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Original Article

ORIGINAL ARTICLE

Analysis of the *ACTN3* heterozygous genotype suggests that α-actinin-3 controls sarcomeric composition and muscle function in a dose-dependent fashion

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Abstract

A common null polymorphism (R577X) in *ACTN3* causes α-actinin-3 deficiency in ~18% of the global population. There is no associated disease phenotype, but α-actinin-3 deficiency is detrimental to sprint and power performance in both elite athletes and the general population. However, despite considerable investigation to date, the functional consequences of heterozygosity for *ACTN3* are unclear. A subset of studies have shown an intermediate phenotype in 577RX individuals, suggesting dose-dependency of α-actinin-3, while others have shown no difference between 577RR and RX genotypes. Here, we investigate the effects of α-actinin-3 expression level by comparing the muscle phenotypes of *Actn3*$^{+/-}$ (HET) mice to *Actn3*$^{+/+}$ [wild-type (WT)] and *Actn3*$^{-/-}$ [knockout (KO)] littermates. We show reduction in α-actinin-3 mRNA and protein in HET muscle compared with WT, which is associated with dose-dependent up-regulation of α-actinin-2, z-band alternatively spliced PDZ-motif and myotilin at the Z-line, and an incremental shift towards oxidative metabolism. While there is no difference in force generation, HET mice have an intermediate endurance capacity compared with WT and KO. The R577X polymorphism is associated with changes in *ACTN3* expression consistent with an additive model in the human genotype-tissue expression cohort, but does not influence any other muscle transcripts, including *ACTN2*. Overall, *ACTN3* influences sarcomeric composition in a dose-dependent fashion in mouse skeletal muscle, which translates directly to function. Variance in fibre type between biopsies likely masks this phenomenon in human skeletal muscle, but we suggest that an additive model is the most appropriate for use in testing *ACTN3* genotype associations.

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Introduction

In skeletal muscle, the sarcomeric α-actinins are a major component of the Z-line where they bind and crosslink the actin thin filaments. α-Actinin-2 is expressed in all muscle fibres, but the highly homologous α-actinin-3 isoform is expressed only in fast, glycolytic fibre types. Importantly, a common null polymorphism (rs1815739, R577X) occurs in the gene coding for α-actinin-3 (*ACTN3*) resulting in its complete absence in homozygous individuals (1). α-Actinin-3 deficiency is detrimental to sprint and power performance in both elite athletes (2,3) and the general population (4–6). Conversely, α-actinin-3 deficiency has been associated with enhanced endurance performance (3,7) and improved response to resistance training (4), but this association is not as strong as with sprint performance and has not consistently been replicated (7).

Over 50% of the global population are heterozygous (HET) for the R577X polymorphism; however, there is no consensus on the functional effects of heterozgosity, nor the appropriate genetic model to test the effect of *ACTN3* on muscle performance in human cohorts. Our studies support strong recent positive selection of the 577X allele in Asian and European populations (3,8). The positive selection also correlates with global latitude gradient, suggesting that environmental variables such as temperature (cold tolerance), and species richness (feast/famine) may have influenced the gene-flow and current R577X allele frequencies worldwide (9,10). It is assumed that the XX genotype confers a phenotypic advantage which drives selection; however, it is possible that an RX phenotype may also contribute to the increase in 577X allele frequency.

Here, we investigate the HET phenotype in our *Actn3* knockout (KO) mouse model, which mimics the phenotype seen in humans; KO mice display reduced muscle mass and strength (11) but display increased endurance capacity, improved recovery from fatigue (8) and enhanced response to training (12). KO muscles do not exhibit a change in fibre type distribution, but fast 2B fibres display a shift towards slow fibre properties including; reduction in 2B fibre size (11), an increase in oxidative metabolism (8,11,13), which corresponds to an alteration in the contractile properties as KO muscles show reduced maximal force (11), slowed relaxation from contraction (14) and an increased recovery from fatigue (15).

α-Actinin-2 is up-regulated in KO muscle (11) and the total amount of sarcomeric α-actinin is not significantly different between wild-type (WT) and KO (12), suggesting that α-actinin-2 directly substitutes for the absent α-actinin-3. Thus, the phenotype in α-actinin-3-deficient muscle is likely a consequence of differences in function and/or protein interactions between α-actinin-2 and α-actinin-3 (16). For example, the Z-line associated proteins, z-band alternatively spliced PDZ-motif (ZASP), titin and vinculin preferentially bind to α-actinin-2 over α-actinin-3, causing the Z-line structure to be altered in KO mice (12). α-Actinin-2 also has increased binding affinity for calsarcin-2, a negative regulator of calcineurin activity, compared with α-actinin-3 (17). In the absence of α-actinin-3, up-regulation of α-actinin-2 results in increased binding of calsarcin-2 and thus reduced inhibition of calcineurin. The increased calcineurin activity is responsible for the observed shift in metabolism towards the slow oxidative phenotype (18). The substitution of α-actinin-2 for α-actinin-3 in KO muscle therefore changes the sarcomeric protein composition, altering the contractile and metabolic properties of the fibre.

In combination, these data suggest that α-actinin-3 may influence muscle function in a dose-dependent fashion; as we would expect the ratio of α-actinin-2 to α-actinin-3 in the sarcomeric pool to determine the muscle's properties. Consistent with this hypothesis, human association studies that involve quantitative analysis of muscle function reveal a dosage-effect pattern in which the RX genotype lies between the RR and XX genotypes. Specifically, in a study examining the muscle performance of healthy Greek adolescents, RX individuals showed an intermediate 40 m sprint time when compared with RR and XX (5). Similar trends were observed when examining the response to strength training in adult women (4) and the elderly (19). In both cases, RX individuals demonstrated intermediate strength gains when compared with RR and XX.

However, other studies have not shown any evidence of a dosage effect associated with *ACTN3* genotype (6,20). The discrepancy in these studies may be attributable to confounding factors such as age, lifestyle and ethnicity, but the influence of α-actinin-3 expression levels remains unclear. In order to remove these confounding factors, we examined HET *Actn3* mice and compared them with their WT and KO littermates to rigorously test the possibility of a dosage effect associated with the α-actinin-3 expression level.

Results

The expression of α-actinin-2 and -3 is inversely regulated at the protein level, but not by mRNA

In order to examine the relationship between *Actn2* and *Actn3* expression, we extracted RNA from the quadriceps of WT, HET and KO mice and compared their transcript levels relative to an experimentally validated housekeeping gene (*Rer*). In HET muscle, we observed a 50% reduction in *Actn3* expression when compared with WT (Fig. 1A). As reported previously, we saw a significant up-regulation of *Actn2* expression in KO muscle (~1.8-fold) when compared with WT (12), but saw comparable *Actn2* expression levels between WT and HET muscle (Fig. 1B).

