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The effect of α-actinin-3 deficiency on muscle aging

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ABSTRACT

Deficiency of the fast-twitch muscle protein α-actinin-3 due to homozygosity for a nonsense polymorphism (R577X) in the *ACTN3* gene is common in humans. α-Actinin-3 deficiency (XX) is associated with reduced muscle strength/power and enhanced endurance performance in elite athletes and in the general population. The association between R577X and loss in muscle mass and function (sarcopenia) has previously been investigated in a number of studies in elderly humans. The majority of studies report loss of *ACTN3* genotype association with muscle traits in the elderly, however, there is some indication that the XX genotype may be associated with faster muscle function decline. To further explore these potential age-related effects and the underlying mechanisms, we examined the effect of α-actinin-3 deficiency in aging male and female *Actn3* knockout (KO) mice (2, 6, 12, and 18 months). Our findings support previous reports of a diminished influence of *ACTN3* genotype on muscle performance in the elderly: genotype differences in intrinsic exercise performance, fast muscle force generation and male muscle mass were lost in aged mice, but were maintained for other muscle function traits such as grip strength. The loss of genotype difference in exercise performance occurred despite the maintenance of some "slower" muscle characteristics in KO muscles, such as increased oxidative metabolism and greater force recovery after fatigue. Interestingly, muscle mass decline in aged 18 month old male KO mice was greater compared to wild-type controls (WT) (−12.2% in KO; −6.5% in WT). These results provide further support that α-actinin-3 deficient individuals may experience faster decline in muscle function with increasing age.

1. Introduction

Muscle aging, or sarcopenia, is defined as the loss of skeletal muscle mass and function with advancing age (Morley et al., 2001). Apart from the loss in mass, aging muscles also display a decline in muscle quality, with changes in muscle structural composition, contractility, fatigability and metabolism (Carmeli et al., 2002). Past studies undertaken on human vastus lateralis muscle have consistently found greater effects of aging in the force producing fast-twitch (type 2) fibres than in slow fibres (type 1), with the average type 2 fibre cross-sectional area diminished by 20–50% and type 1 by up to 25% (Doherty, 2003).

α-Actinin-3, encoded by the *ACTN3* gene, is one of the major structural components of the skeletal muscle Z-disk in fast-twitch muscle fibres and binds to a wide range of structural, metabolic and signalling proteins (Houweling and North, 2009). Deficiency of α-actinin-3 due to homozygosity for the common null *ACTN3* 577X polymorphism occurs in an estimated 16–18% of the global population (North et al., 1999) and is associated with reduced sprint performance and lower muscle strength and power in elite athletes and untrained adolescents and young adults (Yang et al., 2009), suggesting that α-actinin-3 is necessary for optimal forceful and rapid muscle contractions. Interestingly, the *ACTN3* 577XX is also associated with enhanced endurance performance (Eynon et al., 2009; Yang et al., 2003) and significantly lower body mass index and fat free mass (Walsh et al., 2008). Phenotypic analysis of the *Actn3* knockout mouse model has provided a mechanistic explanation for the alterations in muscle function associated with α-actinin-3 deficiency (MacArthur et al., 2007, 2008). Compared to wild-type controls (WT), KO mice showed a reduction in lean mass, muscle mass, and fast-twitch (type 2B) fibre size, as well as a shift in fast fibre metabolic and contractile properties towards those of slower, more oxidative fibres. These changes in fast fibre properties in *Actn3* knockout mice, together with the R577X

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association with muscle function in humans, raise the possibility that *ACTN3* R577X is a genetic variant contributing to variations in skeletal muscle function in older adults.

The association between R577X and muscle function has been investigated in a number of studies in elderly humans, however the results vary between studies, likely due to small sample sizes and variability in the age of the study participants. Contrary to the findings in non-athlete adolescents and young adults, three studies in older Caucasian men and women (aged 60–70 years), and a recent study in Spanish nonagenarians showed no association between *ACTN3* genotype and muscle strength and ambulation ability (Bustamante-Ara et al., 2010; Delmonico et al., 2007; McCauley et al., 2010; San Juan et al., 2006). The effects on muscle power and muscle mass in the elderly are also contentious. One study found significantly greater relative peak power in older XX women (Delmonico et al., 2007), while another demonstrated lower knee extensor shortening and lengthening peak torque in XX women similar to that described in young adults (Walsh et al., 2008). A recent study found no association between *ACTN3* genotype and indices of muscularity such as thigh lean mass (McCauley et al., 2010), contrasting the results of an earlier study that reported a tendency for lower fat free mass in older *ACTN3* XX women compared to RR + RX women (Walsh et al., 2008). Interestingly, another recent study in a group of older Japanese women (aged 50–78 years) also demonstrated significantly lower mid-thigh cross-sectional area by MRI in XX compared with RR + RX genotypes (Zempo et al., 2010), but found no significant genotypic effects on physical activity.

One of the larger studies to date relating to the effect of *ACTN3* genotype in the elderly was a multi-centre study of 2568 well functioning White Caucasian men and women aged 65 years or older, characterised for body composition and muscle traits such as grip strength, quadriceps torque (power), muscle volume and muscle quality (Delmonico et al., 2008). No significant association was found between *ACTN3* genotype and any of the muscle traits at baseline. However, examination of muscle function decline after 5 years revealed significant genotype association; XX men showed a significantly greater 5-year increase in their 400 m walk time compared to RX and RR men, while RR women showed ~35% lower risk for incident persistent lower extremity limitation relative to XX women. Similarly, elderly RX and XX women were found to have a 33% increased risk of falling in two large cohorts of Caucasian menopausal women (total 4163 women) (Judson et al., 2011).

In combination these data suggest that *ACTN3* genotype may affect some aspects of the variation in muscle function in older individuals, although the genotype effects noted in adolescents and young adults for muscle phenotypes such as grip strength, power and muscle volume may not persist with age. On this basis, we aimed to examine the effects of α-actinin-3 deficiency in an aging *Actn3* knockout mouse line where genetic and environmental variables can be controlled. We hypothesised that α-actinin-3 deficiency is detrimental to the function of aging muscle, resulting in lower muscle strength and mass in mature KO mice compared to WT. To test this, we compared the muscle performance and function of aging WT and KO mice and examined their muscle phenotypes at ages 2, 6, 12 and 18 months in male and female groups.

2. Methods

This study was approved by our local Animal Care and Ethics Committee. All tests were performed on mice of 129 genetic background as described previously (MacArthur et al., 2007). Mice were fed food and water *ad libitum*, and were maintained on a 12 h:12 h cycle of light and dark.

2.1. Grip strength

Forearm grip strength was examined using a grip strength meter (Columbus Instruments). Mice were lifted near the base of the tail and allowed to grasp the trapeze with their front paws. During the test, mice were pulled perpendicularly from the meter in one swift and steady motion while keeping the body parallel to the floor. Mean grip strength (recorded in Newtons (N)) for each mouse was derived from ten trials. All mice were tested by operators blinded to genotype.

2.2. Intrinsic exercise capacity test

Baseline intrinsic endurance capacity of WT and KO mice was examined using a modified version of a previously described protocol (Koch and Britton, 2001). Briefly, mice were placed on a motorized treadmill (Columbus Instruments, Columbus, OH) set at a 15° incline and a baseline speed of 10 m/min. Mice were encouraged to continue running on the treadmill by the placement of a negative stimulus (a stiff-bristled brush) at the rear of the treadmill, and by a positive stimulus as represented by a darkened zone at the front of the treadmill. Speed was increased by 1 m/min every 2 min until mice were exhausted. Exhaustion was defined as the inability of the mouse to extricate itself from the negative stimulus three times within a two minute interval. Time of exhaustion and maximum speed achieved were recorded, and total distance calculated. All mice were tested by observers blinded to genotype.

