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ORIGINAL PAPER

The effects of a skeletal muscle titin mutation on walking in mice

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Abstract Titin contributes to sarcomere assembly, muscle signaling, and mechanical properties of muscle. The *mdm* mouse exhibits a small deletion in the titin gene resulting in dystrophic mutants and phenotypically normal heterozygotes. We examined the effects of this mutation on locomotion to assess how, and if, changes to muscle phenotype explain observed locomotor differences. Mutant mice are much smaller in size than their siblings and gait abnormalities may be driven by differences in limb proportions and/or by changes to muscle phenotype caused by the titin mutation. We quantified differences in walking gait among *mdm* genotypes and also determined whether genotypes vary in limb morphometrics. Mice were filmed walking, and kinematic and morphological variables were measured. Mutant mice had a smaller range of motion at the ankle, shorter stride lengths, and shorter stance duration, but walked at the same relative speeds as the other genotypes. Although phenotypically similar to wildtype mice, heterozygous mice frequently exhibited intermediate gait mechanics. Morphological differences among genotypes in hindlimb proportions were small and do not explain the locomotor differences. We suggest that differences in locomotion among *mdm* genotypes are due to changes in muscle phenotype caused by the titin mutation.

Keywords Titin · Locomotion · Morphometrics · Muscle · *Mdm* mouse

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Introduction

Titin is the third most abundant sarcomeric protein (Wang et al. 1984) and has several roles in muscle. Titin is important in sarcomere assembly and structure, plays a role in muscle signaling, and also confers intrinsic muscle properties that affect how muscle functions (e.g. Krüger and Linke 2011; Anderson and Granzier 2012). Titin also exhibits a large size diversity in isoform expression (Neagoe et al. 2003; Guo et al. 2010). This variety is found both among species, and also among muscles within a species (Neagoe et al. 2003; Prado et al. 2005; Ottenheijm et al. 2009). For example, the rabbit psoas expresses a shorter titin isoform than the rabbit soleus (Prado et al. 2005). In cardiac muscle, having different titin isoforms affects cardiac function and biomechanics (Anderson and Granzier 2012) and the same relationship is likely true in skeletal muscle.

Numerous studies have demonstrated that the size of the titin molecule affects the passive tension of single muscle fibers, muscle bundles, and whole muscle (i.e., Wang et al. 1991; Prado et al. 2005; Fukuda et al. 2008; Lopez et al. 2008; Ottenheijm et al. 2009; Cornachione et al. 2016). In general, the longer the titin protein, the lower the titin-based passive stiffness (Prado et al. 2005). For example, the rabbit soleus, with its longer titin protein, has lower passive tension than muscles with shorter titin proteins (Prado

et al. 2005). It varies among muscles whether titin or collagen is the primary determiner of passive tension within the physiological range of muscle length (Prado et al. 2005). Increasing evidence indicates that titin also plays a role in active force generation in muscles, particularly with properties such as force enhancement; although, the exact nature of how force enhancement occurs is currently under debate (e.g. Labeit et al. 2003; Colombini et al. 2009; Leonard and Herzog 2010; Nishikawa et al. 2012; Nocella et al. 2012, 2014; Powers et al. 2014; Cornachione et al. 2016). In addition, components of how force enhancement varies among muscles may be explained by variation in titin (Ramsey et al. 2010; Nocella et al. 2014; Cornachione et al. 2016).

Here, as an example of variation in titin structure and function, we examine the effects of a deleterious titin mutation on locomotion, using the muscular dystrophy with myositis (*mdm*) mutation in mice. The *mdm* mutation consists of a 781 base pair deletion in the N2A region of the titin gene (Garvey et al. 2002), which results in a slightly shorter titin molecule. The N2A region of titin has been postulated to serve mechanical (Leonard and Herzog 2010; Nishikawa et al. 2012; Powers et al. 2016) and regulatory functions (Krüger and Linke 2011). Mutant skeletal muscle exhibits several phenotypic changes including higher passive tension (Lopez et al. 2008), lower active stiffness (Taylor-Burt et al. 2015; Powers et al. 2016), and moderately increased collagen (Lopez et al. 2008). The disease is progressive with the pathology initiating in the distal limb musculature and over time moving to more proximal musculature.

While mutant mice can be identified from littermates by their small size and "stiff" gait, no studies have quantified how mutant gaits differ from wildtype and heterozygote gaits. Although there is superficially no phenotypic difference between wildtypes and heterozygotes, subtle differences in gait have been observed (Huebsch et al. 2005), and heterozygotes do express both forms of titin (Garvey et al. 2002). One indirect effect of the titin mutation is that *mdm* mutants are smaller than the other genotypes. Therefore, it is possible that their gait abnormalities are driven by morphological differences (such as limb proportions) rather than by changes to the muscle phenotype induced by the titin mutation. However, gait differences between morphologically similar wildtype and heterozygote mice suggest that variation in locomotion corresponds to variation in muscle phenotype, rather than gross morphological changes.