We then obtained protein lysates from these muscles to investigate the relationship between mRNA transcript levels and α-actinin-2 and -3 protein expression. We quantified protein levels of α-actinin-2 and -3 using a method established previously (12); a standard curve generated with purified recombinant α-actinin-2 and -3 was used to quantify the level of expression in quadriceps samples from WT, HET and KO normalized to the total muscle protein solubilized in the lysate. We saw a slight, but not statistically significant reduction in α-actinin-3 expression in HET muscle (Fig. 1C) compared with WT. We again saw a significant up-regulation in α-actinin-2 expression in KO muscle which we propose is in compensation for the loss of α-actinin-3 (17,21). Interestingly we also see a significant up-regulation of α-actinin-2 in HET muscle when compared with WT (Fig. 1D), such that HET muscles show an intermediate level of α-actinin-2 protein when compared with WT and KO.

Using this technique, we can compare the pool of sarcomeric α-actinin relative to total muscle protein across the genotypes by combining the calculated α-actinin-2 and -3 levels. Overall, we saw no significant differences in the total amount of combined α-actinin-2 and -3 between WT, HET and KO (data not shown), indicating that the overall amount of sarcomeric α-actinin remains constant irrespective of *Actn3* genotype.

α-Actinin-3 influences sarcomeric composition and muscle metabolism in a dose-dependent fashion

We have previously shown that α-actinin-3 deficiency results in altered expression of a number of proteins associated with the Z-line (12). Most prominent is the up-regulation of α-actinin-2

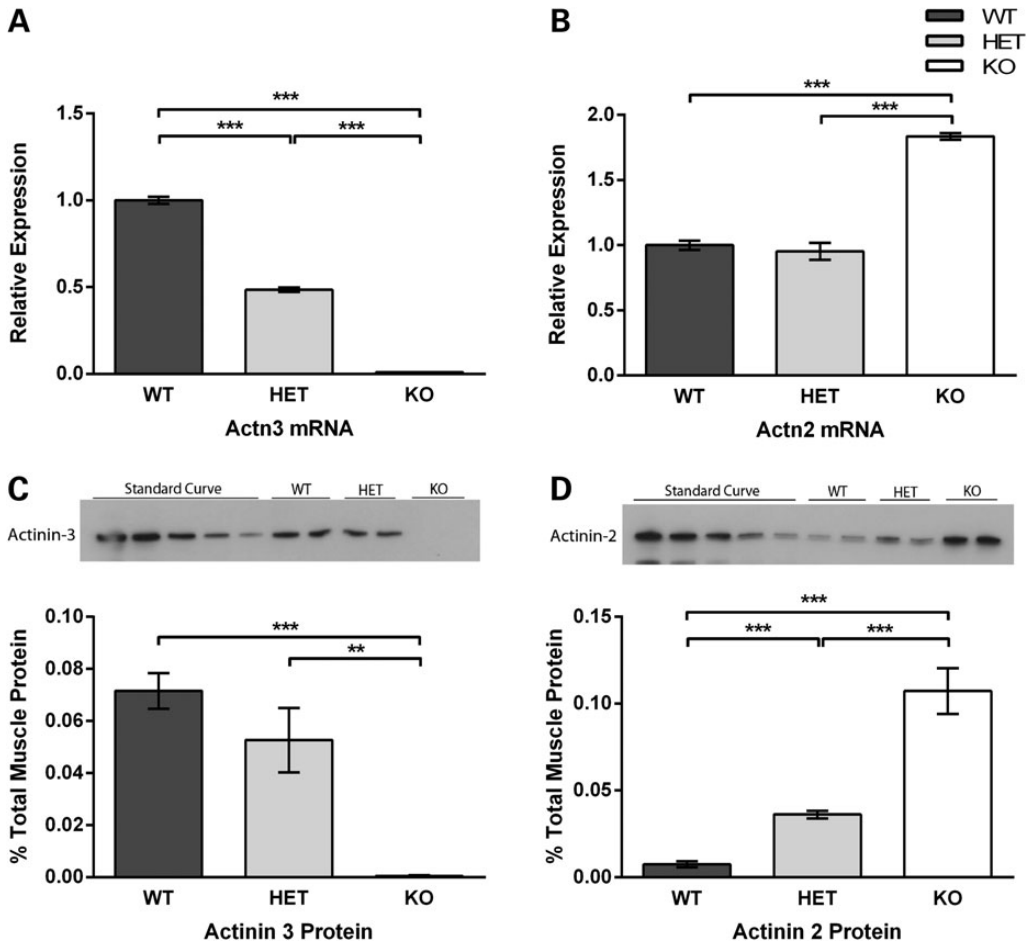

Figure 1. The expression of α-actinin-2 and -3 is tightly regulated in mouse skeletal muscle. (**A**) Quadriceps isolated from HET mice show a 50% reduction in *Actn3* expression compared with WT littermates when normalized to an experimentally validated housekeeping gene (*Rer*). (**B**) The expression of *Actn2* is no different between WT and HET muscles, but is significantly up-regulated in KO (1.8-fold compared with WT). (**C**) Quantification of α-actinin-3 protein expression in mouse quadriceps using a standard curve of purified recombinant α-actinin-3. Muscles from HET mice showed a slight, but not significant reduction in α-actinin-3 as a percentage of total muscle protein in the lysate (HET: 0.053 ± 0.021% versus WT: 0.071 ± 0.014%, $P = 0.13$). (**D**) Quantification of α-actinin-2 using a purified recombinant protein standard curve shows a significant increase in HET muscles (0.036 ± 0.004%) compared with WT (0.007 ± 0.003%). KO muscle lysates had significantly increased α-actinin-2 protein levels compared with both WT and KO (0.107 ± 0.026%). Data presented as mean ± SEM, $n = 4$ for each group, $^{**}P < 0.01$, $^{***}P < 0.001$.

in KO muscle, but this is accompanied by significant increases in the expression of the myofibrillar proteins desmin, myotilin, γ-filamin and ZASP. Given that HET muscle shows an apparent intermediate level of both α-actinin-2 and -3, we investigated the expression levels of these Z-line associated structural proteins by western blot (Fig. 2A). We saw intermediate levels of ZASP and myotilin in HET muscle, but no significant alteration in the expression of desmin (Fig. 2B).

Increased α-actinin-2 expression in KO muscle alters the regulation of calcineurin, increasing its activity and causing a shift towards oxidative metabolism (17). HET muscles show an intermediate level of RCAN1.4 expression, a direct reporter of calcineurin activity (18), and a significant increase in the expression of cytochrome c oxidase (COX IV) when compared with WT (Fig. 2C). The mitochondrial protein porin also showed a trend for intermediate expression in HET muscle.

HET mice show intermediate muscle mass and 2B fibre size

Given the apparent dose-dependent effect of α-actinin-3 on the protein composition of the sarcomere, we examined the relationship between *Actn3* genotype and muscle architecture by isolating individual skeletal muscles of the hindlimb and spine. We did not detect significant differences in the smaller hindlimb muscles; the tibialis anterior (TA) extensor digitorum longus (EDL) or soleus (SOL), although we did observe trends for decreased TA and EDL mass, and increased SOL mass in KO compared with WT (Fig. 3A), which is consistent with what has previously been published in the R129 strain (11).

