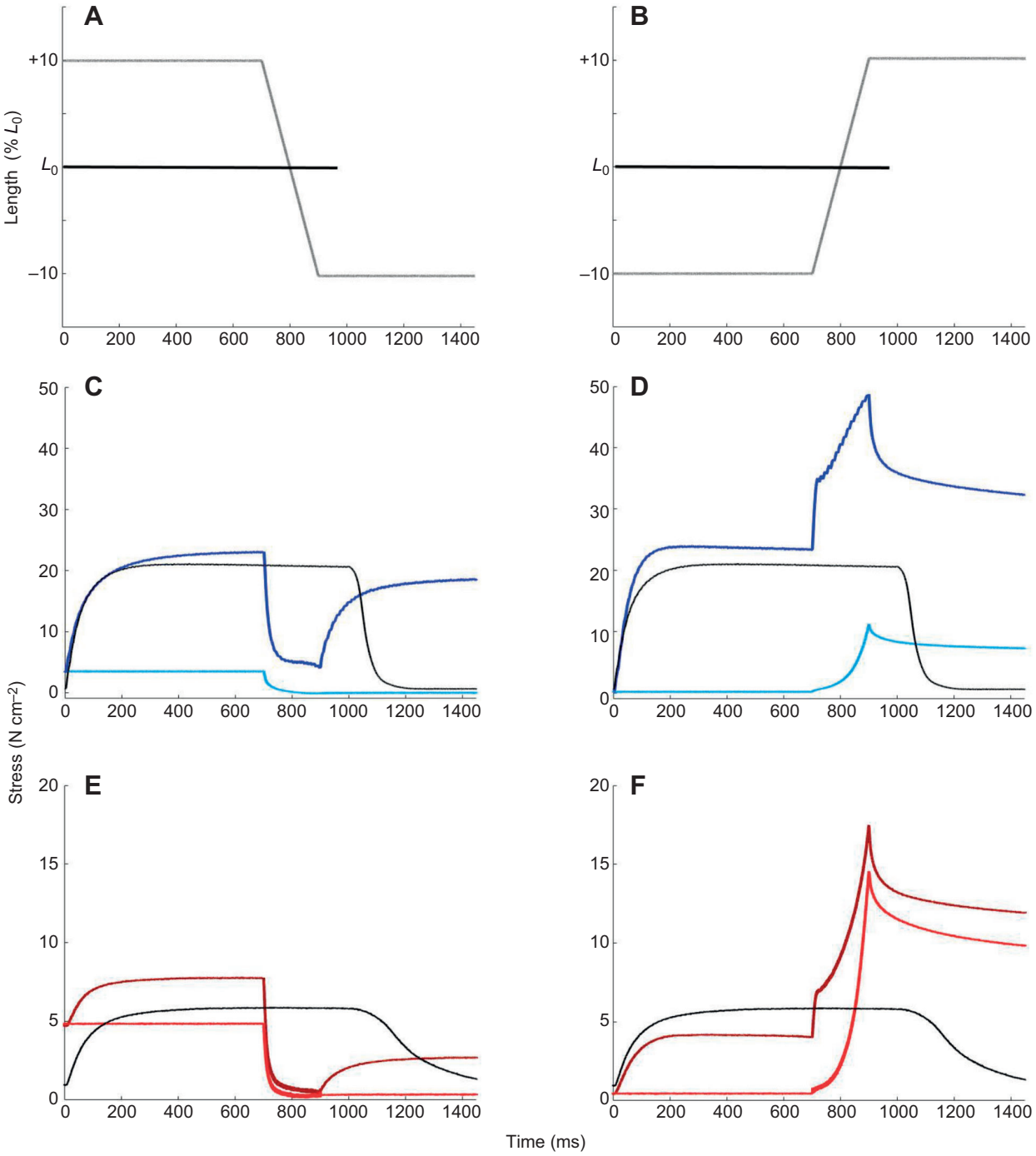

Fig. 2. Length and stress during isovelocity stretch and shortening in wild-type and *mdm* soleus. (A,B) Length (gray lines) of soleus muscles during isovelocity shortening (A) and stretch (B) as well as during isometric contraction (black lines in A and B). (C–F) Stress from a single representative wild-type (blue lines; C,D) and *mdm* (red lines; E,F) soleus muscle during isovelocity shortening (left) and stretch (right). Dark lines indicate active stretch whereas light lines indicate passive stretch or shortening. Isometric muscle stress at L_0 is shown in black. Note the difference in scale between graphs for wild-type and *mdm* muscles.

muscles (Fig. 3C). Maximum shortening velocity (V_{max}) estimated from isovelocity experiments did not differ statistically between wild-type (1.69±0.14 ML s^{-1}) versus *mdm* soleus (1.73±0.23 ML s^{-1}; *t*-test, P=0.52). The Hill velocity constant b (normalized to L_0) was also similar between genotypes (wild-type 0.66±0.10 ML s^{-1}, *mdm* 0.47±0.06 ML s^{-1}; *t*-tests, P=0.11). The Hill force constant a was lower in *mdm* soleus (2.08±0.71 N cm^{-2}) than in wild-type (9.62±1.31 N cm^{-2}; *t*-tests, P=0.0003), likely because *mdm* muscles on average produced 67% lower stress than wild-type muscles. Finally, the curvature (a/F_0) of the force–velocity relationship was similar between genotypes (wild-type 0.46±0.04 ML s^{-1}, *mdm* 0.29±0.09 ML s^{-1}; *t*-test, P=0.12).