In vitro muscle preparations have been used to study the effects of the *mdm* mutation on muscle function; however, the effects of this mutation on locomotion remain to be fully investigated. Therefore, we examined how titin-induced muscle phenotypic differences among genotypes contribute to locomotor biomechanics in the *mdm* mouse. Our research has two main goals: (1) to determine if the *mdm* genotypes vary in limb morphology, and whether

differences in limb proportions can account for differences in locomotion; (2) and to determine whether, and in what way, *mdm* genotypes differ in their walking kinematics.

Methods

Mice of the B6.B6C3Fe-Ttn^mdm-J/Cx strain were maintained in a temperature-controlled room with a 12:12 light:dark cycle and were fed ad libitum. For limb morphometrics, frozen euthanized specimens from previous experiments were used. Because heterozygous and wildtype mice are indistinguishable, ear punches and tail snips were used to differentiate them via genotyping using methods of Lopez et al. (2008).

Limb morphometrics

Measurements were taken on euthanized wildtype ($n = 27$; 11♂, 16♀), heterozygous ($n = 25$; 8♂, 17♀), and mutant ($n = 36$; 19♂, 17♀) mice. For each genotype, individuals ranged in age from 25 to 100 days. In addition to mass and total length (measured from nose to anus and abbreviated TL), the hindlimbs of each mouse were measured in lateral view as a series of four segments: toes (from toe tip to the joint between the toes and metatarsals); metatarsals (from the metatarsal joint to the center of the ankle); shank (from the center of the ankle to the center of the knee); thigh (from the center of the knee to the center of the hip). To account for measurement error, each limb segment on each hind limb was measured twice and all four measurements were averaged. Limb length was calculated by summing the measurements for each limb segment. Each limb segment was converted into a percentage of total limb length (%LL). Data was analyzed using an ANCOVA model in JMP 9.0 with age as the covariate, and genotype and sex as fixed factors. Age, mass, and the four limb segment variables were log-transformed for the analysis.

Walking kinematics

Walking data were collected from 9 wildtype mice, 10 heterozygous mice, and 8 mutant mice that were 28–44 days old. Body mass ranged from 13 to 21.8 g in wildtype mice, 15.1 to 21.6 g in heterozygous mice, and 6.6 to 8.4 g in mutant mice. It is impossible to simultaneously size match and age match mutant mice with the other genotypes, as at all post-weaning ages mutant mice are smaller (Fig. 1). However, by using age-matched young mice we have minimized the size difference as much as possible (Fig. 1). Mice were filmed in lateral view at 250 Hz , walking across a flat surface using a Vision Research Phantom V5.1 digital imaging system.