2.3. Tissue collection

Mice were euthanized by cervical dislocation immediately prior to tissue collection. Muscles were removed, weighed, and immediately snap-frozen in liquid nitrogen (for metabolic enzyme analysis) or covered in cryo-preservation medium (Tissue-Tek) and snap frozen in partially-thawed isopentane for protein and muscle fibre analyses. Tissues were stored in liquid nitrogen until use.

2.4. Fibre analyses

A transverse 8 μm section was cut from the mid-section of the frozen mouse quadriceps muscle. AffiniPure Fab fragment goat anti-mouse IgG (1:25 dilution; Jackson ImmunoResearch) was used to block the section for 1 h at room temperature to prevent cross-reaction with endogenous mouse antibodies. Detection of myosin heavy chain (MyHC) type 2B was performed using a monoclonal antibody raised from hybridoma culture (BF-F3; developmental study hybridoma bank). To identify the sarcolemma and define muscle fibre borders, the section was also co-stained with dystrophin (dys6–10; kindly supplied by L. Kunkel). Secondary incubation was performed at room temperature using Alexa Fluor 555 goat anti-mouse IgM and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes). To detect MyHC type 2A and type 1 fibres within the same section, we used the Zenon mouse IgG labeling kit (Molecular Probes) as previously described (Gregorevic et al., 2008). Antibody against MyHC 2A (SC71) was pre-labeled with Zenon Alexa Fluor 488; the antibody against MyHC type 1 (MAB1628, Chemicon) was tagged with the Zenon Alexa Fluor 350 as per manufacturer's instructions. MyHC type 2X fibres could not be identified on the same section with antibody labeling since the only antibody available is also an IgM antibody raised in mouse (Lucas et al., 2000); no labeling kit for mouse IgM is currently available. Non-staining fibres are therefore assumed to be type 2X fibres, which was confirmed in a sequential muscle section with an antibody against MyHC 2X (results not shown). All images were captured using the Olympus BX50 microscope attached to a Jenoptik ProgRes digital camera and ProgRes software (SciTech). Fibre analysis was performed using *MetaMorph®* software (Molecular Devices) using previously described protocol (Garton et al., 2010).

2.5. Western blot

Equal sample loading was evaluated using intensity of myosin and actin bands on pre-cast mini-gels (Invitrogen) stained with Coomassie

BlueBrilliant (Sigma-Aldrich). Samples adjusted for loading were separated by SDS-PAGE on pre-cast mini-gels, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore), probed with antibodies against mitochondrial proteins porin (20B12; 1:5000; Molecular Probes) and cytochrome *c* oxidase (COX IV) (20E8; 1:2000; Molecular Probes). Following probing, PVDF membranes were stained with Coomassie BlueBrilliant (Sigma-Aldrich) to generate final myosin loading controls. Densitometry was performed using Quantity-One software (Biorad); porin and COX IV expression levels were presented relative to total myosin.

2.6. Enzyme assays

The activities of lactate dehydrogenase (LDH, EC 1.1.1.27), 3-hydroxyacyl-CoA dehydrogenase (BHAD, EC 1.1.1.35), citrate synthase (CS, EC 4.1.3.7) and hexokinase (HK, EC 2.7.1.1) in WT and KO muscles were determined using previously described methods (MacArthur et al., 2008). For all assays, the linearity with time and dependence on the amount of extract added was confirmed. All assays for a particular enzyme were performed in triplicates on the same day. Activities for each sample in the absence of substrate were measured for each sample and were subtracted from these values.

2.7. Contractile properties

Mice used in the muscle contractile studies were in four age groups: young males (age 2 months), aged males (age 19–20 months), young females (age 6 months) and aged females (age 19–22 months). The protocols used have previously been described (MacArthur et al., 2008).

2.8. Statistics

Most comparisons reported in this study involved small sample sizes to which standard tests for normality could not be applied. As such, those pair-wise comparisons were performed using the non-parametric Mann–Whitney U test; comparisons between groups where sample size $n>25$ were performed using the student's t-test. Differences were considered significant at $P<0.05$ ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). All histograms show mean values, with error bars indicating 95% confidence intervals (95% CI) unless otherwise stated. Two-way ANOVA was also performed to determine the effect of *Actn3* genotype on the response to aging. Statistics were performed using SPSS.

3. Results

3.1. Muscle performance

Grip strength and intrinsic exercise performance were examined in aging male and female mice at 2, 6, 12 and 18 months. The lifespan of the 129 strain is ~22 months for males and ~21 months for females; there is a high incidence of testicular teratomas in the 129 strain (Storer, 1966) and a number of female mice were euthanized due to the formation of malignant ovarian tumours at 22 months (J.T. Seto, unpublished observations). We have thus opted not to age our mice beyond 18–20 months to avoid confounding the resulting phenotypes by the development of tumours, and to avoid losing members of the cohort as they neared the limit of their life-span. Comparisons of WT and KO mice showed lower grip strength in KO mice at all ages in both male and female groups, suggesting an on-going effect of genotype on this functional trait (Fig. 1). These differences were significant between genotypes for all ages in the female group, and at all ages except at 18 months for the males. Regardless of genotype or gender, grip strength peaked at age 6 months and showed a steady decline at 12 and 18 months in all mice, suggesting that WT and KO mice have

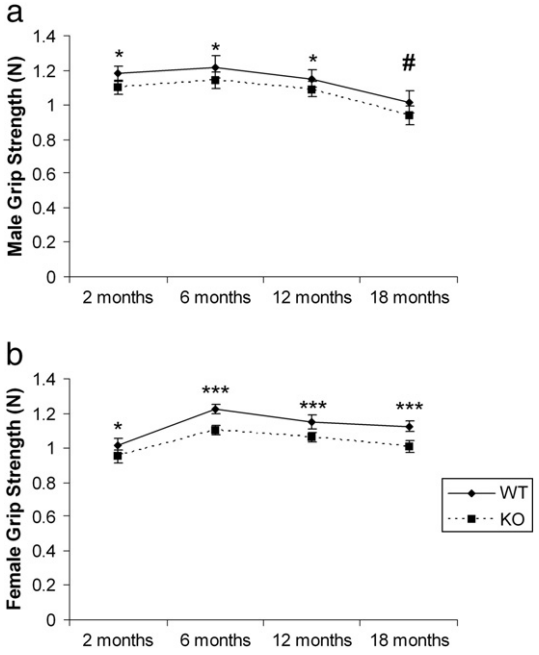

Fig. 1. Grip strength is reduced in aging WT and KO mice. Both male (a) and female (b) mice showed a steady decline in grip strength with age after 6 months. Differences in grip strength between WT and KO were maintained in male and female mice for all ages, with significance achieved at all ages for both genders, except for 18 month old males. Grip strength at 18 months compared to 2 months was significantly lower in male mice ($^{\#}P<0.001$), but remained significantly higher in female mice, suggesting less severe age-related grip strength losses in female mice. Mean ± 95% CI; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, student's t-test. *n* ranged from 19 to 33 in males; from 22 to 84 in females.

similar rates of decline in grip strength with age. This was confirmed by a lack of significant genotype and age interaction for male and female grip strength by two-way ANOVA (data not shown). The decline in grip strength with age was more pronounced in aging males compared to females, with 18 month WT and KO male mice showing significantly lower grip strength compared to age 2 months (WT: −14.1%, $P=0.0001$; KO: −14.7%, $P<0.0001$).