In contrast, parameters of the force–velocity relationship differed significantly between maximal and submaximal activation in both wild-type and *mdm* soleus. V_{max} was higher when muscles were activated submaximally compared with maximal activation in both

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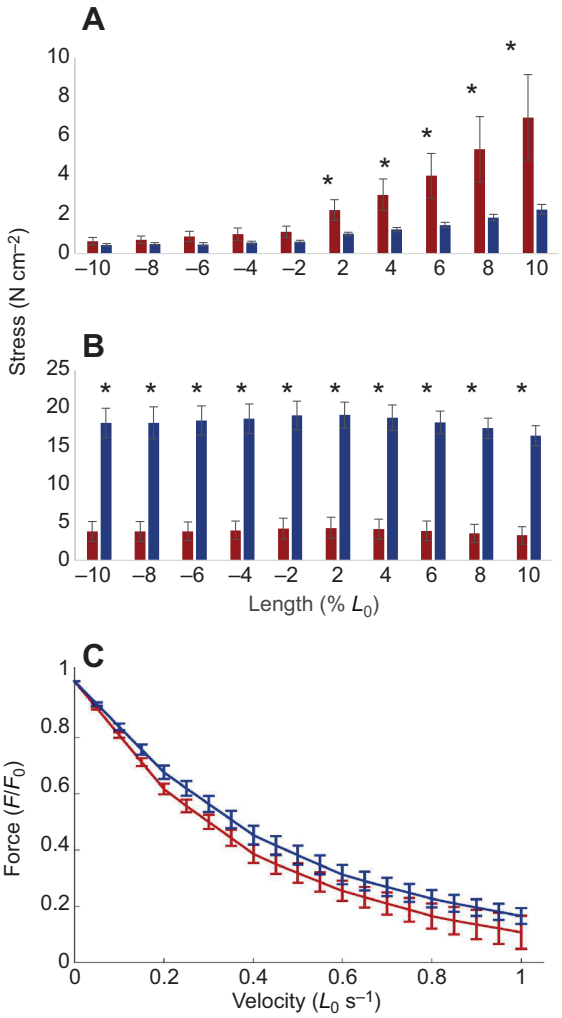

Fig. 3. Passive and active stress and force–velocity relationships for wild-type and *mdm* soleus. (A,B) Passive (A) and active (B) isometric stress of wild-type (blue) and *mdm* soleus (red). (C) Normalized force–velocity relationship for wild-type (blue) and *mdm* (red) soleus at maximal activation. *Statistically significant difference between genotypes using Steel–Dwass non-parametric tests (α=0.05).

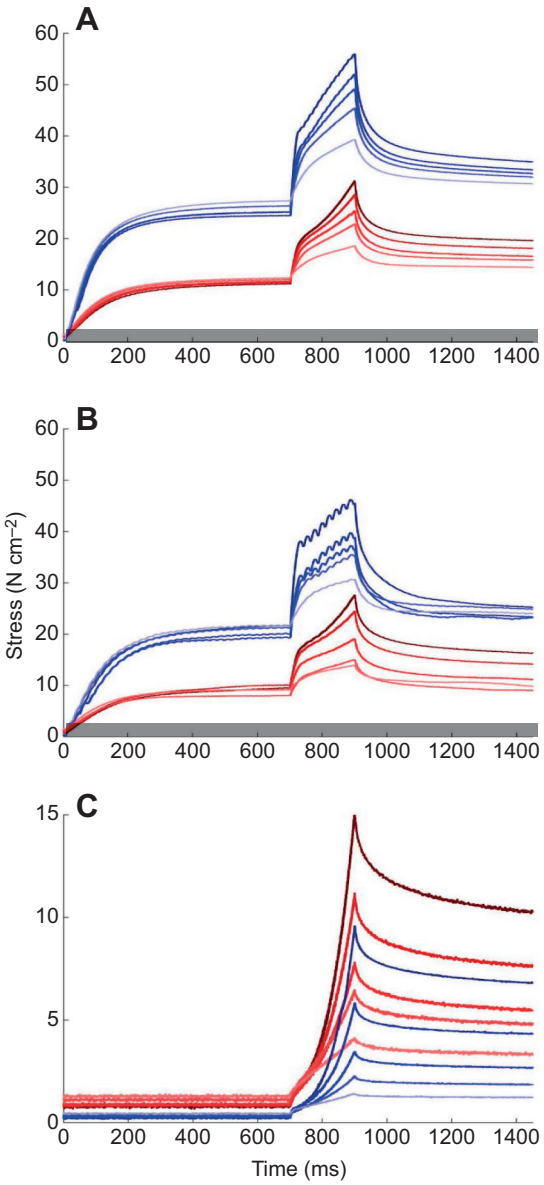

Fig. 4. Stress during isovelocity stretch experiments in a single representative wild-type (blue) and *mdm* (red) soleus muscle. (A) Maximally stimulated, (B) submaximally stimulated and (C) passive muscles. Active stress was lower in *mdm* muscles at maximal (A) and submaximal (B) activation levels. Passive stress (C) was higher in *mdm* muscles than in wild-type muscles. Darker lines denote larger amplitude and velocity of stretch. Gray bar denotes activation duration (1450 ms). Note: *Y*-axis scale is smaller for passive than for active graphs.

wild-type (maximal: 1.69±0.14 ML s^{-1}, submaximal: 15.83±1.1 ML s^{-1}; *t*-test, P=0.001) and *mdm* soleus (maximal: 1.73±0.23 ML s^{-1}, submaximal: 12.84±3.77 ML s^{-1}; *t*-test, P=0.012). There was also a decrease in the shape (a/P_0) parameter with decreasing activation in both genotypes (wild-type: maximal 0.59±0.06, submaximal 0.003±0.0006; *t*-test, P<0.001; *mdm*: maximal 0.50±0.11, submaximal 0.0007±0.000016; *t*-test, P=0.0012). Similarly, the force constant a decreased and the velocity constant b increased with submaximal compared with maximal activation in both genotypes (all P<0.01).

Residual force enhancement after isovelocity stretch

When wild-type and *mdm* soleus muscles were stimulated maximally (Fig. 4A) or submaximally (Fig. 4B), muscle stress increased rapidly during the first 200 ms after activation and then gradually from 200 to 700 ms after activation. During stretch (700–900 ms after activation), stress increased non-linearly with an initial rapid increase followed by a slower increase. After stretching, stress approached a steady-state value that was greater than the isometric stress at the stretched length. During passive stretch (Fig. 4C), stress increased exponentially and then settled to a higher force after stretching.