We selected a walking gait for analysis because at faster speeds the mutant mouse gait becomes idiosyncratic and difficult to compare to the other genotypes. Gathering data at
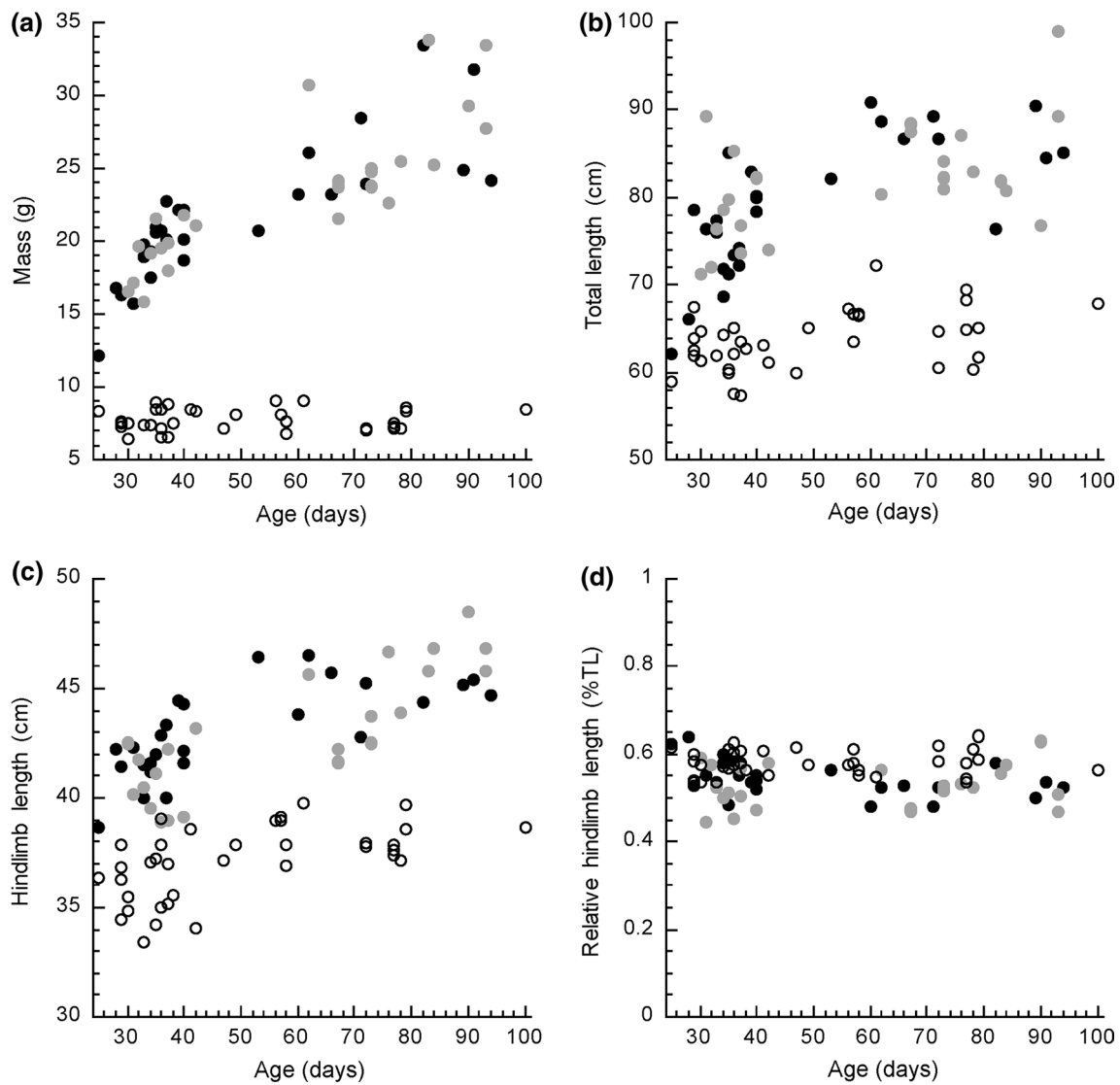
Fig. 1 In all four panels wildtype values are *black*, heterozygote values are *grey*, and mutant values are *white* and every point depicts a different individual. **a, b** Depict body mass and total length vs. age. Mutants are smaller and shorter than the other genotypes, while heterozygotes and wildtypes do not differ. **c, d** Show how mutant hindlimbs are shorter than the other genotypes, but when size differences are accounted for mutant hindlimbs are slightly longer

these slower locomotor speeds was more tractable to do with free walking than with a treadmill. Therefore, we controlled for speed by filming many locomotor trials per mouse, calculated the speed of each trial, and then analyzed only those trials that were speed matched. As can be seen in Table 1 average speed was not significantly different among genotypes.

Walking was encouraged by providing a dark tube at the opposite end of the filming chamber for mice to walk to and hide in. Per individual, walking was characterized from the mean of 2–3 speed-matched locomotor trials. From each trial two complete sequential strides of the left hindfoot were quantified (of 63 trials, in 5 only a single stride was quantified). Sequences were longer than that analyzed,

but usually the rest of the strides were accomplished off camera. Analyzed strides began at the start of stance phase of the left hindfoot, ended at the end of swing phase, and never included strides where the mouse started, or stopped, walking.

After filming, image sequences were imported into *Didge* (A. Cullum, Creighton University) for data analysis. Three points were digitized for each frame: a reference point at the base of the tail; the tip of the toes; and the ventral tip of the heel. The *X, Y* coordinates acquired from digitizing were used to calculate stride length (in cm and %TL) as well as average speed (in both cm/s and TL/s). For each stride, TIFFS were selected that corresponded with

Table 1 Kinematic and timing variables for walking among the *mdm* genotypes

Genotype	Wildtype	Heterozygote	Mutant	*P* value
Speed (cm/s)	16.70 ± 0.84	15.26 ± 1.00	13.85 ± 0.92	0.1305
Speed (TL/s)	1.94 ± 0.10	1.75 ± 0.13	2.03 ± 0.11	0.2207
Total stride (s)	0.35 ± 0.02	*0.36 ± 0.02*	0.3 ± 0.02	0.0361
Stance (s)	0.24 ± 0.02	*0.26 ± 0.02*	0.19 ± 0.02	0.0031
Swing (s)	0.11 ± 0.003	0.1 ± 0.004	0.11 ± 0.004	0.0611
Duty factor	0.69 ± 0.01	0.71 ± 0.01	0.62 ± 0.02	<0.0001
Stride frequency	2.92 ± 0.13	2.91 ± 0.19	3.43 ± 0.22	0.1314
Stride length (cm)	5.88 ± 0.11	5.45 ± 0.09	3.99 ± 0.14	<0.0001
Stride length (%TL)	68.33 ± 1.28	*62.30 ± 1.61*	58.74 ± 1.48	0.0021
Ankle angle Min (°)	35.4 ± 2.3	35.2 ± 0.8	47.5 ± 4.0	<0.0016
Ankle angle Max (°)	117.2 ± 2.8	113.6 ± 3.6	84 ± 4.5	<0.0001