In the larger hindlimb muscles, KO mice showed a significant reduction in the mass of both the gastrocnemius (GST) and quadriceps compared with WT (Fig. 3B). The spinalis, a postural muscle which runs along the dorsal aspect of the vertebrae, was also significantly smaller in KO mice. Gastrocnemius muscles isolated from HET mice (120 ± 15.1 mg) were significantly larger than KO (104 ± 9.8 mg, $P < 0.05$), although there was not a statistically significant difference when compared WT (127 ± 6.4 mg). The quadriceps from HET mice appeared to have an intermediate mass, significantly increased compared with KO (167 ± 21.5 mg versus 146 ± 11.3 mg, $P < 0.05$), and showed a trend for being reduced compared with WT (182 ± 9.3 mg, $P = 0.08$). In the spinalis, which was the largest muscle examined, HET muscle mass was intermediate between WT and KO; there was a significantly

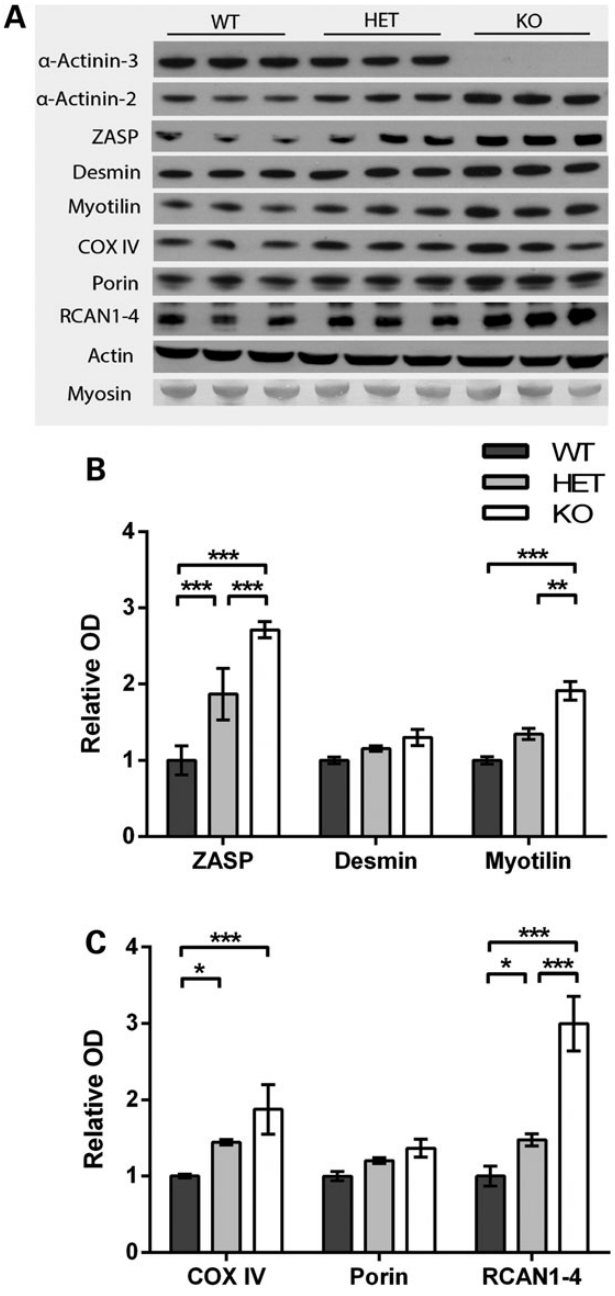

Figure 2. α-Actinin-3 expression influences sarcomeric composition and muscle metabolism. (**A**) Western blot analysis of muscle lysate from the quadriceps of WT HET and KO mice, with α-sarcomeric actin and myosin used as loading controls. (**B**) Dose-dependent up-regulation of the Z-line associated structural proteins ZASP and myotilin according to the loss of α-actinin-3. (**C**) Dose-dependent up-regulation of the metabolic and signalling proteins COX IV and RCAN1.4 according to the loss of α-actinin-3. Data presented as mean ± SEM, $n = 3$ for each group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

decrease in mass compared with WT (196 ± 33.0 mg versus 218 ± 18.7 mg, $P < 0.01$), and a significant increase compared with KO (152 ± 11.5 mg, $P < 0.001$).

The results from the larger muscles suggest that α-actinin-3 may influence muscle mass in a dose-dependent fashion; hence, we also investigated the individual fibre type and size profiles of the muscle. Using antibodies against myosin heavy chains (MyHCs) 2B, 2A, 2X and type 1, we labelled whole cross-sections of the quadriceps from WT, HET and KO mice to analyse the fibre size and number (Fig. 3C). We saw no shift in fibre type associated with *Actn3* genotype, as WT, HET and KO muscles show no change in fibre type proportion (Fig. 3D). As previously reported, there was no difference in size of fibres which do not typically express α-actinin-3 (type 1, 2A or 2X fibres); however, type 2B fibres were altered with *Actn3* genotype (Fig. 3E). HET muscles showed an intermediate 2B fibre size (1729 ± 446 μm^2) with ~19% smaller 2B fibre size compared with WT (2132 ± 255 μm^2, $P < 0.01$) and ~45% larger 2B fibre size compared with KO (1180 ± 297 μm^2, $P < 0.001$). These results suggest that α-actinin-3 directly influences the size of 2B fibres in a dose-dependent fashion without an associated shift in fibre type.

HET mice show intermediate endurance capacity, but display comparable strength to WT

The most prominent functional consequences of α-actinin-3 deficiency reported to date in *Actn3* KO mice are a reduction in forelimb grip strength and enhanced endurance capacity (11). Here, we analysed these phenotypes in HET mice to test for a possible dosage effect of α-actinin-3. As in our previous studies, we examined the endurance capacity of the mice with a run to exhaustion on a motorized treadmill. KO mice run significantly further (945 ± 219 m) than their WT littermates (729 ± 218 m, $P < 0.01$) before reaching exhaustion (17). Interestingly, HET mice show an intermediate phenotype (835 ± 159 m), but his was not significantly different to either WT ($P = 0.1$) or KO ($P = 0.08$) (Fig. 4A). *Actn3* genotype also significantly altered grip strength (Fig. 4B), with reduced grip strength in KO mice (1.01 ± 0.14 mN) compared with WT (1.14 ± 0.15 mN, $P > 0.01$) or HET (1.134 ± 0.11 mN, $P > 0.01$). There was no difference between WT and HET mice, which suggests that grip strength may be a multifactorial phenotype and that is not influenced by α-actinin-2/3 expression level alone.