When measured relative to isometric force at the final length, both wild-type (Fig. 5A) and *mdm* (Fig. 5B) muscles demonstrated residual force enhancement (Table 2). There was no effect of genotype on residual force enhancement (P=0.6680). Residual force enhancement increased with amplitude of stretch (ANCOVA, amplitude, P<0.0001) in both genotypes (ANCOVA, genotype×amplitude, P=0.1550) and across all activation levels (activation×amplitude, P=0.4372). However, genotype and activation level showed a significant interaction (P=0.0002;

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Table 2. Effects of genotype, amplitude and activation level on residual force enhancement (RFE) before and after accounting for the contribution of passive stress in wild-type and *mdm* soleus muscles

	RFE		RFE after subtracting the contribution of passive stress	
Model effects	*F*-ratio	*P*-value	*F*-ratio	*P*-value
Genotype	0.19	0.6680	10.00	**0.0075**
Activation	6.90	**0.0013**	4.74	**0.0312**
Amplitude	132.43	**<0.0001**	10.52	**0.0015**
Genotype×activation	8.92	**0.0002**	14.72	0.0868
Genotype×amplitude	2.04	0.1550	7.31	**0.0078**
Activation×amplitude	0.83	0.4372	0.42	0.5176
Genotype×activation×amplitude	0.72	0.4872	1.82	0.1797

Results of three-way ANCOVA after best Box–Cox transformation. See Figs 4 and 5 for means±s.e.m. *N*=8 wild-type and *N*=7 *mdm*. Bold indicates significance.

Table 2), demonstrating that activation level affected the genotypes differently. For *mdm* muscles, there was no effect of activation level on force enhancement (Tukey's HSD, $P>0.05$). Additionally, the increased force after passive stretch was not statistically different between *mdm* and wild-type muscles (Tukey's HSD, $P>0.05$). The results of non-parametric tests were similar to those for parametric tests (Fig. 5).

Because *mdm* muscles had higher passive force after stretch than wild-type muscles, we also analyzed force enhancement after subtracting the change in passive stress after stretch (i.e. the difference between passive stretch after active stretch and the isometric passive stretch at the same final length; see Fig. 1). After correcting for the contribution of passive stress after stretch, wild-type soleus had increased force enhancement compared with *mdm* soleus (Fig. 5C,D, Table 2; $P=0.0075$). Force enhancement increased with amplitude of stretch ($P=0.0015$) and activation level ($P=0.0312$). The genotype×activation interaction was not significant (ANCOVA, $P=0.0868$). Force enhancement increased with amplitude of stretch in wild-type soleus muscles, but not in *mdm* muscles (ANCOVA, genotype×amplitude, $P=0.0078$; Table 2). The results of non-parametric tests were similar to those of parametric tests (Fig. 5).

Force depression after isovelocity shortening

When wild-type and *mdm* soleus muscles were shortened over 200 ms, muscle stress decreased non-linearly with an initial rapid decrease followed by a slower rate of decrease. Muscle stress then redeveloped after shortening, approaching a level that was lower than the isometric stress at the final, shorter length (Fig. 6).

When measured relative to isometric force at the final length (Table 3), both wild-type (Fig. 7A) and *mdm* (Fig. 7B) muscles demonstrated residual force depression (Fig. 7A,B). *mdm* soleus demonstrated reduced force depression compared with wild-type soleus (ANCOVA, genotype, $P=0.0413$). Additionally, force depression increased with amplitude of shortening in wild-type but not *mdm* muscles (ANCOVA, genotype×amplitude, $P=0.0096$). In wild-type and *mdm* muscles, force depression was similar in maximally and submaximally (Fig. 6A,B) activated muscles and was reduced in muscles shortened passively (ANCOVA, $P<0.0001$; Tukey's HSD, $P<0.05$). The results of non-parametric tests were similar to those of parametric tests (Fig. 7).

After accounting for the contribution of passive tension (see Fig. 1), wild-type soleus had significantly higher force depression compared with *mdm* soleus (Fig. 7C,D; ANCOVA, genotype, $P=0.0066$; Table 3). There was no effect of activation on force depression (ANCOVA, $P=0.3166$) and no difference between genotypes in the effect of activation (ANCOVA, genotype×activation, $P=0.632$). Force depression increased with increasing amplitude of shortening (ANCOVA, $P<0.0001$) in wild-type muscles, but the effect of amplitude on force depression was abolished in *mdm* soleus (Tukey's HSD, $P>0.05$). The results of non-parametric tests were similar to those of parametric tests (Fig. 7).