All values are mean ± s.e. Heterozygote variables that are intermediate (when using ANCOVA) between wildtypes and mutants are indicated in italics

Table 2 Statistical results for factors and interactions in the limb morphometrics

Variable	Whole model	Genotype	Sex	Age	Genotype*sex	Genotype*age	Sex*age	Genotype *sex*age
Mass	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	0.0343	0.9854
	302.760_{11}	1457.797_2	25.470_1	161.720_1	10.084_2	38.612_2	4.648_1	0.015_2
Total length	<0.0001	<0.0001	0.0865	<0.0001	0.2994	0.0032	0.8053	0.2842
	33.453_{11}	132.024_2	3.016_1	50.570_1	1.225_2	6.190_2	0.061_1	1.280_2
Limb length	<0.0001	<0.0001	0.9056	<0.0001	0.5047	0.0549	0.9764	0.3147
	37.824_{11}	138.722_2	0.014_1	80.086_1	0.690_2	3.016_2	0.001_1	1.174_2
Limb length (%TL)	<0.0001	<0.0001	0.1246	0.1534	0.7420	0.0485	0.6524	0.0429
	5.153_{11}	18.223_2	2.412_1	2.080_1	0.300_2	3.15_2	0.205_2	3.284_2
Toes (%LL)	0.0001	<0.0001	0.8834	0.0007	0.7412	0.9828	0.3374	0.8487
	4.0539_{11}	11.639_2	0.0217_1	12.612_1	0.301_2	0.017_2	0.932_2	0.164_2
Metatarsals (%LL)	<0.0001	<0.0001	0.0986	0.0001	0.3879	0.8930	0.9104	0.9484
	4.858_{11}	12.089_2	2.796_1	16.721_1	0.959_2	0.113_2	0.013_2	0.053_2
Shank (%LL)	<0.0001	0.0003	0.0043	0.0077	0.0505	0.1699	0.0300	0.4817
	4.771_{11}	9.152_2	8.645_1	7.507_1	3.106_2	1.815_2	4.889_2	0.738_2
Thigh (%LL)	<0.0001	<0.0001	<0.0001	<0.0001	0.0014	0.1621	0.0022	0.4974
	16.065_{11}	25.843_2	26.396_1	21.106_1	7.187_2	1.864_2	10.023_2	0.705_2

For each variable, *p* values are shown on the top row and *F* ratios (with degrees of freedom in subscript) are shown on the bottom row

0–100% of the stride in 10% increments and were used to calculate ankle angle using the angle tool in Image J (NIH freeware). The angle tool calculates an angle created by any three sequential points. In this case all points were from the lateral view with the first point placed on the metatarsal joint, the second point (the angle vertex) on the center of rotation of the ankle joint, and the final point on the center of rotation of the knee. In addition, for each trial, five timing variables were measured: stride duration; swing duration; stance duration; duty factor; stride frequency.

Because speed affects gait parameters (e.g., Heglund and Taylor 1988), we controlled for speed by selecting only slow-speed-matched walking trials for analysis as noted above. While average speed was not significantly different among genotypes (Table 1), we suspected that subtle differences in speed from trial to trial might influence our genotype comparison. Therefore, for all timing and kinematic variables, we used an ANCOVA model with average speed (TL/s) as the covariate, genotype as a fixed factor, and the interaction of genotype*average speed (TL/s).

Results

Mass and total length

At all ages, mutant mice had the smallest body mass and shortest total length (Fig. 1; Table 2). Wildtype and
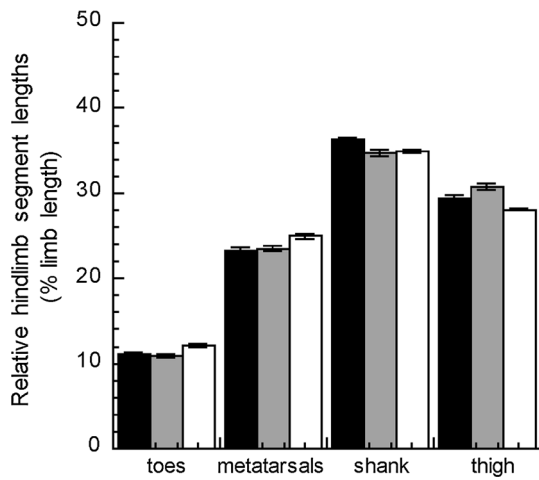
Fig. 2 Average limb segment length as a percentage of limb length ± s.e. for each genotype. Wildtype values are *black*, heterozygote values are *grey*, and mutant values are *white*. Overall mutants have slightly larger feet than the other genotypes