In contrast to grip strength, genotype differences in baseline forced treadmill running capacity between WT and KO were not maintained in mature mice. Although KO mice ran further than WT at age 2 months, no significant difference in distance running ability was detected between genotype groups at other time points. Both WT and KO male and female mice demonstrated a decline in intrinsic exercise capacity from 2 months of age (Fig. 2).

3.2. Muscle mass

We removed and weighed the quadriceps of WT and KO mice of various ages and examined for changes in the mass of the quadriceps with age. We specifically focused on the quadriceps muscle for the majority of the remaining studies because the quadriceps is a weight bearing muscle and is most commonly active, thus changes with age are likely to be more evident. We found marked differences in the effect of *Actn3* genotype in aging female (Fig. 3) and male (Fig. 4) mice. In females, the quadriceps mass of KO mice were significantly lower compared to WT mice at all ages examined (Fig. 3a), suggesting an ongoing effect of *Actn3* genotype on female muscle mass. Age appears to have similar effects on female quadriceps mass regardless of genotype; maximum muscle mass in both WT and KO mice was reached at 6 months, with no appreciable decline at 18 months. Examination of quadriceps mass relative to total body mass however showed significant decline in both WT (−15%, $P<0.0001$) and KO (−13.6%, $P<0.0001$) at 18 months compared to their peak at 6 months (Fig. 3b); this is due to the persistent and significant

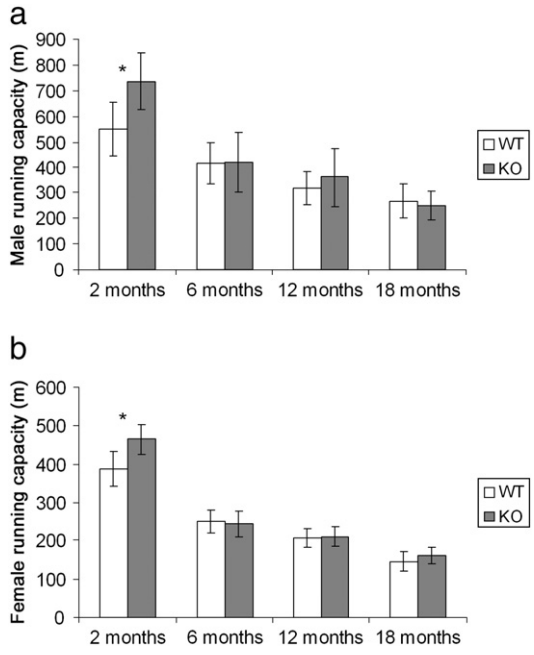

Fig. 2. Intrinsic exercise capacity declines with age for WT and KO mice. Both male (a) and female (b) mice showed decreased endurance capacity with age regardless of genotype. At age 2 months, KO mice ran significantly greater distance than WT, however, the effect of genotype was lost in aging mice. Mean ± 95% CI; *$P<0.05$, Mann–Whitney U test, $n>17$ for all groups.

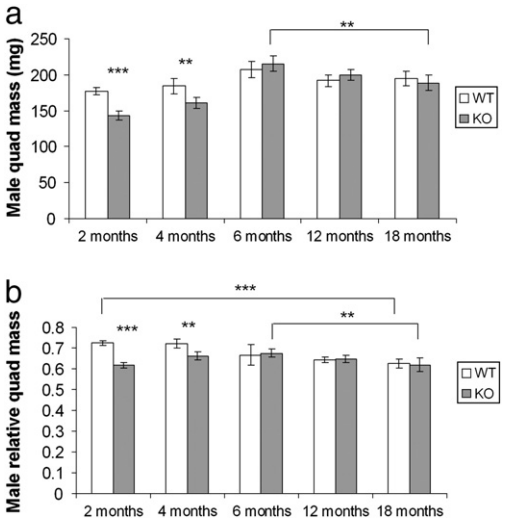

Fig. 4. Mean quadriceps mass in male WT and KO mice. At 2 and 4 months, male WT mice showed greater (a) absolute and (b) relative quadriceps mass than KO, but genotype difference was lost by age 6 months due to a significant increase in KO quadriceps mass between 4 and 6 months. Both WT and KO muscles showed decreases in absolute muscle mass from age 6 months, but significance was only reached in the KO. Peak relative muscle mass was at 2 months for WT and at 6 months for KO. Both WT and KO mice showed significant declines in relative muscle mass at 18 months compared to their respective peak. Mean ± 95% CI; $n>13$ for most samples; $n>3$ for age 6 month samples. **$P<0.01$, ***$P<0.001$.

increases in body weight in both female WT and KO mice between 6 and 18 months (data not shown).

The effect of *Actn3* genotype with age was vastly different in male mice compared to females. Although peak muscle mass was also reached in male WT and KO mice at age 6 months (Fig. 4a), in contrast to female mice, the effect of *Actn3* genotype on male quadriceps mass was not maintained at all time points, with WT and KO mice demonstrating similar quadriceps mass from age 6 months and onwards. Examination of an intermediate time point (4 months) showed significantly lower quadriceps mass in KO compared to WT, suggesting that the effect of *Actn3* genotype in male quadriceps muscle is lost upon reaching peak muscle mass. Between ages 4 and 6 months, male KO mice showed a greater increase in quadriceps mass (34.0%, $P<0.0002$) compared to WT (12.7%, $P=0.018$), thus accounting for the loss of genotype difference in muscle mass at 6 months and thereafter. Interestingly, male KO quadriceps mass also showed greater levels of decline at age 18 months compared to 6 months (KO: −12.2%, $P=0.002$; WT: −6.5%, $P=0.179$), indicating increased muscle atrophy in KO mice. Likewise, relative muscle mass in male KO mice was significantly lower at age 18 months compared to their peak at 6 months (−9%, $P<0.001$) (Fig. 4b). The relative quadriceps mass of male WT mice at 18 months was also significantly lower compared to their peak at age 2 months (−13.4%, $P<0.0001$). In contrast to the female group, the decline in male relative muscle mass was due to the decrease in quadriceps mass alone; body weight was unchanged between 6 and 18 months for both WT and KO mice (data not shown). The different ages of decline in relative muscle mass suggest subtle differences exist between male WT and KO mice in their response to muscle aging. This was confirmed by two-way ANOVA analysis of quadriceps mass and the mass of other lower limb muscles, which demonstrated significant age and genotype interaction for the mass of all fast-twitch muscles examined in males but not in females (Table 1).