Isolated EDL muscles from HET mice show comparable physiology to WT

In order to investigate these phenotypes in greater detail, we isolated EDLs to test muscle force and fatigue independent of behaviour and neural input. We stimulated EDLs from WT, HET and KO mice at increasing force frequencies to determine absolute force output (Fig. 5A). Consistent with previous findings (11), KO muscles produced significantly less (~25%) force than WT. HET muscle produced greater force than the KO (~34%, 214 ± 12 mN versus 159 ± 23 mN, $P < 0.001$), but not to the extent of the WT (201 ± 8 mN). However, when we applied correction for cross-sectional area to calculate specific force, there were no significant differences in maximum specific force across the three genotypes (Fig. 5B). Analysis of the force-frequency curves showed no difference in the EC 50, Hill coefficient and time to peak across WT, HET and KO muscles (Table 1), which is a powerful indicator that there is no change in myosin ATPase activity or the calcium sensitivity of the troponin complex associated with *Actn3* genotype; this was not surprising as no change in fibre type was observed in these muscles. Consistent with previous findings (22), KO muscles showed longer half-twitch relaxation times than WT, but HET muscles were not significantly different to either.

Following the force measurements, the EDL was fatigued via 30 s of repeated stimulation, and then allowed to recover with five stimulations intermittently over 10 min to record recovery of muscle force (Fig. 5C). KO muscles showed some resistance to fatigue during the 30 s protocol; WT muscles declined to 33% of their original 100 Hz force on average, whereas KO declined to 42% ($P = 0.07$) (Fig 5D). The force produced by HET muscles

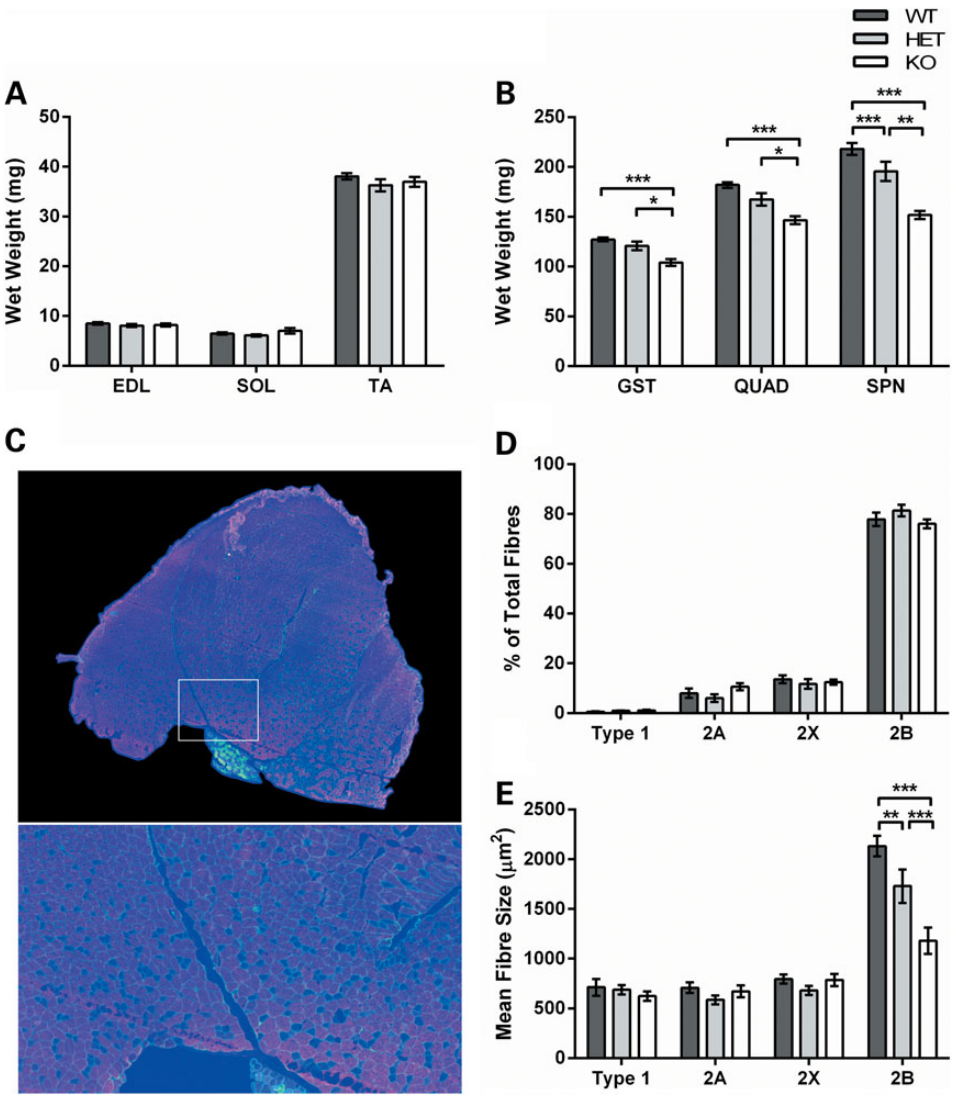

Figure 3. α-Actinin-3 influences muscle mass and 2B fibre size in a dose-dependent fashion. (**A**) No significant differences were observed in the isolated masses of the EDL, SOL or TA. (**B**) The HET muscle mass of the GST, quadriceps (QUAD) and spinalis (SPN) was significantly greater than KO mice, both the quadriceps and spinalis were also significantly reduced in HET compared with WT. (**C**) Cross sections of the whole mouse quadriceps were labeled with antibodies against myosin heavy chain type 1 (green), type 2A (blue) and type 2B (red) while type 2X fibres were left unstained. (**D**) No change was observed in the fibre type composition of the quadriceps with *Actn3* genotype. (**E**) Dose-dependent reduction in 2B fibre size according to the loss of α-actinin-3, but no change in the size of type 1, type 2A or type 2X fibres with *Actn3* genotype. Data presented as mean ± SEM, $n = 10$ for each group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

declined to 41% and was no different from WT or KO muscle immediately post fatigue. KO muscles showed a trend for greater recovery from fatigue producing 83% of their original 100 Hz force after resting for 10 min, compared with WT which recovered to 74% ($P = 0.053$), replicating previously published data (11,15). HET muscles were directly comparable to WT as they recovered 75% of their original 100 Hz force, although they seemed to follow a different recovery profile than WT (Fig. 5C).

*ACTN3*genotype does not result in observable changes in the level of α-actinin-2 expression in human skeletal muscle

α-Actinin-2 and -3 appear to be co-regulated in mouse muscle and this relationship directly influences both muscle architecture and function. Hence, we investigated the expression patterns in human skeletal muscle to test for a similar relationship. Utilizing human skeletal muscle expression and genome data from the genotype-tissue expression (GTEx) consortium (23,24), we performed an unbiased genome-wide scan in human skeletal muscle to examine which transcripts were altered with *ACTN3* R577X (rs1815739) (trans-eQTL scan). To characterize the extent to which the observed distribution followed the expected (null) distribution, we assumed an additive model and plotted a quantile–quantile (Q–Q) plot (Fig. 6A). Observed transcripts followed an expected (null) linear association; however, *ACTN3* significantly deviated from this assumption and was the only transcript that survived a Bonferroni correction (Supplementary Material, Table S1).