heterozygous mice increased in mass with age; whereas, mutant mice showed little change in mass with age. The genotype*sex interaction was also significant for body mass such that mice formed three statistically different groups from largest to smallest: wildtype and heterozygous males, wildtype and heterozygous females, and mutants (there was no difference between mutant males and females). Non-mutant males and females had similar masses at 30 days, but as mice aged, the males gained mass more quickly than the females (Table 2).

Hindlimb morphometrics

For all genotypes, the hindlimbs increased in length with age, although wildtype and heterozygous mice always had longer hindlimbs than mutant mice (Fig. 1c; Table 2). However, when hindlimb length was normalized by total length, mutants had the longest hindlimbs (Fig. 1d; Table 2). For all mice, the shank was the longest hindlimb segment, followed by the thigh, metatarsals, and toes, respectively (Fig. 2). The relative lengths of all hindlimb segments changed with age (Table 2). Segments comprising the foot (metatarsals and toes) became relatively shorter with age, the thigh became relatively longer and the shank changed

little. No genotype*age interactions were significant, indicating that the slopes of growth with age were the same for all genotypes (Table 2).

Limb proportions differed slightly among *mdm* genotypes (Table 2). Distal limb segments (toes and metatarsals) were relatively longer in mutants than in wildtypes and heterozygotes (Fig. 2), resulting in a proportionally longer foot. There were no significant interactions among factors for the distal limb segments (Table 2). Genotype, sex, and age effects on proximal limb segments were more complicated. For both the shank and thigh, genotype, sex, and age were significant, as were genotype*sex and sex*age (Table 2). Wildtype and heterozygous mice (but not mutant) showed a difference between sexes in proximal limb segment lengths. In particular, female heterozygotes had the shortest shanks (particularly as they aged) of any gender or genotype (Table 3). Additionally, wildtype and heterozygous females had slightly longer thighs than their male counterparts (Table 3). However, all genotype and sex differences in limb proportions were very small, only 1–3% of limb length.

Walking kinematics: stride variables

Walking kinematics differed significantly among genotypes. Genotype had a significant effect on all variables except speed, swing duration, and stride frequency (Table 1). Average speed (%TL) had a significant effect on stance duration, total stride duration, duty factor, and stride frequency. The genotype*speed (%TL) interaction term was not significant for any variable, indicating that all genotypes were affected similarly by variation in speed.

Wildtype mice had the longest stride durations, mutants the shortest, and heterozygotes were intermediate (Table 1). Differences in stride duration were due to corresponding differences in stance duration. The mutant's short stance duration resulted in a walking gait that often looked like a hop or a limp. Although it appears that heterozygotes had a slightly longer (rather than intermediate) stance duration than the other genotypes (Table 1), this is because their walking speed was actually slightly slower than the other genotypes. This example illustrates why it was necessary to use average speed (%TL) as a covariate to account for slight variations in speed. Corresponding to the increase

Table 3 Tukey's post hoc test results for the genotype*sex interaction for shank (%LL) and thigh (%LL) limb segments

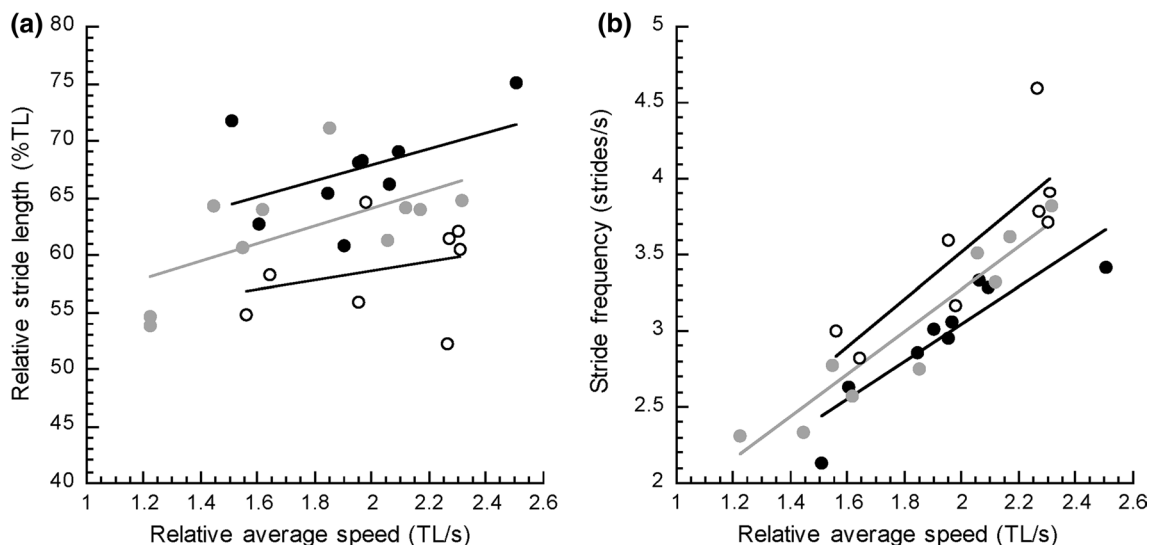|	Wildtype (+/+)		Heterozygote (+/−)		Mutant (−/−)	
	♂	♀	♂	♀	♂	♀
Shank	A	AB	AB	C	BC	BC
Thigh	C	AB	BC	A	C	C