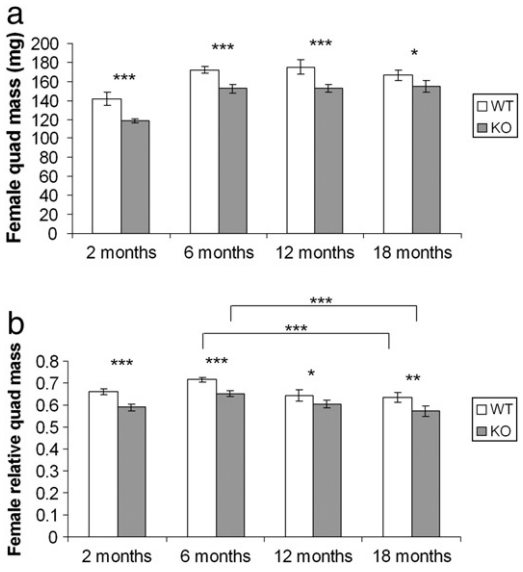

Fig. 3. Mean quadriceps mass in female WT and KO mice. At all time points examined, female WT mice showed greater (a) absolute quadriceps (quad) mass and (b) relative quadriceps mass, than KO. In WT and KO mice, peak muscle mass was reached at age 6–12 months and was maintained at age 18 months for most muscles. Mean ± 95% CI; $n>6$ for all samples. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

3.3. Fibre size and number

We next examined WT and KO quadriceps muscle for changes in fibre size and number at "younger" (2, 4, 6, 12 months) and "older" (18 months) time points in female and male mice, since the loss of fast fibres and fibre type shift towards slower fibre types are established hallmarks of muscle aging (Pette and Staron, 2000). We examined this by automated fibre analysis on muscle cross-sections stained with antibodies against myosin heavy chain isoforms 2B, 2A and type 1; all remaining non-staining fibres were considered to be type 2X fibres. Again, we detected differences between males and females, and thus present these results separately.

In female quadriceps muscle (Fig. 5), there was a strong trend towards smaller 2B fibres (in which α-actinin-3 is usually expressed) in KO compared to WT at all time points examined, however, due to

Table 1
Two-way ANOVA for muscle weights in aging WT and KO.

Muscle	Male (2, 4, 6, 12, and 18 months)			Female (2, 6, 12, and 18 months)		
	Genotype	Age	Interaction	Genotype	Age	Interaction
TA	0.261	<0.001	0.001	0.220	<0.001	0.614
EDL	<0.522	<0.001	<0.001	<0.001	<0.001	0.212
SOL	<0.001	0.485	0.566	<0.001	<0.001	0.835
GST	<0.001	<0.001	<0.001	<0.001	<0.001	0.305
QUAD	0.008	<0.001	<0.001	<0.001	<0.001	0.371
SPN	<0.001	<0.001	0.014	<0.001	0.209	0.690
HRT	0.129	<0.001	0.136	0.832	<0.001	0.913

Significant age and genotype interaction was found in the male group for the weights of all fast-twitch muscles: tibialis anterior (TA), extensor digitorum longus (EDL), gastrocnemius (GST), quadriceps (QUAD) and spinalis (SPN), indicating an effect of *Actn3* genotype on muscle mass changes with age. No genotype and age interaction was found for the weights of the predominately slow-twitch muscle soleus (SOL) and heart (HRT), which do not normally express α-actinin-3, nor for the weights of any muscles in the female group.

small sample sizes ($n=2$–3) for each group, we were unable to reach formal statistical significance in our analyses. There was no difference in the size of 2X, 2A or type 1 fibres (which do not usually contain α-actinin-3) between WT and KO at any time point examined; the average size for each fibre type at 18 months were similar to the earlier time points. Similarly, fibre type proportions were similar at 18 months compared to "younger" time points, suggesting that the effects of aging have yet to set in on female mice at age 18 months. There was also no difference between WT and KO in fibre type proportions at 2, 6, 12 or 18 months.

In males (Fig. 6), we examined WT and KO muscles only at age 4 months for comparison at 18 months since the effect of *Actn3* genotype in male quadriceps mass was lost from age 6 months and muscle mass was near its peak at age 4 months. At 18 months, the proportion of fast 2B fibres was significantly and similarly reduced in both male WT and KO quadriceps compared to 4 months (WT: −10.3%, $P=0.009$; KO: −10.2%, $P=0.015$), while the proportion of 2X fibres

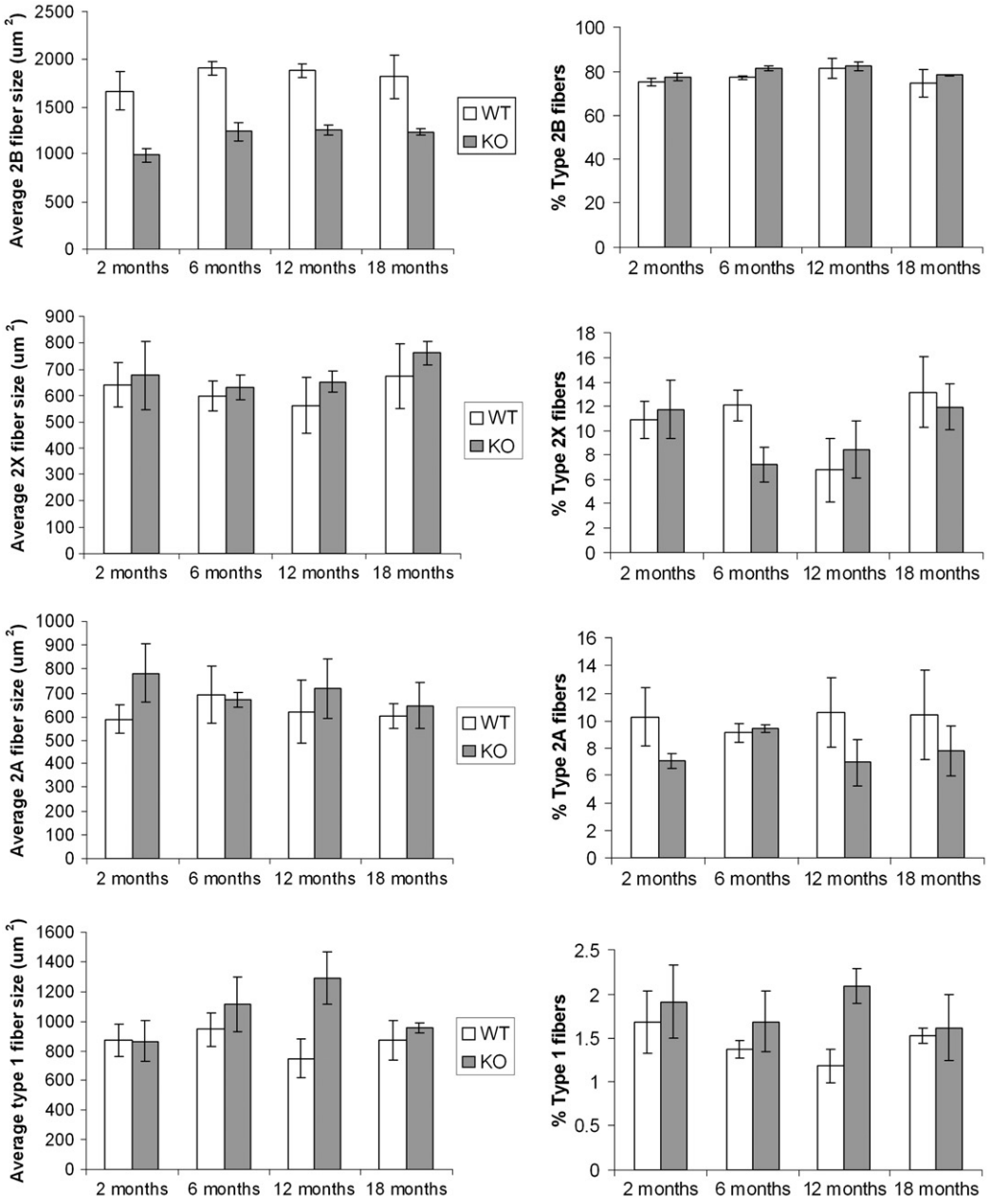

Fig. 5. Fibre size and fibre type proportions in female WT and KO quadriceps. Fibre type proportions were similar between WT and KO muscles and were not different between time points. WT muscles showed a trend for larger 2B fibres at 2, 6, 12 and 18 months but significance was not reached due to low sample numbers. Mean ± SEM; $n=3$ for all samples.