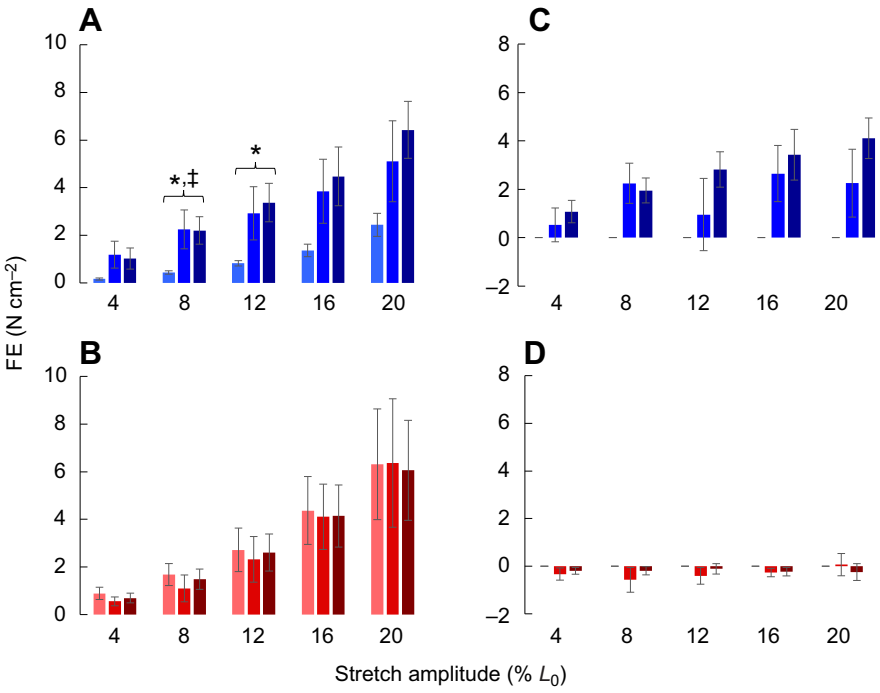

Fig. 5. Residual force enhancement in wild-type and *mdm* soleus. (A,B) Residual force enhancement (RFE) increases with activation in wild-type (A, blue) but not *mdm* (B, red) soleus muscles (see Table 2). Stress was not significantly different between wild-type and *mdm* muscles that were stretched passively (ANCOVA, genotype×activation, $P<0.0001$ and Tukey's HSD, $P>0.05$). (C,D) The contribution of passive stress was subtracted from RFE (see Fig. 1 for details) for wild-type (C, blue) and *mdm* (D, red) soleus muscles. Darker bars denote larger amplitude and velocity of stretch. *Statistically significant difference using Steel–Dwass non-parametric tests ($\alpha=0.025$). ‡Statistically significant differences between passive and submaximal activation; Steele–Dwass tests ($\alpha=0.025$).

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Table 3. Effects of genotype, amplitude and activation level on residual force depression (RFD) in wild-type and *mdm* muscles before and after accounting for the contribution of passive stress

	RFD		RFD after subtracting the contribution of passive stress	
Model effects	*F*-ratio	*P*-value	*F*-ratio	*P*-value
Genotype	5.13	**0.0413**	10.41	**0.0066**
Activation	62.52	**<0.0001**	1.01	0.3166
Amplitude	22.49	**<0.0001**	30.79	**<0.0001**
Genotype×activation	14.72	**<0.0001**	0.23	0.6320
Genotype×amplitude	6.84	**0.0096**	5.40	**0.0217**
Activation×amplitude	6.16	**0.0025**	0.11	0.7433
Genotype×activation×amplitude	1.14	0.3213	0.12	0.7251

Results of three-way ANCOVA after best Box–Cox transformation. See Figs 5 and 7 for means±s.e.m. *N*=8 wild-type and *N*=7 *mdm*. Bold indicates significance.

DISCUSSION

The main results from this study demonstrate that: (1) active stress was reduced in *mdm* compared with wild-type muscles, although maximum shortening velocity estimated during isovelocity experiments was similar in wild-type and *mdm* muscles; (2) after accounting for the contribution of passive tension, residual force enhancement and depression were negligible in *mdm* soleus muscles.

Contractile properties of *mdm* versus wild-type muscles

Active contractile stress was reduced in *mdm* compared with wild-type soleus muscles, similar to results from previous studies using *mdm* myofibrils (Powers et al., 2016), single fibers (Lopez et al., 2008; Powers et al., 2017) and whole muscles (Hessel and Nishikawa, 2017; Monroy et al., 2017). One previous study found that reduced muscle force was not associated with a reduction in actin or myosin content in *mdm* fibers (Powers et al., 2017).

Although *mdm* muscles generated lower active stress, their force–velocity relationship, as measured from isovelocity experiments, was similar to that of wild-type muscles. A previous study found differences between isovelocity and isotonic force–velocity curves (Bullimore et al., 2010) especially at higher forces. However, using the Hill equation (Hill, 1938; Sandercock and Heckman, 1997), we found no difference between force–velocity relationships estimated using isotonic versus isovelocity methods (data not shown). At maximal and submaximal levels of activation, maximum shortening velocity (V_{max}) was not statistically different between genotypes. Both wild-type and *mdm* muscles demonstrated an increase in maximal shortening velocity with submaximal activation. Additionally, the curvature of the force–velocity relationship (a/F_0) was similar between genotypes at maximal activation. However, in both genotypes the curvature of the force–velocity relationship decreased with submaximal activation similar to previous studies (Chow and Darling, 1999; Gilliver et al., 2011). The results suggest that the decrease in active stress of *mdm* soleus muscles is not due to impaired activation and imply that cross-bridge kinetics may be relatively unaffected by *mdm* (Hill, 1938; Huxley, 1957; Joyce et al., 1969).

To determine how *mdm* affects passive force (Magid and Law, 1985; Maruyama, 1976; Wang et al., 1991, 1993), we compared the passive force of wild-type and *mdm* soleus muscles before and after stretch. *mdm* muscles had higher passive forces on the descending limb of the force–length curve than wild-type muscles (see Fig. 3A). In contrast, there was no difference between wild-type and *mdm* muscles in the average force after passive stretch (see Table 2). Previous studies found no difference in passive force after stretch in myofibrils from wild-type and *mdm* muscles (Powers et al., 2017, 2016). However, single fibers from *mdm* muscles showed increased passive stress before and after stretch compared with wild-type fibers (Powers et al., 2017). The increase in passive stress was likely