Within a row, when a column shares a letter with another column it indicates that those categories are not different from each other
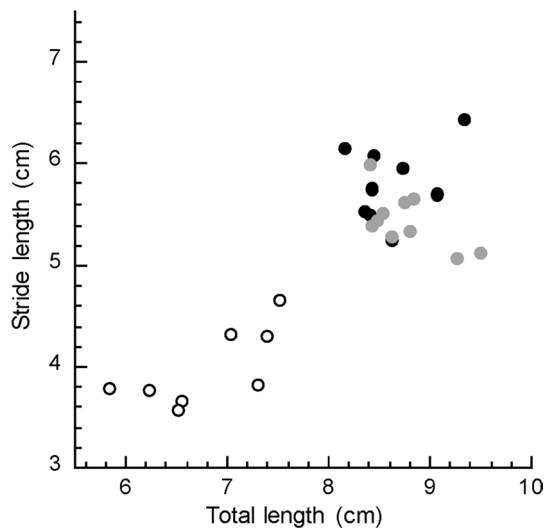
Fig. 3 Average stride length versus total length for all three genotypes where every point depicts a different individual. Wildtype values are *black*, heterozygote values are *grey*, and mutant values are *white*

in stance duration, the duty factors for wildtype and heterozygous mice (~0.70) were significantly larger than for mutants (~0.61).

Wildtype mice had longer stride lengths (both in cm and %TL) than mutant mice (Table 1; Fig. 3). Heterozygous mice exhibit intermediate stride lengths when stride length is standardized for body length (Table 1; Fig. 3). When stride length (%TL) and stride frequency were plotted

against average speed, all genotypes displayed corresponding increases in stride length and stride frequency as speed increased (Fig. 4). Although mutant mice trend towards higher stride frequencies than the other genotypes, the difference is not statistically significant (Table 1).

Walking kinematics: ankle angle

For all genotypes, during the stance phase the ankle was first flexed and then extended, reaching maximum extension at the end of stance. The ankle starts swing phase at its maximum extension and then is greatly flexed as the foot is lifted off the ground and repositioned. Near the end of the swing phase, ankle angle increases again as the foot is extended toward the ground. While the overall pattern is the same for all the genotypes, wildtype and heterozygous mice have a significantly greater range of movement at the ankle than mutant mice during both swing and stance phases (Fig. 5).

Discussion

We quantified differences in walking kinematics and hindlimb morphometrics among the *mdm* genotypes. Genotypes differ in titin expression, which results in numerous changes to the mutant mouse (Garvey et al. 2002; Witt et al. 2004; Huebsch et al. 2005; Lopez et al. 2008). Kinematic differences in walking among the genotypes cannot be explained by morphological differences in limb

Fig. 4 For each panel wildtype values are *black*, heterozygote values are *grey*, mutant values are *white* and every point represents a different individual. **a** Depicts relative stride length (%TL) vs. relative average speed (TL/s) and **b** depicts stride frequency vs. relative

average speed (TL/s). In **a** wildtype and mutant mice differ from each other, while heterozygotes are intermediate. In **b** all genotypes show similar increase in stride frequency with increases in speed and genotypes do not differ
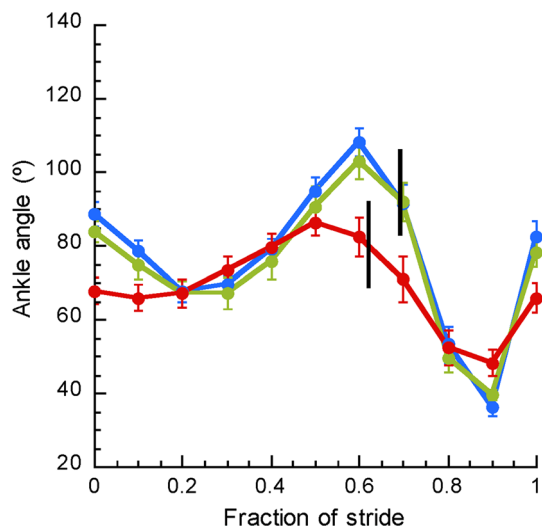
Fig. 5 Average ankle angle ± s.e. versus fraction of stride duration for wildtype mice (*blue*), heterozygotes (*green*), and mutants (*red*). Separate *vertical black bars* on the mutant trace and the wildtype/heterozygote trace demarcate the transition from stance to swing phase in the stride. Wildtype and heterozygote ankle angles do not differ from one another, whereas mutants exhibit a decreased range of motion and both flex and extend their ankle less than the other genotypes