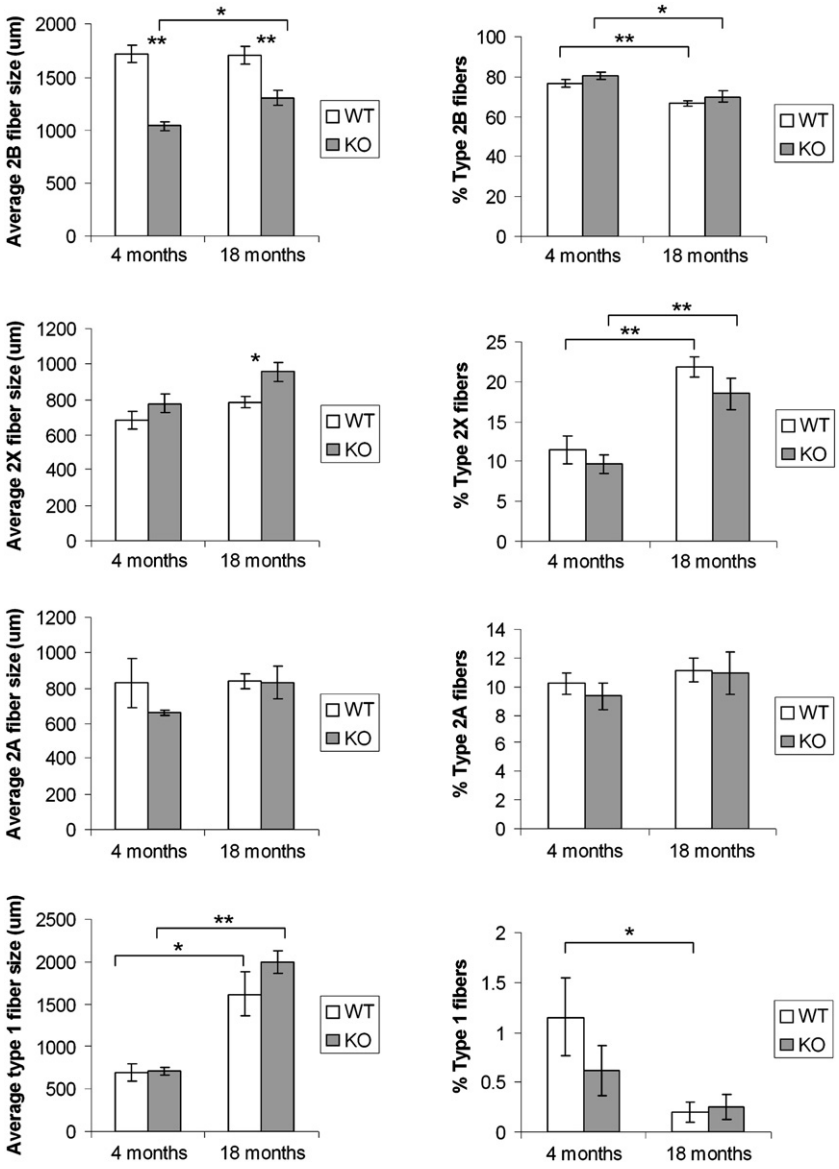

Fig. 6. Fibre size and fibre type proportions in male WT and KO quadriceps. WT and KO muscles showed decreased 2B and type 1 and increased 2X fibre proportions at age 18 months compared to young muscle at 4 months. Aging also resulted in slight increase in fast 2B fibre size in KO muscles as well as type 1 fibre atrophy in both WT and KO muscles. At both 4 months and 18 months, KO muscles demonstrated smaller fast 2B and larger 2X fibres compared to WT, however fibre type proportions were similar between WT and KO muscles at both time points. Mean ± SEM; $n = 6$ for all samples. $^{*}P<0.05$, $^{**}P<0.01$.

was increased (WT: 10.3%, $P = 0.002$; KO: 8.9%, $P = 0.009$), suggesting a fibre type shift in both male WT and KO muscles at age 18 months that may be indicative of the onset of sarcopenia. There was also a trend for decreased proportion of type 1 fibres, however this was only significant in WT (WT: −0.9%, $P = 0.04$; KO: −0.4%, $P = 0.309$).

We also detected subtle differences between male WT and KO quadriceps in age-related changes in fibre size. Fast 2B and type 1 fibres from KO muscles were significantly larger at 18 months compared to 4 months; there was also a trend for increased 2X fibre size in KO mice at 18 months ($P = 0.06$). This effect of age on fibre size is in marked contrast with WT quadriceps, where only type 1 fibres showed significant increases in size at 18 months compared to age 4 months. Despite these differences, KO muscles continued to show significantly smaller fast 2B fibres compared to WT as well as a trend for larger 2X fibres at both 4 months and 18 months, consistent with previously published results at age 2 months (MacArthur et al., 2008). Overall, these results suggest that the effect of α-actinin-3 deficiency on the size of fast 2B fibres is maintained throughout life in both male and female muscles.

3.4. Metabolism

Since we have previously demonstrated significant increases in mitochondrial protein expression and aerobic enzyme activities in KO muscles compared to WT at age 2 months (MacArthur et al., 2008; MacArthur et al., 2007), we examined for differences between WT and KO in muscle metabolism in 18 month male and female quadriceps (Fig. 7). In males, the expression of mitochondrial proteins porin and cytochrome oxidase subunit IV (COX IV) continues to be higher in 18 month KO muscles compared to WT (Fig. 7a). Interestingly, there was an increased expression of COX IV in both WT and KO muscles with increasing age; WT muscles also showed increased expression of porin with age.

In females, KO muscles also showed higher levels of porin and COX IV compared to WT at age 2 months, however the same trends were not observed at 18 months (Fig. 7b). Moreover, age did not alter the expression of porin or COX IV in WT mice, and KO mice showed decreased expression of these mitochondrial proteins at 18 months compared to 2 months.