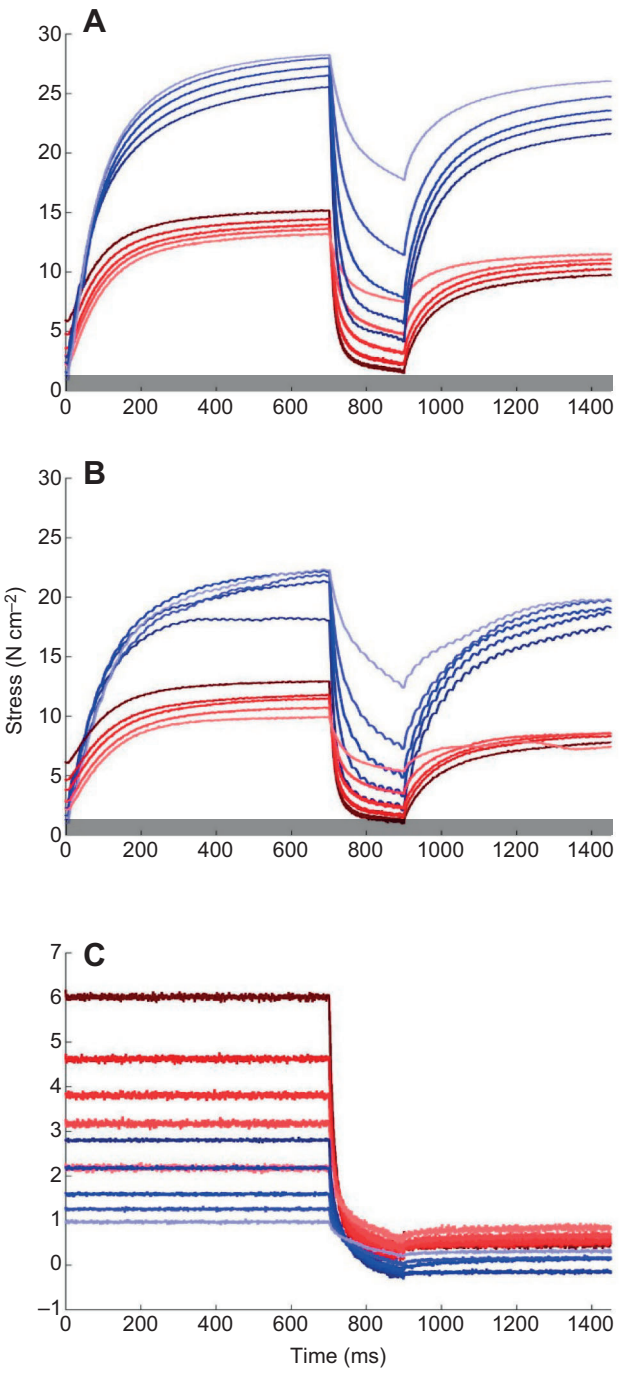

Fig. 6. Stress during isovelocity shortening experiments in a single representative wild-type (blue) and *mdm* (red) soleus muscle. (A) Maximally stimulated, (B) submaximally stimulated and (C) passive muscles. Active stress was lower in *mdm* muscles at maximal (A) and submaximal (B) activation levels compared with wild-type muscles. Passive stress (C) was higher in *mdm* muscles than in wild-type muscles. Darker lines denote larger amplitude and velocity. Gray bar denotes activation duration (1450 ms). Note: *Y*-axis scale is smaller for passive than for active graphs.

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due to increased collagen (Lopez et al., 2008; Powers et al., 2017) and not to the very small 83 amino acid deletion in *mdm* muscles. Although not investigated previously, intermediate filaments or microtubules could also potentially contribute to increased passive force of *mdm* soleus on the descending limb of the force–length relationship (see Fig. 3A).

Residual force enhancement is impaired in active soleus muscles from *mdm* mice

After accounting for the contribution of passive tension, wild-type soleus muscles exhibited force enhancement that increased with stretch amplitude as observed in many previous studies (Bullimore et al., 2007; Edman et al., 1982; Hisey et al., 2009; Julian and Morgan, 1979; Noble, 1992; Schachar et al., 2002). Additionally, force enhancement was higher in active compared with passive wild-type muscles, as observed in other studies (Oskouei and Herzog, 2005; Pinniger and Cresswell, 2007). Previous studies also found that force enhancement was reduced in submaximally compared with maximally stimulated (50% P_0) muscles (Meijer, 2002). However, we found no statistical difference in force enhancement between maximally and submaximally activated wild-type muscles, likely because the reduction in activation was relatively small in the present study (~80% F_0).

In contrast to wild-type muscles, *mdm* muscles failed to exhibit force enhancement after accounting for passive stress after stretch to the same final length, similar to observations from previous studies on *mdm* fibers (Powers et al., 2017) and myofibrils (Powers et al., 2016). In contrast to wild-type soleus, there was also no effect of activation or stretch amplitude on force enhancement in *mdm* soleus. Previous studies using wild-type psoas myofibrils stretched beyond cross-bridge overlap showed that titin-based force and stiffness were ~4 times higher in calcium-activated myofibrils than in passive myofibrils (Leonard and Herzog, 2010; Powers et al., 2014). In contrast, results from single myofibrils and fibers from *mdm* psoas demonstrated only a small increase in force with activation and no increase in stiffness, indicating that titin activation (i.e. the increase in titin stiffness with calcium activation) is impaired in *mdm* myofibrils (Powers et al., 2016). By extending these observations to whole muscles, the present study demonstrates that force enhancement is abolished in active, intact *mdm* soleus muscles.

The present study also highlights problems with the existing methodology that has been applied to force enhancement when tested in muscles with high passive tension. Although we found residual force enhancement – traditionally defined as an increase in force after stretch compared with the isometric force at the stretched length (Edman et al., 1982) – in both wild-type and *mdm* soleus, the comparison of force enhancement relative to the passive force after stretch to the same final length produced different results. Based on the traditional definition, it appears that *mdm* muscles demonstrate residual force enhancement. However, actively stretched *mdm* muscles demonstrate no increase in stress relative to the passive