elements. Instead, our data suggests that differences in walking among the genotypes are primarily driven by differences in muscle phenotype caused by the mutation to the titin molecule.

Differences in morphology and locomotion

An indirect effect of the *mdm* titin mutation is that mutant mice are smaller than the other genotypes. Mutants also exhibit subtle differences in their limb proportions, having slightly longer distal limb segments. However, it seems unlikely that these small (1–3%) differences in limb proportions can explain the observed locomotor differences. For example, while mutants have longer hindlimbs (%TL), they have shorter stride lengths than the other genotypes, the exact opposite pattern expected from their morphology. Additionally, because hindlimb length is derived from summation of the four limb segments, if the normalized length of one segment is smaller, other segments must be correspondingly larger, which can inflate differences among genotypes. Finally, there are almost no morphological differences between wildtype and heterozygous mice, yet there are locomotor differences. This suggests that differences in locomotion between genotypes stem primarily from differences in muscle function, not limb morphology.

Mutant mice display kinematic changes in the ankle joint kinematics, as well as a shorter hindlimb stride length, stance duration, total stride duration, and duty factor than

the other genotypes. During locomotion, mutant mice exhibit a reduced range of motion in the ankle; however, manipulations of the ankle joint in mutant mice showed that their ankle is structurally capable of making larger excursions. Mutants have shorter stride lengths as well as strides of shorter duration than the other genotypes. It is unknown if these kinematic changes limit the range of motion at the ankle joint, if the ankle joint has limited mobility because of increased muscle stiffness, or some combination of the two. It is unlikely that the ankle joint is the only joint affected. A complete kinematic study of walking among the *mdm* genotypes would determine the full impact of the titin mutation on walking and how mutants may compensate for changes to muscle phenotype.

Walking in heterozygotes generally appears similar to walking in wildtype mice. However, our data show that heterozygotes actually exhibit some kinematics that are intermediate between wildtypes and mutants. Therefore, despite appearances, when walking at the same speeds as wildtypes, heterozygote hindlimbs undertake smaller strides with shorter periods of ground contact resulting in shorter total stride duration. Unlike the mutant mice, who exhibit different locomotor patterns at the ankle joint in addition to overall stride timing differences, heterozygote differences are more subtle and their ankle kinematics are the same as the wildtype mice. In contrast to the results reported here, Huebsch et al. (2005) found that heterozygous mice had longer stance phases and stride durations than wildtype mice. There are several possible methodological reasons for our differing results. For example, Huebsch et al. (2005) averaged hindlimb and forelimb data, whereas this study did not, and stance and swing durations vary between hind and forelimbs (Clarke and Still 1999). Huebsch et al. (2005) also collected data using a treadmill, and as our study included mutants we did not use a treadmill. This could be important because subtle stride differences exist between overland vs. treadmill walking in mice (Herbin et al. 2007). Lastly, although our methodology differs slightly than Huebsch et al. (2005) our data is internally consistent in that variables such as stride length, stride frequency, and stance duration vary together in a predictable manner for each genotype.

Potential mechanisms for altered muscle function

There are several ways by which the *mdm* mutation could affect muscle function and it is likely that there are multiple causal mechanisms for the observed differences in locomotion among the genotypes. First, the section of the N2A region of titin that is deleted likely plays a role in muscle signaling (Garvey et al. 2002; Witt et al. 2004; Huebsch et al. 2005). At both the transcriptome and protein levels there have been differences found in potential titin-related

signaling proteins between mutant and wildtype mice (Haravuori et al. 2001; Garvey et al. 2002; Witt et al. 2004; Hayashi et al. 2008) that likely affect aspects of whole muscle phenotype either directly or indirectly. Interestingly, changes in signaling protein levels were not found in heterozygote mouse muscle (Witt et al. 2004). Additionally, both wildtype and heterozygote gastrocnemius muscle responded to an overloading experiment in a similar fashion by increasing mass and not exhibiting muscle damage (Huebsch et al. 2005). This demonstrates that although heterozygotes express a percentage of mutant titin, it does not appear to interfere with the signaling for promoting activity-induced adaptation (Huebsch et al. 2005).