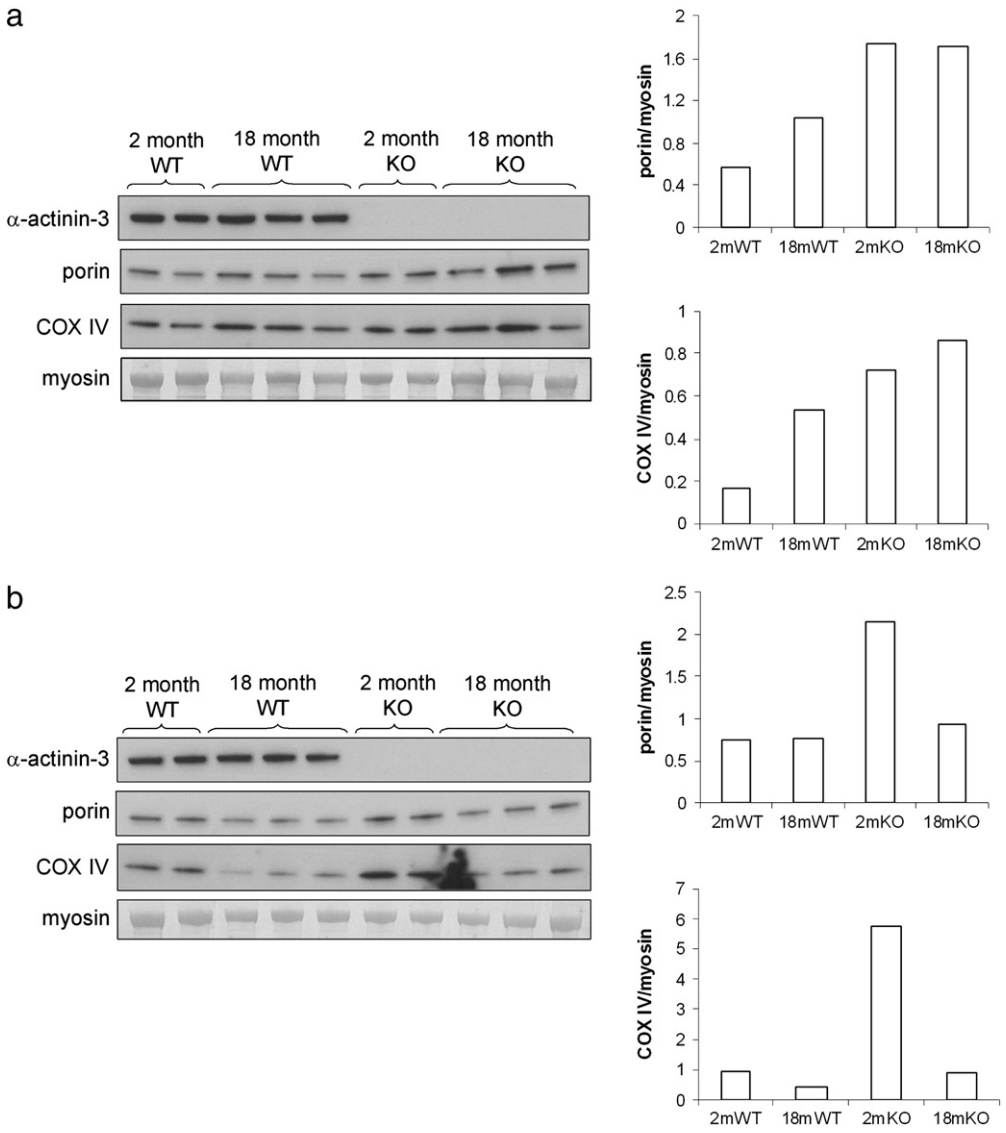

Fig. 7. Mitochondrial protein expression in young and mature WT and KO quadriceps. (a) In males, KO quadriceps consistently showed increased porin and cytochrome *c* oxidase subunit IV (COX IV) levels compared to WT in both young (2 months) and mature (18 month) time points. There was also a trend for increased porin in WT muscles as well as increased COX IV in both WT and KO muscles with age. (b) In females, porin and COX IV levels were higher in young KO quadriceps compared to WT, but levels were similar in mature WT and KO quadriceps. In contrast to male muscles, porin and COX IV levels were unchanged and decreased in both WT and KO groups.

To better quantitate the changes in muscle metabolism with age in female WT and KO mice, we used a more sensitive assay and analysed the activities of various metabolic enzymes in female quadriceps muscles aged 2, 6, and 18 months; these include the glycolytic enzyme hexokinase (HK), the anaerobic pathway enzyme lactate dehydrogenase (LDH), the fatty acid oxidation pathway enzyme 3-hydroxyacyl-CoA-dehydrogenase (HAD), and the TCA cycle enzyme citrate synthase (CS) (Fig. 8). Many of these comparisons did not achieve formal statistical significance due to the small sample number ($n=6$), however, KO muscles showed a consistent trend for higher activities for the glycolytic and oxidative enzymes HK, HAD and CS compared to WT, while WT muscles showed higher or similar activity of the anaerobic enzyme LDH compared to KO at all ages. There were no changes in the levels of mitochondrial enzyme activities in either WT or KO with age. Overall, these results suggest a consistent effect of α-actinin-3 deficiency on muscle metabolism across the life-span of both male and female mice.

3.5. Contractile properties

We next examined the contractile properties of EDL muscles from mature WT and KO mice to determine if the contractile differences with α-actinin-3 deficiency that were previously reported in 8 week old muscles are maintained with age. Compared to WT, young male and female KO mice showed significantly lower absolute force (Fig. 9), however there was no significant difference in absolute force between mature WT and KO muscles in either male or female groups. Interestingly, mature male KO muscles demonstrated significantly greater absolute force than young male KO muscle, while mature female WT and KO muscles both showed a trend for lower absolute force compared to young female muscles. Specific force in mature WT and KO muscles however was significantly lower compared to young muscles in both male and female groups. There was also no significant difference in specific force between WT and KO muscles in either young or mature age groups, suggesting that differences in absolute force in young muscles can be accounted for by differences in muscle cross-sectional area.

Isolated EDL muscles were also subjected to a strenuous fatigue protocol to determine the force recovery ability of mature WT and KO muscles. Young 8 week old EDL muscles from KO mice have previously been shown to demonstrate enhanced force recovery following fatigue (MacArthur et al., 2008); similar results were also achieved in this study in young and mature muscles in both male and

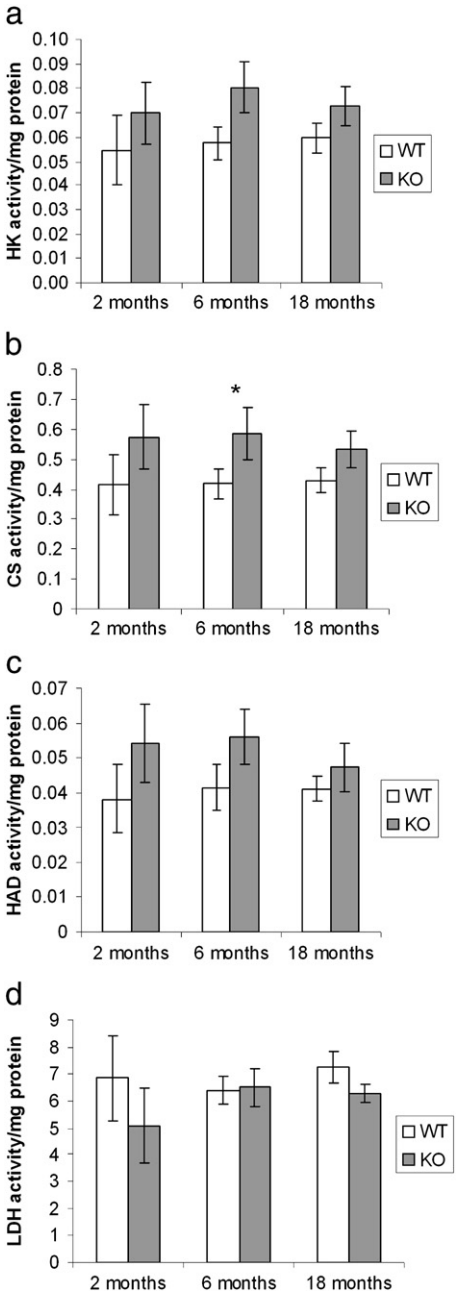

Fig. 8. Metabolic enzyme activity differences between female WT and KO are maintained with age. Activities of (a) hexokinase (HK), (b) lactate dehydrogenase (LDH), (c) 3-hydroxyacyl-CoA-dehydrogenase (HAD) and (d) citrate synthase (CS) were increased with age in both WT and KO muscles. Differences in enzyme activity between WT and KO mice were maintained at all ages tested, but did not always reach significance. Mean ± 95% CI; $n = 6$ for all samples. $^{*}P<0.05$.