We quantitated the *ACTN3* transcript level with R577X genotype (Fig. 6B), and saw an expression pattern consistent with an additive gene model ($P = 2.19 \times 10^{-14}$). It should be noted however, that applying a recessive model also returned a significant association ($P = 5.29 \times 10^{-16}$ Supplementary Material, Data).

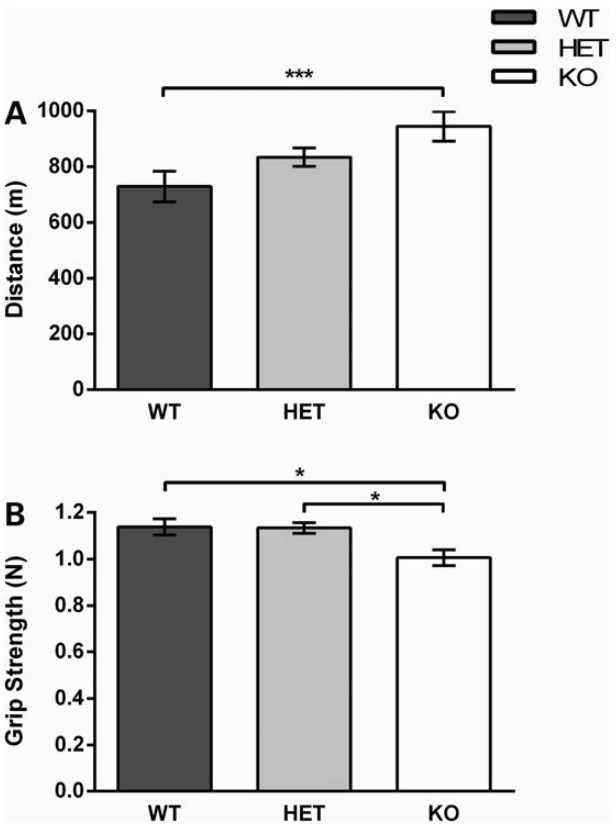

Figure 4. HET mice show an intermediate endurance phenotype, but comparable grip strength to WT. (**A**) *In vivo* endurance capacity measured as the distance run before exhaustion on a motorized treadmill. HET mice show an intermediate endurance capacity (835 ± 159 m), but are not significantly different to either WT (729 ± 218, $P = 0.1$) or KO (945 ± 219 m, $P = 0.08$). (**B**) Forelimb grip strength *in vivo*.. *Actn3* genotype significantly alters grip strength, with reduced grip strength in KO mice (1.01 ± 0.14 mN) compared with WT (1.14 ± 0.15 mN) or HET (1.134 ± 0.11 mN). Data presented as mean ± SEM, $n \geq 18$ for each group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Performing a similar analysis for *ACTN2* revealed no alteration in transcript across RR, RX or XX (Fig. 6C).

In cohort of 21 human biopsies, we examined protein levels of α-actinin-3 in RR ($n = 6$), RX ($n = 8$) and XX ($n = 7$) skeletal muscle. While RX muscle showed a reduction in α-actinin-3 expression compared with RR, the difference was not significant [0.74 ± 0.22 versus 1.0 ± 0.34 95% confidence interval (CI), $P = 0.28$]. Both RR and RX showed significantly greater α-actinin-3 levels than XX homozygotes, which (as expected) had no detectable α-actinin-3 ($P = 0.0003$) (Fig. 6D). We saw no effect of *ACTN3* genotype on the expression of either α-actinin-2 or ZASP in the biopsies tested. We did see a significant up-regulation in RCAN1.4 in XX muscle [previously reported by Seto *et al.* (17)], but no difference between RR and RX.

Discussion

α-Actinin-3 deficiency occurs at high frequency in the general population (1), and has a significant effect on muscle function. The HET genotype accounts for >50% of the general population; however, little is currently known about the phenotypic response in *ACTN3* 577RX individuals. Here, we present evidence that the expression of α-actinin-2 and -3 is tightly linked and that the relationship between the two isoforms is directly related to both muscle structure and function.

Sarcomeric α-actinins are post-transcriptionally regulated

In muscle from HET mice, we observed a 50% reduction in *Actn3* mRNA, but no change in the level of *Actn2* mRNA expression when compared with WT. However, at the protein level, while we saw a reduction in α-actinin-3 in line with the reduced mRNA, we also saw a significant up-regulation of α-actinin-2 despite *Actn2* transcript levels being unchanged. This suggests that the observed up-regulation of α-actinin-2 occurs post-transcription. It is difficult to determine the mechanism which underpins this phenomenon; however, α-actinin-2 is expressed during embryonic development, while α-actinin-3 expression is not evident in mouse skeletal muscle until 1–2 weeks of age (13,25). This coincides directly with the period of postnatal fibre type diversification according to MyHC isoform expression (26). It is, therefore, possible that during the process of fast fibre specification, α-actinin-3 is expressed and substitutes for the α-actinin-2 present during development. In this scenario, HET muscle would have a proportion of α-actinin-2 which is not substituted for and hence persists as a result of the reduced α-actinin-3. In mature HET muscle, this persistent α-actinin-2 would be seen as an apparent 'up-regulation' of the protein independent of a change in *Actn2* expression.

Z-Line composition and fibre metabolism are altered in a dose-dependent fashion

Differences in functional protein interactions between α-actinin-2 and -3 are thought to underlie the phenotype associated with α-actinin-3 deficiency (27). In this study, we show that the reduction in α-actinin-3 levels in HET muscle are associated with a dose-dependent alteration of fast fibre Z-line protein composition, consistent with this hypothesis. HET muscle shows an intermediate level of α-actinin-2, ZASP and myotilin when compared with WT and KO, suggesting that sarcomeric remodelling occurs in a dose-dependent fashion according to the level of α-actinin-3 expression. Interestingly, desmin did not show a significant difference across the genotypes; however, we did see a trend for an up-regulation in KO muscle ($P = 0.08$) which is consistent with what we have reported previously in other genetic backgrounds (12). There is a suggestion for an intermediate desmin expression level in HET muscle, but the small difference between WT and KO makes this difficult to detect relative to myotilin and ZASP.

We have previously demonstrated that an alteration in the regulation of calcineurin underpins the shift towards a slow fibre metabolic phenotype in α-actinin-3-deficient muscle (17). HET muscles showed an intermediate level of RCAN1.4, a reporter of calcineurin activity (18), which is highly suggestive of an inverse relationship between α-actinin-3 expression and calcineurin activity. Consistent with this hypothesis, the intermediate level of COX IV in HET muscle is indicative of a partial shift towards the oxidative metabolic profile classically associated with slow fibres. Overall, these findings suggest that the ratio of α-actinin-2 to α-actinin-3 in the sarcomeric pool (which is linked to *Actn3* genotype) directly influences both the structural and metabolic properties of fast fibres.