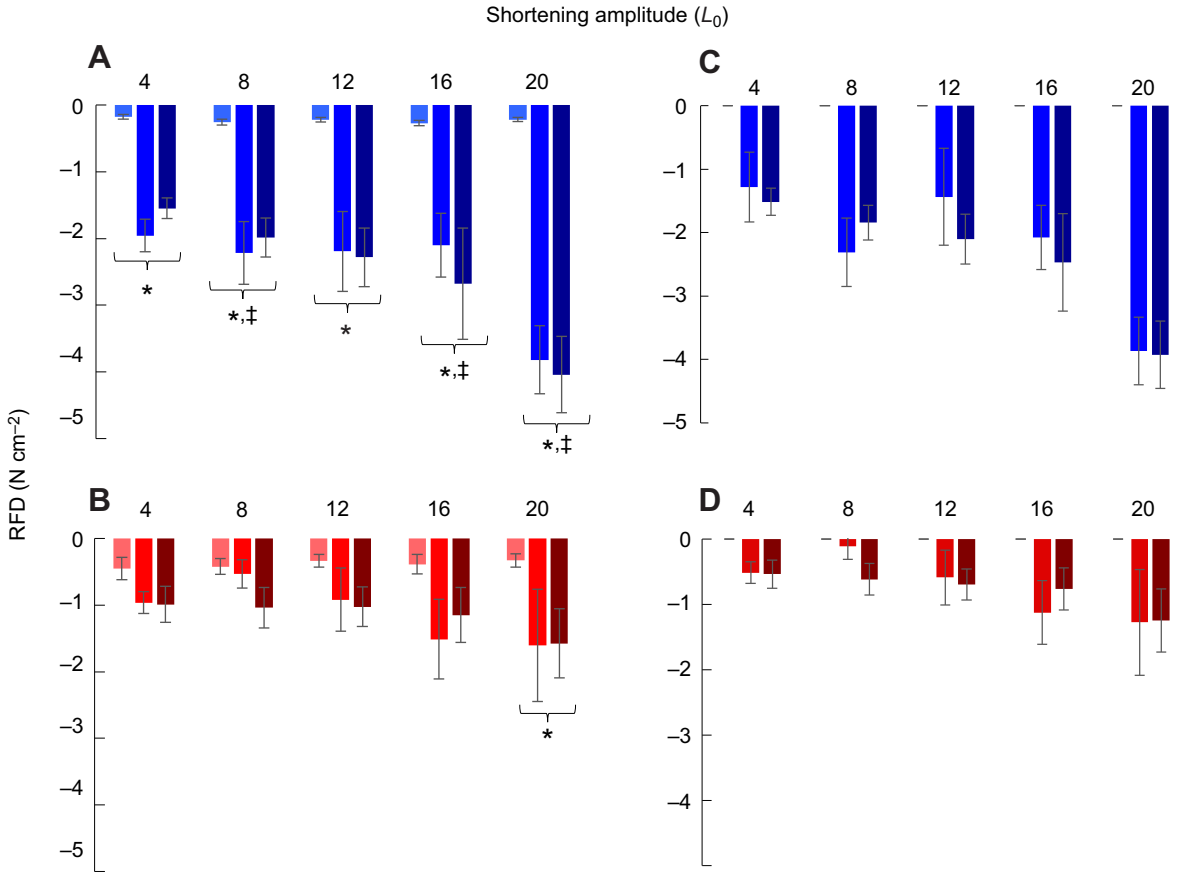

Fig. 7. Residual force depression after isovelocity shortening. (A,B) Residual force depression (RFD) increases with activation in wild-type (A, blue) but not *mdm* (B, red) soleus muscles. (C,D) The contribution of passive stress was subtracted from RFD (see Fig. 1 for details) for wild-type (C, blue) and *mdm* (D, red) soleus muscles. RFD was not significantly different between wild-type and *mdm* muscles that were shortened passively (ANCOVA, genotype×activation, $P<0.0001$ and Tukey's HSD, $P>0.05$). *Statistically significant differences between passive and maximal activation; ‡statistically significant differences between passive and submaximal activation; Steele–Dwass tests ($\alpha=0.025$).

force after stretch to the same final length, as also observed in single fibers from *mdm* psoas (Powers et al., 2017).

The problem is that the traditional definition leads to the inference that residual force enhancement is present in *mdm* soleus, when in fact these muscles produce the same additional force after active or passive stretch. In the case of *mdm* muscles, this problem with defining residual force enhancement arises specifically because the passive forces following stretch can exceed isometric forces on the descending limb of the force–length relationship (see Figs 2 and 3). Therefore, we suggest that caution should be observed in future studies when comparing residual force enhancement in muscles that differ in passive or active tension, or both.

Force depression is impaired in active soleus muscles from *mdm* mice

In the present study, we observed that force depression increased in proportion to the distance shortened in active wild-type muscles as previously observed in whole muscles (Abbott and Aubert, 1952; De Ruiter et al., 1998; Herzog and Leonard, 1997; Maréchal and Plaghki, 1979; Morgan et al., 2000) as well as single fibers (Edman, 1975; Sugi and Tsuchiya, 1988). Force depression increases with activation in wild-type soleus muscles compared with passive muscles (De Ruiter et al., 1998). During voluntary contractions, force depression was reduced by ∼20% at 30% of maximal voluntary effort compared with maximally activated muscles (Rousanoglou et al., 2007). However, in our study, force depression was similar in wild-type muscles activated maximally or submaximally, likely due to the small difference in active force (20%) between maximal and submaximal activation.

In contrast to wild-type soleus, *mdm* soleus showed no statistical difference in force depression relative to the passive force after shortening to the same final length. In the present study, maximally activated isometric force at L_0 in *mdm* soleus muscles decreased by 66.9% compared with wild-type muscles. However, force depression after isovelocity shortening decreased disproportionally by up to 120% at the largest shortening amplitude. We also found that force after passive shortening did not differ between wild-type and *mdm* muscles, suggesting that passive whole muscle shortening is unaffected by the *mdm* mutation.