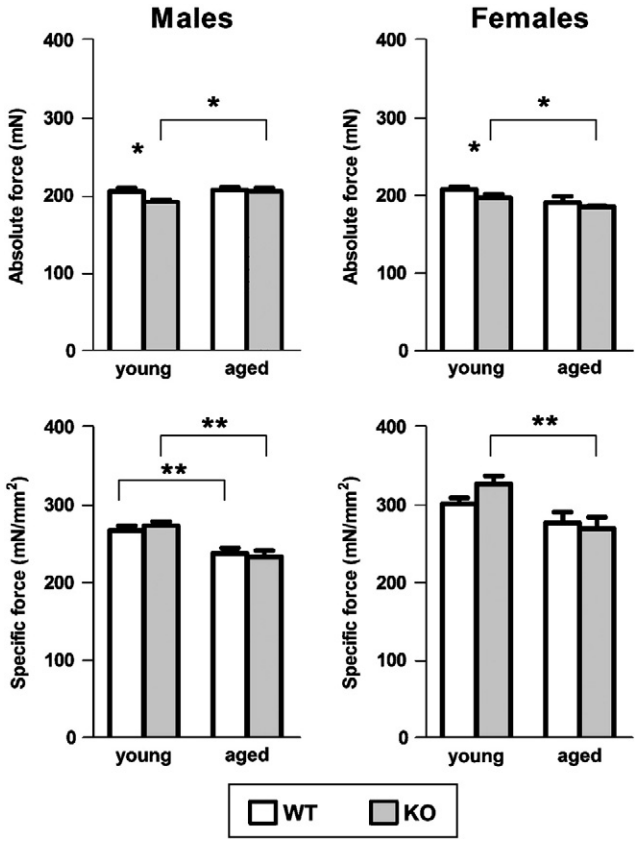

Fig. 9. Genotype difference in maximal EDL force is lost in aged mice. With aging, absolute force generation is increased in male KO muscle and is decreased in female WT and KO muscles, however, both male and female mice demonstrated decreased specific force generation with age, regardless of genotype. Although young male and female KO muscles showed significantly lower maximal force compared to WT, this genotype difference is lost when examined in aged mice. There was no genotype difference in specific force in young or aged groups for both male and female mice. Mean ± SEM; $n = 4–8$ for all samples. $^{*}P<0.05$, $^{**}P<0.01$.

female groups (Fig. 10). Comparisons between WT and KO showed no significant differences in the level and rate of force decline, both having lost about 50% of their original force. However, over the 30 minute recovery period, the force generated by KO muscles was greater than WT in all groups, indicating faster force recovery in both young and mature KO muscles. After 30 min, the force produced by the muscles as a percentage of their pre-fatigue force was greater in KO than in WT, and this difference was statistically significant in all age groups. These results indicate that α-actinin-3 deficiency is associated with improved recovery from muscle fatigue, and that this characteristic persists with aging in both male and female groups.

4. Discussion

In this study, we have examined the effect of α-actinin-3 deficiency in aging skeletal muscle from male and female mice. At age 18 months, both WT and KO mice demonstrated decreased intrinsic exercise capacity, decreased grip strength, reduced relative muscle mass and reduced isolated muscle specific force compared to mice at age 2 months, suggesting that *Actn3* WT and KO mice are demonstrating signs of muscle aging and functional decline at 18 months. In addition, older male mice showed significant fibre type shifts from 2B to 2X, consistent with the reported increase in fast-to-slow muscle fibre type transition with age (Pette and Staron, 2000).

We also found that age affected the influence of *Actn3* genotype on certain muscle traits, but not others, and this may explain some of the variability seen in studies of *ACTN3* genotype effects in older humans. Previously observed genotype differences for some traits were maintained in mature mice, for example, for grip strength, muscle metabolism, fast 2B fibre size and force recovery post-fatigue. Genotype differences were lost for other traits such as running performance, muscle mass (in males) and force measurements in isolated muscle fibres. A lack of genotype difference in muscle function and muscle traits is consistent with the findings from the majority of the published *ACTN3* association studies in elderly humans (Bustamante-Ara et al., 2010; Delmonico et al., 2008; McCauley et al., 2010; San Juan et al., 2006). The loss of genotype difference in some traits and not others indicate firstly, that other aging related factors may outweigh the effects of *Actn3* genotype, and secondly, that the