In addition to regulatory or signaling function there is also evidence that the titin mutation affects mechanical properties of muscle. For example, the *mdm* mutation results in muscles that are stiffer than expected based on the deletion alone (Lopez et al. 2008; Monroy et al. 2008; Taylor-Burt et al. 2015). It has been suggested that the *mdm* mutation may lead indirectly to an even shorter titin protein via post-transcriptional processing (Lopez et al. 2008). This idea is supported by a recent study on mice with a deletion in a different region of titin that demonstrated differential splicing of the titin protein, leading to increased passive stiffness of the soleus (Buck et al. 2014). However, in psoas myofibrils passive stress did not differ between mutant and wildtype myofibrils (Powers et al. 2016). This suggest either that muscles differ in how the *mdm* mutation affects passive tension (i.e. psoas vs. soleus and diaphragm) or that the increase in passive tension seen in whole muscle studies comes from increases in collagen in the muscle that is not present in the myofibril. Lopez et al. (2008) found that in addition to changes in titin, mutant diaphragm muscles did have increased collagen. However, diaphragm muscle passive stiffness was higher at all lengths including the range of muscle lengths at which titin, rather than collagen, is the main contributor to passive force (Prado et al. 2005). For a given muscle, how much variation in titin (vs. collagen) directly affects muscle properties may depend on the role titin plays for that specific muscle at physiological ranges of muscle length in generating stiffness. For behaviors that are produced by many muscles, such as walking, it is possible that changes to both titin and collagen are affecting the behavior.

Variation in titin likely affects active stiffness of muscles as well (Leonard and Herzog 2010; Nishikawa et al. 2012; Rassier 2012; Nocella et al. 2014; Powers et al. 2016). Although the exact mechanism is debated, several studies have suggested that titin stiffness increases upon muscle activation and contributes to active muscle properties (Colombini et al. 2009; Leonard and Herzog 2010; Nishikawa et al. 2012; Nocella et al. 2012, 2014; Cornachione et al. 2016; Powers et al. 2016). It has also been

demonstrated that the effect of activation on titin varies depending on the muscle (Nocella et al. 2014; Cornachione et al. 2016). It has been shown that for wildtype and *mdm* psoas myofibrils there is over a threefold increase in titin based active vs. passive stiffness in wildtype mice that is not observed in *mdm* mutant mice (Powers et al. 2016). Therefore, the *mdm* mutation appears to prevent titin from increasing stiffness upon activation, resulting in low active muscle stiffness, which has been observed in *mdm* psoas myofibrils (Powers et al. 2016) and *mdm* whole soleus muscle (Monroy et al. 2008; Taylor-Burt et al. 2015).

One of the great challenges in studying the physiology of movement is how to apply information gathered using reductionist methods to what is occurring in the whole organism. At this juncture it is impossible to ascertain exactly what changes to the muscle phenotype are primarily responsible for the observed locomotor changes in the mutant and heterozygous mice. It is likely to be a combination of multiple aspects of muscle physiology as there is strong evidence for disruption to both titin-based muscle signaling (Garvey et al. 2002; Witt et al. 2004; Huebsch et al. 2005) and to titin-based muscle mechanical properties (Lopez et al. 2008; Monroy et al. 2008; Taylor-Burt et al. 2015; Powers et al. 2016). Identifying how locomotor patterns change as muscle phenotype changes is a critical step in understanding how muscles work in the whole organism. Heterozygote mice may prove particularly interesting for examining muscle compensatory mechanisms as in most instances they have been found to not differ from wildtype mice; yet, they express mutant titin and subtle locomotor differences have been observed both by Huebsch et al. (2005) and in this study.

Conclusions

Intrinsic muscle properties play an important role in locomotion, because they may be important in adjusting leg stiffness (Ferris et al. 1998; Lindstedt et al. 2002). Changes in titin expression may be one way that muscle is modified. For example, eccentrically trained rats demonstrated an increase in passive stiffness (likely attributable to titin) in their triceps brachii muscle (Reich et al. 2000). Human athletes demonstrate different titin isoforms in their vastus lateralis muscles when compared to non-athletes (McBride et al. 2003). Mice have shown increased expression of titin in their gastrocnemius muscle in response to endurance training (Bellafiore et al. 2007). This study shows that the *mdm* mutation in titin changes walking kinematics in both mutant and heterozygote mice, possibly by affecting both the signaling and mechanical properties of titin resulting in a changed muscle phenotype. This suggests that variation in titin among muscle groups is important for the execution

of complicated motor tasks, such as walking. The *mdm* mouse model is a valuable model system to address questions about titin, locomotor plasticity, and locomotor compensatory mechanisms.

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