Mechanisms of force enhancement and depression

Many previous studies have suggested that force enhancement and force depression are caused by fundamentally different mechanisms (Bullimore et al., 2007). For example, force depression is widely thought to result from stress-induced cross-bridge inhibition (Corr and Herzog, 2016; Maréchal and Plaghki, 1979; Sugi and Tsuchiya, 1988). Indeed, the decrease in muscle stiffness in the force-depressed state is assumed to represent a decrease in the number of attached cross-bridges (Ford et al., 1981; Joumaa et al., 2017). In addition to cross-bridge mechanisms (Herzog et al., 1998), force enhancement has long been speculated to result from engagement of an elastic structure upon muscle activation (Edman et al., 1982), now thought to be titin (Herzog and Leonard, 2002; Leonard and Herzog, 2010; Minozzo and Lira, 2013; Powers et al., 2016, 2014).

The present finding that the *mdm* mutation reduces both force enhancement and depression in skeletal muscle suggests either that a basic function of skeletal muscle is compromised in *mdm* muscles or that a common underlying mechanism of force enhancement and depression is affected, or both. Either possibility is surprising given the very small size of the *mdm* deletion (83 amino acids) in relation to the very large size (∼38,000 amino acids) of the titin molecule (Garvey et al., 2002).

Two mechanisms have been proposed that purport to explain both force enhancement and force depression. These are sarcomere length non-uniformity (Edman et al., 1993; Edman, 2012) and calcium-dependent binding of titin to actin (Nishikawa et al., 2012; Schappacher-Tilp et al., 2015). In some respects, observations on *mdm* muscles reported here and elsewhere appear to support sarcomere length non-uniformity as a mechanism for both force enhancement and depression. Passive tension is higher in skeletal muscles from *mdm* mice (Powers et al., 2017; Hessel and Nishikawa, 2017) and *mdm* muscles fail to show a shift in optimal length from twitch to tetanus (Hessel et al., 2017), which has been attributed to shortening against series elastic elements. Thus, it is possible that sarcomere length non-uniformity is reduced in *mdm* muscles, although no direct measurements are available and this should be investigated in future studies.

Observations on *mdm* muscles reported here and elsewhere are also consistent with predictions of calcium-dependent binding of titin to actin: (1) that titin stiffness increases upon muscle activation (Leonard and Herzog, 2010; Nishikawa et al., 2018); (2) that during concentric contractions, titin slackens, which leads to force depression in proportion to the distance shortened (Nishikawa et al., 2018; Schappacher-Tilp et al., 2015); and (3) that these phenomena fail to occur in skeletal muscles from *mdm* mice carrying a deletion of 53 amino acids in Ig83 of the N2A region, implicated to be necessary for calcium-dependent titin–actin binding in previous studies (Dutta et al., 2018).

There is no question that sarcomere length non-uniformities exist within myofibrils and single muscle fibers (Edman et al., 1993; Johnston et al., 2016). The question is whether they are causally related to force enhancement or depression. Edman et al. (1993) showed that the coefficient of variation of sarcomere length along fibers was correlated with the amount of force depression, and that a model of non-uniform sarcomere shortening could predict the results. They also showed that 'length-clamp' of fibers eliminated force depression. In contrast, Granzier and Pollack (1989) found that force depression persisted in similar experiments in which shortening was prevented using servo length control.

However, Trecarten et al. (2015) demonstrated that even single sarcomeres demonstrate force depression, suggesting that sarcomere length non-uniformities do not cause force depression. Furthermore, Pun et al. (2010) demonstrated that force depression occurs on the ascending limb of the force–length relationship where sarcomere instability and length non-uniformity should be minimal. Therefore, the balance of evidence fails to support a causal relationship between non-uniform shortening and force depression.

Several observations fail to support the predictions of force enhancement based on the sarcomere length non-uniformity theory. These include the following observations (for review, see Minozzo and Lira, 2013): (1) even when sarcomeres were clamped and kept stable to prevent non-uniformities, force enhancement was still observed (Abbott and Aubert, 1951; Joumaa et al., 2008; Pun et al., 2010); (2) although sarcomeres do not stretch uniformly on the descending or ascending limb of the force–length curve, they remain stable after stretch, indicating that force enhancement can occur even in the absence of instability (Pun et al., 2010; Rassier et al., 2003); (3) force enhancement occurs on the ascending limb of the force–length relationship (Peterson et al., 2004); (4) force enhancement can exceed the maximum isometric force at optimal length (Herzog and Leonard, 2002; Rassier et al., 2003); (5) force enhancement can occur in a single sarcomere (Leonard et al., 2010); and (6) in single myofibrils in which the length of every sarcomere in series can be measured, the distribution of sarcomere lengths is

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more uniform in the force-enhanced state after stretch than in isometric contractions at the stretched length (Joumaa et al., 2008).

An alternative hypothesis is that titin binds to thin filaments in calcium-activated skeletal muscles (Leonard and Herzog, 2010; Nishikawa et al., 2012; Schappacher-Tilp et al., 2015). While titin force increases marginally in the presence of calcium ions (Labeit et al., 2003), this effect is much smaller than the increase in titin-based stiffness observed in single myofibrils stretched beyond overlap of the thick and thin filaments (Leonard and Herzog, 2010; Powers et al., 2014). By decreasing titin free length and increasing titin stiffness (Nishikawa et al., 2012), the binding of actin and I-band titin could potentially underlie both force enhancement and depression (Herzog, 2019; Nishikawa et al., 2019). A recent model (Schappacher-Tilp et al., 2015) demonstrates that calcium-dependent binding of titin to actin can predict both force enhancement and depression.

Until recently, only data from *in vitro* motility assays supported the hypothesis that titin binds to actin at low pCa (pCa<6.0) (Kellermayer and Granzier, 1996). These studies demonstrated reduced motility of actin on heavy meromyosin in the presence of the large T2 fragment of titin under high calcium conditions. More recently, *in vitro* single molecule force spectroscopy further demonstrated that N2A titin binds to actin at pCa<5.0 (Dutta et al., 2018). This study showed an increase in both the association constant and rupture force between N2A titin and F-actin in the presence of calcium.