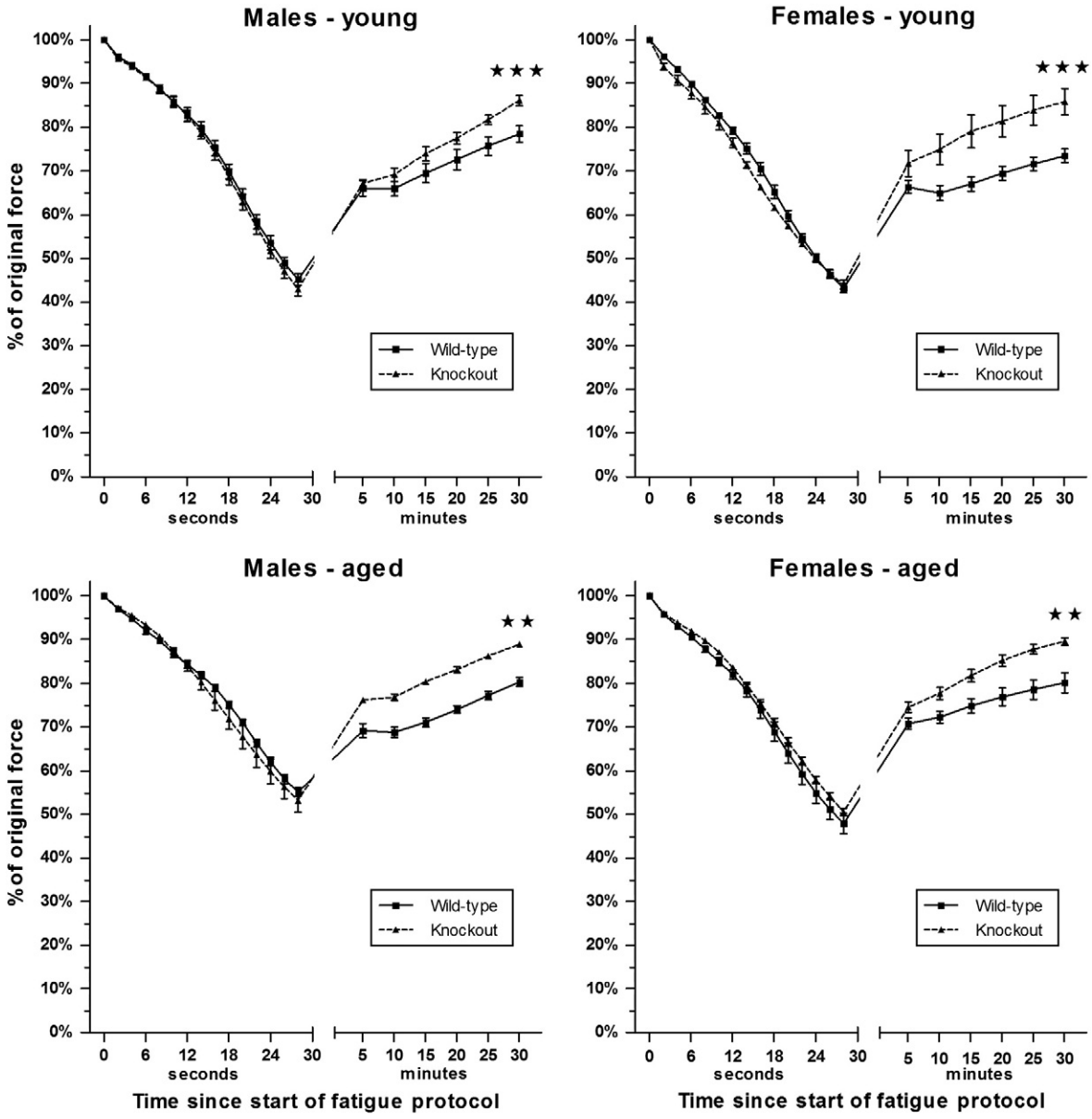

Fig. 10. Aged KO muscles continue to show greater force recovery following fatigue. Muscles were subjected to a strenuous fatigue protocol of repeated 100-Hz tetani over 30 s, resulting in decline in force production. Over the 30 minute recovery period, KO muscles recovered significantly more of their pre-fatigue force than WT in all age groups. Mean ± SEM; $n = 4–8$ for all samples. $^{***}P<0.001$, $^{**}P<0.01$.

effects of *Actn3* genotype with aging could be gender dependent. The ongoing influence of *Actn3* genotype in most female muscle traits is consistent with previous reports that show greater effects on muscle related phenotypes in women (Clarkson et al., 2005; Yang et al., 2003), but could also reflect a lesser effect of sarcopenia in females compared to males at 18 months, as evidenced by the smaller declines in female grip strength and muscle mass.

The presence of a continued trend for increased aerobic enzyme activity and mitochondrial protein expression, as well as significantly higher force recovery post-fatigue in KO muscles and reduced fast 2B fibre size indicates that deficiency of α-actinin-3 continues to result in "slower" fast fibre properties even at age 18 months. However, despite this, genotype association with intrinsic exercise performance is lost after age 2 months, suggesting that changes in other body systems with age, such as diminished cardio-respiratory fitness, likely outweigh the effect of *Actn3* genotype on exercise capacity. The loss of genotype difference in forced treadmill running performance could also be partially attributed to a reduced effect of α-actinin-3 deficiency on other muscle contractile properties (Chan et al., 2011). We have previously demonstrated slower twitch and tetanus relaxation times in KO EDL muscles compared to WT (MacArthur et al., 2008), which is consistent with a slowing of fast fibre properties with α-actinin-3 deficiency, however, we have recently found that these differences in twitch and tetanus relaxation are diminished in aged muscles (Chan et al., 2011). These intrinsic muscle changes could also explain the loss of genotype difference in maximal isolated muscle (EDL) force generation. Interestingly, similar results have also been reported in humans. In a recent study examining associations between *ACTN3* genotype and muscle contractile properties in 100 Caucasian men aged 60–70 years, no association was found for time to peak tension, half relaxation time or peak rate force development (McCauley et al., 2010). Furthermore, we have found that the slower relaxation in young KO muscles following twitch and tetanus may be due to slower calcium re-uptake by the sarcoplasmic reticulum (Chan et al., 2011). The loss of difference in these contractile properties in aged muscles could therefore also be related to differential changes in

calcium dynamics between aged WT and KO, but this has yet to be experimentally tested.

Although our results in the mouse and association studies in humans so far indicate a diminished influence of *ACTN3* genotype on muscle function in the elderly, there is some evidence to suggest that *ACTN3* genotype could influence the process of muscle function deterioration with aging. In the 4-year follow-up within their large multi-center study, Delmonico and colleagues found that elderly XX men and women demonstrated greater declines in muscle performance: XX men showed significant increases in their 400 m walk time compared to RR and RX men, and elderly XX women were found to have 35% greater risk of incident persistent lower extremity limitation compared to RR women (Delmonico et al., 2008). A similar pattern could also be observed in the forced treadmill running performance of KO mice. KO mice demonstrated enhanced intrinsic exercise capacity compared to WT at age 2 months, but this genotype difference was lost with time and both WT and KO mice demonstrate decreases in running capacity with increasing age. In this instance, the deficit in performance is greater in the KO. The same pattern is seen with KO muscle mass, which appears to be more sensitive to the effects of aging than WT, as demonstrated by significant genotype and age interaction by two-way ANOVA (Table 1). Between ages 4 and 6 months, male KO mice also showed a greater increase in quadriceps mass (34.0%, $P=0.0002$) compared to WT (12.7%, $P=0.018$), which accounted for the loss of genotype difference in muscle mass at 6 months and thereafter. At age 18 months, average KO quadriceps mass was 12.2% lower than at 6 months ($P=0.002$), while average WT quadriceps only demonstrated 6.5% decrease ($P=0.179$). Whether these greater changes in KO muscle mass contributed to the greater deficits in KO running performance is unknown. However our data does support the findings from the Delmonico study that although genotype differences in muscle function and muscle traits may be lost in the elderly, XX individuals may experience faster declines in muscle function over time compared to RR or RX individuals.

In contrast to intrinsic exercise performance, α-actinin-3 deficiency consistently influenced the grip strength of KO mice at all ages examined, with male and female KO mice showing reduced forearm grip strength compared to WT mice, regardless of age. Although these results differed from the EDL maximal force results in aged WT and KO mice, the discrepancy between the two results could be explained by the differences in protocol: rather than examining the maximal force of a single muscle, grip strength represents the average force produced by multiple muscles that are synergistically activated during the test, involving forearm muscles, biceps as well as abdominal muscles. The contractile results from the EDL are thus representative of the specific intrinsic changes in a single muscle with age, while grip strength presents a general representation of the changes in upper body strength with age. The persisting influence of *Actn3* genotype on grip strength, but not running performance in mature mice emphasises the importance of α-actinin-3 in optimal muscle force generation and suggests that some phenotypes may be more susceptible to the influences of aging. The maintenance of *Actn3* genotype association with muscle strength in the aged mice may have clinical ramifications: RX and XX elderly women have recently been shown to have 33% increased risk of falls (Judson et al., 2011), and this may be associated with lowered muscle strength with α-actinin-3 deficiency influencing the speed of response in the elderly to prevent falls.

In conclusion, our results in the aging *Actn3* mouse model support the findings of the majority of *ACTN3* human association studies and demonstrate a loss of *Actn3* genotype association in intrinsic exercise performance, isolated muscle (EDL) force generation and male muscle mass. The loss of genotype difference in running performance occurred despite the persistently "slower" α-actinin-3 deficient muscle characteristics, with reduced fast 2B fibre size, increased oxidative metabolism and greater force recovery after fatigue, suggesting that other age-related factors outweigh the influence of *Actn3* genotype on muscle traits. Furthermore, significantly greater decline in male KO muscle mass at 18 months is consistent with the increase in muscle function decline in elderly XX humans. These results provide further support that α-actinin-3 deficient humans may experience faster decline in muscle function with increasing age.

Author contributions

J.T.S. and N.Y. tested the muscle performance and analysed the muscle mass; S.C. and S.I.H. performed the muscle contractile property studies; J.T.S, N.T. and G.J.C. performed the metabolic analyses; J.T.S performed the fibre typing analyses; D.G.M., K.G.Q., J.M.R. and Y.D.B. contributed to the experimental design; J.T.S., N.Y. and K.N.N. designed the study and wrote the paper.

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