α-Actinin-3-dependent protein interactions influence muscle architecture and function

The dose-dependent alteration of sarcomeric composition associated with *Actn3* genotype translates directly to muscle fibre

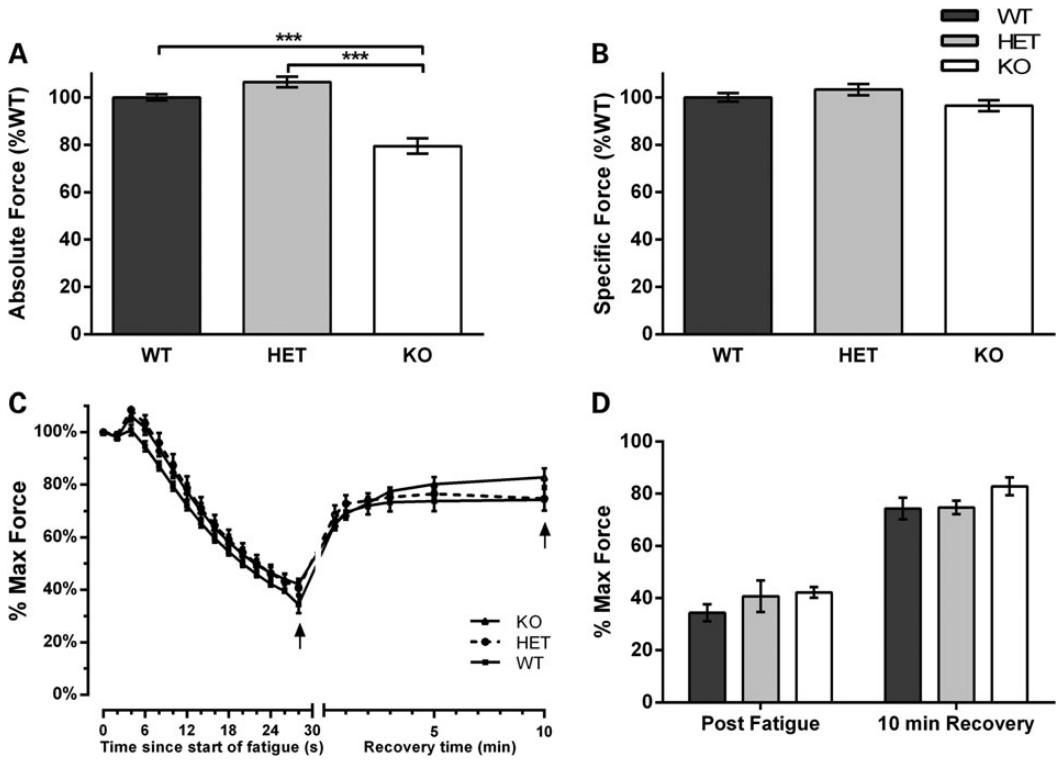

Figure 5. EDL muscles isolated from HET mice show comparable electrophysiology to WT. (**A**) Both WT (201 ± 8 mN) and HET (214 ± 12 mN) muscles show significantly greater maximal tetanic force compared with KO (159 ± 23). (**B**) When average cross sectional area is taken into account, there is no significant difference in maximal specific force associated with *Actn3* genotype. (**C**) Muscles were subjected to a fatigue protocol consisting of a 1 s, 100 Hz tetanus every 2 s for 30 s. The descending part of the curve shows the decline in 100 Hz force over the duration of the fatigue protocol. Muscles were then allowed to recover for 10 min, shown by the ascending part of the curve. (**D**) No significant differences were observed in either post-fatigue force or recovery at 10 min [timepoints indicated by arrows in (**C**)] according to *Actn3* genotype, although KO muscles did show a trend for increased recovery at 10 min (83 ± 11% recovery) compared with both HET (75 ± 3%) and WT (74 ± 14). Data presented as mean ± SEM, $n \geq 6$ for each group, $^{*}P < 0.05$, $^{***}P < 0.001$.

Table 1. Physiological properties of isolated EDLs from WT, HET and KO mice

Parameter	WT	HET	KO
Maximum force (mN)	201.0 ± 8.2	214.0 ± 12.8	159.7 ± 22.7**
Maximum specific force (mN/mm^2)	283.5 ± 18.1	292.6 ± 20.0	273.5 ± 27.4
EC50 (Hz)	59.4 ± 0.5	54.9 ± 1.1	55.5 ± 0.8
Hill co-efficient	4.1 ± 0.1	4.0 ± 0.3	3.9 ± 0.2
Twitch time to peak (ms)	21.3 ± 2.4	21.1 ± 1.8	19.3 ± 2.2
Twitch half-relax time (ms)	16.2 ± 3.2	16.0 ± 1.8	18.6 ± 3.7*

*P > 0.01 versus WT; **P > 0.01 versus HET.

architecture as HET muscles show intermediate 2B fibre size. As we have reported previously, α-actinin-3 has no influence on fibre type determination; i.e. there is no shift in MyHC profile associated with *Actn3* genotype at baseline. Given the predominance of 2B fibres in most mouse skeletal muscles, it is reasonable to assume that this dose-dependent effect of α-actinin-3 on 2B fibre size directly underlies the relationship between *Actn3* genotype and muscle mass which follows an additive model in the larger muscles analysed.

The influence of the α-actinin-3 level on muscle function is less clear. The *in vivo* treadmill experiments suggest that HET mice may have intermediate endurance capacity; however, their acute recovery from fatigue was not altered in the *ex vivo* muscle studies. Similarly, no differences in strength were observed between WT and HET, both *in vivo* (grip strength) and *ex vivo* when corrected for muscle size (EDL maximal specific force). This suggests that the metabolic properties which underlie the endurance phenotype may be more sensitive to a reduction in α-actinin-3, whereas the complete absence of α-actinin-3 is required before any difference in muscle force output is evident. However, it is clear that any changes in function associated with the HET phenotype are likely to be subtle and difficult to detect.

α-Actinin3 expression is strongly associated with *ACTN3* genotype

Interestingly, the R577X single nucleotide polymorphism (SNP) (rs1815739) showed strong associations with the ACTN3 expression level using both additive and recessive gene models in the GTEx consortium dataset. The recessive model returned a slightly lower *P*-value, but this is likely a product of the improved statistical power obtained by combining RR and RX. However, unlike the mice, there was no association with the R577X SNP (rs1815739) and the expression of any other transcripts, including *ACTN2*. This finding correlated with analysis of human muscle protein expression, where we saw no change in the levels of α-actinin-2 or ZASP with *ACTN3* genotype. Interestingly, a recent study showed altered mRNA levels coding for a number of sarcomeric proteins (including *ACTA1*, *MYOT* and *TTN*), but not *ACTN2*, according to *ACTN3* genotype in a cohort of healthy individuals (28). The reason for the apparent discrepancy between this study and the data from the GTEx consortium is unclear; when performing a genome-wide