If N2A titin binds to actin upon muscle activation, then two mechanical consequences should also be observed. The equilibrium length of active muscles should decrease and the titin-based stiffness should increase when muscles are activated (Nishikawa et al., 2019). In fact, both of these phenomena are observed in active versus passive muscles (Monroy et al., 2017) and myofibrils stretched beyond filament overlap (Leonard and Herzog, 2010; Powers et al., 2016). Recent studies demonstrate that the decrease in equilibrium length (Monroy et al., 2017) and the increase in titin-based stiffness are reduced in *mdm* muscles with a deletion of 53 amino acids in Ig83 in the N2A region. These results are consistent with the idea that N2A-actin binding increases titin stiffness upon muscle activation and plays an important role in force enhancement (Hessel et al., 2017; Nishikawa, 2016) and force depression (Forcinito et al., 1998; Nishikawa et al., 2012; Schappacher-Tilp et al., 2015).

Titin adaptation to training and exercise

Although titin has long been speculated to play a role in eccentric contraction and force enhancement (Herzog and Leonard, 2002), there is surprisingly little evidence to suggest that titin stiffness or isoform expression adapts with training or exercise in skeletal or cardiac muscle. Although studies have suggested that exercise, and in particular eccentric exercise, might be expected to increase titin stiffness in skeletal muscle (Hidalgo et al., 2014) and to decrease it in the heart (Lalande et al., 2017), most studies have not quantified muscle stiffness directly (e.g. Ochi and Westerfield, 2007). Only Reich et al. (2000) directly quantified the passive and active stiffness of whole triceps brachii muscles before and after 8 weeks of chronic eccentric training. They found that both passive and active force and stiffness increased after training, suggesting the possibility that titin stiffness might have increased in response to eccentric training. Likewise, the passive tension of a soleus ghost treated to remove sarcomeric proteins other than titin also showed an increase in passive tension after exercise (Yamaguchi et al., 1985). The only other evidence suggesting adaptive changes in titin stiffness in response to exercise comes from the Heritage Family Study of ∼750 individuals from ∼200 families, which found that the response of stroke volume to endurance training mapped to sequence variants in the titin gene (Rankinen et al., 2003).

Many studies have used mRNA expression, phosphorylation assays and/or gel electrophoresis to quantify changes in titin in response to exercise or training. However, no studies report a change in titin isoform expression as a result of eccentric exercise (Ochi and Westerfield, 2007), fatiguing exercise (Lehti et al., 2009) or plyometric training (Pellegrino et al., 2016). In contrast, studies have found that the ratio of T1:T2 titin fragments appears to decrease with plyometric training and decreased energy cost of running, perhaps due to increased protein turnover (Krüger and Kötter, 2016). Likewise, no studies of cardiac muscle found changes in titin isoform expression in response to exercise or training. A few studies found that titin expression increased either absolutely or relative to myosin heavy chain (MHC) expression (Li et al., 2019).

In the diaphragm of IG domain knock-out mice (Hidalgo et al., 2014), voluntary wheel running increased the titin:MHC ratio and decreased titin phosphorylation, both of which should increase titin-based stiffness. In the heart, increased phosphorylation of titin was observed in the ventricles of IG KO mice (Hidalgo et al., 2014), which would tend to decrease titin passive stiffness and improve exercise tolerance (Lalande et al., 2017). However, Müller et al. (2014) observed decreased titin phosphorylation and increased passive tension in the ventricles of rats after a single 15 min bout of treadmill exercise (Müller et al., 2014).

Rather than direct effects of exercise on titin isoform expression, it appears that titin-based signaling plays an important role in muscle adaptation to exercise (Krüger and Kötter, 2016). For example, titin kinase is phosphorylated in heart tissues in association with exercise (Guo et al., 2017) and stress-sensing proteins associated with titin signaling are upregulated after exercise in skeletal muscle (Koskinen et al., 2017). It is important to keep in mind, however, that finding changes in isoform expression in response to exercise in the giant titin molecule may be like looking for a needle in a haystack, so that absence of evidence is not necessarily evidence of absence. Gel electrophoresis is likely not sensitive enough to detect changes in titin isoform expression, and while exon arrays are perhaps the most sensitive tools for investigating changes in isoform expression (Buck et al., 2014), they are also expensive and not widely used. Thus, while it remains possible that titin isoform expression contributes to exercise adaptation, available data suggest that other mechanisms including post-translational processing (e.g. phosphorylation) and signaling functions that modulate hypertrophy and protein turnover appear more likely roles for titin in adaptation to exercise.

Conclusions

In summary, both force enhancement and force depression are reduced in *mdm* soleus muscles, which are characterized by a small deletion in the N2A region of titin. Following active stretch, *mdm* soleus produced no additional force relative to the passive force after stretch to the same final length. Following active shortening, *mdm* soleus produced less force depression than wild-type muscles relative to the passive force after shortening to the same final length. The result that the *mdm* mutation reduced both force enhancement and force depression suggests that titin contributes to these phenomena.

Acknowledgements

The authors would like to acknowledge Erik Dillingham for the LabVIEW code used to collect data. We are grateful to Beth Dennison for assistance with tissue collection

and to Lindsay Piwinski for help with collecting data. We also thank Natalie Holt, Stan Lindstedt and the anonymous reviewers whose suggestions helped to improve the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: U.T., J.A.M., K.C.N.; Methodology: U.T., J.A.M., K.C.N.; Formal analysis: U.T., N.A.R.; Investigation: U.T., N.A.R.; Data curation: U.T., N.A.R.; Writing - original draft: U.T.; Writing - review & editing: U.T., N.A.R., J.A.M., K.C.N.; Visualization: U.T.; Supervision: K.C.N.; Funding acquisition: U.T., K.C.N.

Funding
This research was supported by the W. M. Keck Foundation, the National Science Foundation [IOS-0732949, IOS-1025806, IOS-1456868 and BIOTEC 0742483], and the Technology Research Initiative Fund of Northern Arizona University.

Data availability
All data and analyses reported in the paper are available from the Dryad digital repository (Tahir et al., 2019): dryad.3vm2818

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