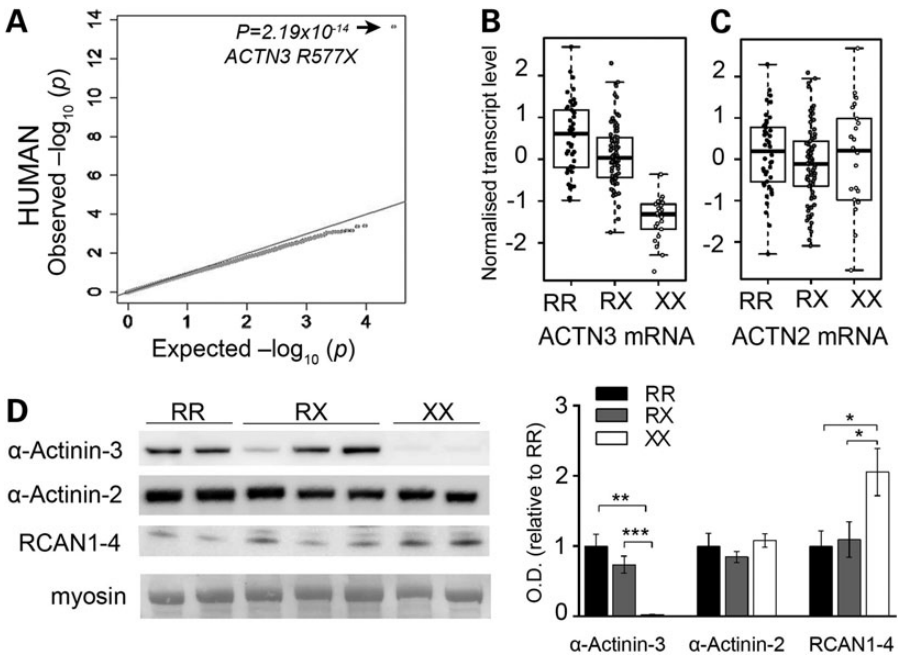

Figure 6. Relationship between *ACTN2* and *ACTN3* expression in human skeletal muscle. **(A)** In human skeletal muscle expression data from the GTEx consortium, the R577X variant (rs1815739) is significantly associated with *ACTN3* transcript expression ($P = 2.19 \times 10–14$), but all other skeletal muscle genes tested showed no deviation from expected/observed value of 1. **(B)** The *ACTN3* transcript level is significantly different in RR ($n = 40$), RX ($n = 70$) and XX ($n = 22$) genotypes, which can be explained by both an additive and recessive model. **(C)** The expression of ACTN2 is not significantly altered by R577X. **(D)** Western blot analysis of muscle lysates from human control muscle biopsies, using myosin as a loading control. A slight, but not statistically significant reduction in α-actinin-3 expression was observed in RX individuals compared with RR (~26%, $P = 0.28$), but no difference was observed in the α-actinin-2 protein level with *ACTN3* genotype. RCAN1.4 protein levels were ~2-fold higher in XX muscle compared with both RR and XX. Data presented as mean ± SEM, $n \geq 5$ for each group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

trans-eQTL scan in the GTEx dataset, a Bonferroni correction for multiple comparisons reduces the *P*-value for significance to 1.6×10^{-6}, which may obscure any small changes in expression as a result of the *ACTN3* R577X polymorphism. However, individually testing each of the genes reported by Riedl *et al.* revealed no significant associations between expression level and rs1815739 in the GTEx dataset, suggesting that further investigation is needed to understand the putative effect that *ACTN3* genotype has on the expression of other muscle genes.

That we see a disparity between the phenotype and protein response in HET mice and humans is possibly not surprising. α-Actinin-3 is highly expressed in mice based on the predominance of 2B fibres in most skeletal muscles (including the quadriceps used in this study), and thus presumably requires significant up-regulation of α-actinin-2 to compensate for its absence. The proportion of fast fibres in human skeletal muscle is much lower than mice; therefore, the relative expression level of *ACTN3* is lower and hence a compensatory signal from *ACTN2* is likely to be difficult to detect. In addition to this, we can sample the entire cross-sectional area of a mouse muscle, but in humans we are limited to a small biopsy. This becomes a problem because fibre types are not homogenously distributed within the muscle, and hence the fibre type proportion in the biopsy sample used for expression profiling may obscure the detection of any change in *ACTN2*. Norman *et al.* (20) showed a significant increase in *ACTN2* mRNA in *ACTN3* 577XX biopsies when the percentage of 2B fibres was used as a co-variate in the analysis, which is strong evidence for a similar relationship between *ACTN2* and *ACTN3* expression in human skeletal muscle as what we have shown. Unfortunately, this study did not include *ACTN3* 577RX individuals and we are unable to perform a similar analysis with the current GTEx dataset.

Significance of the *ACTN3*HET genotype

Altered protein levels are the basis of various diseases (29), which highlights the biological importance of the expression level of a gene product rather than simply its presence or absence. Here, we show that a single functional *Actn3* allele is not sufficient to drive WT levels of α-actinin-3 expression in mouse skeletal muscle. Although organisms HET for a loss-of-function allele typically show no discernable phenotype due to the redundancy of cellular physiology (30) haploinsufficiency is observed in genes that are required at high levels and are, therefore, more sensitive to a reduction in dose (31). The sarcomeric α-actinins fit this profile as they represent a critical component of the Z-line assembly in skeletal muscle.

While α-actinin-3 deficiency does not result in disease (due to compensation by α-actinin-2), we believe that differences in protein/protein interactions between α-actinin-2 and -3 cause dose-dependent remodelling of the Z-line according to the α-actinin-3 expression level, which has a direct effect on muscle architecture and function. Interestingly, studies in zebrafish suggest that the reverse is not true; α-actinin-3 does not compensate for the loss of α-actinin-2, even when over-expressed (32), which highlights the functional diversity between these two isoforms and may account for the apparently grave consequences of the α-actinin-2 deletion as no mutation resulting in either complete absence or significant reduction in α-actinin-2 has been identified. The effect of *ACTN3* dosage in human muscle is less clear; we show that *ACTN3* expression levels fit an additive gene model according to *ACTN3* R577X genotype in human skeletal muscle, but see no evidence of compensation by α-actinin-2 for the loss of α-actinin-3. As discussed earlier, this is likely a product of the slower fibre profile in human muscle compared with mice.

Conclusions

Positive selection for loss-of-function variants is rare in evolution (33), but recently it was suggested that alterations in the calcium handling properties of α-actinin-3-deficient muscle may confer a thermogenic benefit to *ACTN3* 577XX individuals and drive expansion of X allele frequency as ancient humans migrated out of Africa to colder climates (22). Here, we show evidence for dose-dependency of α-actinin-3, which suggests that heterozygosity could possibly impart a partial phenotypic advantage and the RX genotype may also contribute to positive selection of the X allele.

Understanding the molecular impacts of a loss-of-function allele also has significant implications in the analysis of human association data. Investigators often choose the appropriate genetic model for analysis retrospectively according to the 'best-fit' model, but this exposes data to multiple testing and increases the chance of type I error. Although an HET phenotype is not always evident in published studies on muscle function in humans, we suggest that an additive model is most appropriate when investigating the effect of *ACTN3* genotype in human association studies, consistent with previous evidence suggesting most genetic contributions to heritable traits are primarily additive.

The work we present here is important in the context of muscle biology, as the current paradigm suggests that MyHC isoform is the major determinant of muscle fibre properties and is used to classify fibre types (34). While this is generally true, we show that α-actinin-3 also influences the properties of fast fibres in a dose-dependent fashion without signalling for a change in fibre type, indicating that the properties of each fibre type may be on a spectrum and not as clearly defined as previously thought. Understanding the molecular effects of *ACTN3* genotype therefore offers considerable insight into the biological basis of natural variation in skeletal muscle function, and may have implications for therapeutic development as we enter into the age of personalized medicine given that fast fibres appear more susceptible to atrophy both with age (35) and in muscular dystrophy (36).

Materials and Methods

Study approval

This study was performed in strict accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes published by the National Health and Medical Research Council. All animal experiments were approved by the Animal Care and Ethics Committee of the Children's Medical Research Institute and The Children's Hospital at Westmead (application number K199/11). Ethical approval for the use of human samples was obtained from the Human Research Ethics Committees of the Children's Hospital at Westmead (10/CHW/45).

Animals

C57Bl/10ScSnJ *Actn3*$^{-\backslash -}$ mice were generated by crossing the R129 *Actn3* KO mouse model developed in this laboratory with the C57Bl/10 strain (Jackson Laboratories, Bar Harbor, MI, USA) and backcrossing for 10 generations. HET breeding pairs were employed to obtain male 8 ± 1-week old littermate *Actn3*$^{+/+}$ (WT), *Actn3*$^{+/-}$ (HET) and *Actn3*$^{-/-}$ (KO) mice for this study. All mice had ad libitum access to food and water, and were maintained in a 12:12 h cycle of light and dark.

Mouse functional tests

Mouse forelimb grip strength was tested using a grip strength meter (Columbus Instruments) according to a previously described method (11). Mice were lifted by their tail and allowed to grasp the bar with their forelimbs, then pulled in a linear fashion away from the force meter. This process was repeated 15 times for each mouse, with both the highest two readings and lowest two readings omitted to account for operator effects.

The exercise capacity of the mice was tested using a modified version of a previously described protocol (37), using an AccuPacer mouse treadmill (AccuScan Instruments). Mice were placed on the treadmill at a speed of 10 m/min and an incline of 15°. A negative stimulus (electrical shock of 0.5 mA) was placed at the rear of the treadmill, and the front of the treadmill housed a darkened box as positive reinforcement to encourage running. The speed of the treadmill was increased by 1 m/min every 2 min until the mice were exhausted (defined as the point where mice are unable to extricate themselves from the negative stimulus without assistance inside 3s). The speed and time to exhaustion were recorded, and the total distance run calculated.

Tissue collection

Mice were euthanized by cervical dislocation immediately prior to tissue collection. Muscles were removed, their wet weight was recorded, and then were immediately snap frozen in partially thawed isopentane for protein and muscle fibre analyses. Tissues were stored at −80°C until use.

MyHC isoforms staining and measurements

Fibre measurements and fibre typing were performed on immunostained muscle sections using the MetaMorph software (Molecular Devices), as previously described (38). A transverse 8 μm section was cut from the mid-section of the frozen mouse quadriceps muscle and blocked for 1 h at room temperature to prevent cross-reaction with endogenous mouse antibodies using AffiniPure Fab fragment goat antimouse IgG (1:25 dilution; Jackson ImmunoResearch). Sections were then incubated for 1 h at room temperature with primary antibodies against myosin heavy chain 2B (used neat, BF-F3; developmental studies hybridoma bank (DSHB), University of Iowa, Iowa City, IA, USA), 2A (used neat, SC71; (DSHB), University of Iowa), and type 1 (1:300; MAB1628; Chemicon), which were prelabelled using the Zenon labelling kit (Molecular Probes) according to the manufacturer's instructions. A final 15 min incubation step with wheat germ agglutinin 488 (WGA) was used to mark fibre borders before washing and fixing the sections as previously described. Images were captured using an Olympus BX50 microscope and ProgRes software (SciTech). Individual fibre analysis was performed via METAMORPH software using the sarcolemmal WGA stain to delineate fibres (Molecular Devices). The whole quadriceps was analysed from each mouse to assess fibre type area and proportions while being blinded to genotype.

Total RNA isolation

Total RNA was extracted from ∼100 mg of Actn3 WT, HET and KO mouse quadriceps, lysed with 2 ml of Tri-Reagent (Molecular Research Center) as per manufacturer's conditions. Briefly, the lysates were transferred to a 2 ml Eppendorf tube and 0.2 ml of chloroform was added. The homogenate was vortexed for 1 min and then centrifuged at 12 000 g for 15 min at 2–8°C. The upper aqueous solution was transferred to a fresh tube, and total RNA was precipitated with 0.5 ml of isopropyl alcohol per 1 ml of Tri-Reagent used in the initial lysate. The RNA was pelleted by centrifugation at 12 000 g for 10 min and washed with 1 ml of 70% ethanol, the RNA pellet was dissolved in 0.35 ml of RLT buffer from RNeasy Mini Kit (Qiagen). Further purification of total RNA was achieved using the RNeasy Mini Kit (Qiagen) following the

manufacturer's instruction. The total RNA quality was analysed using the Bioanalyser 6000 RNA kit (Agilent Technologies) according to the manufacturer's protocol.

Quantitative real-time PCR

For quantitative real-time polymerase chain reaction (qPCR), cDNA was synthesized from 2 μg of total RNA using SuperScriptTM III reverse transcriptase (Invitrogen) and random oligos (dN6, Roche), as per manufacturer's instructions. cDNA were diluted 1/10 in DECP treated water (Bioline) prior to qPCR. Taqman probes (Applied Biosystems) for *Actn2* (assay ID Mm00473657_m1), *Actn3* (Mm00496495_m1) and the reference gene, *Rer* (Mm00471267_m1) were used to quantitate expression levels in Actn3 WT ($n = 4$), HET ($n = 4$) and KO ($n = 2$) muscle. Standard curves were performed in each analysis and consisted of a serial (1/10) dilution of total cDNA from a WT sample. All samples were analysed in triplicate using the TaqMan assay master mix (1×) and selected probes (1×) with 2 μl of cDNA per reaction in a final volume of 10 μl; under the following conditions; 50°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. A melt curve analysis was performed following amplification which consisted of a 1°C/s increase in temperature (68°–90°C). The amplification and melt analyses were performed using a Rotor-Gene 6000 (Qiagen) machine. *Rer* was used as a reference gene as it was identified through microarray to be stably expressed between WT and KO at a level similar to the studied genes, and subsequently confirmed by qPCR in three different mouse strains (39).

Human expression analysis

Utilizing the GTEx database, we examined the effect of *ACTN3* R577X genotype on the expression level of all skeletal muscle transcripts. We also tested for associations with specific candidate genes including: *ACTN3*, *ACTN2*, Desmin (*DES*), Myotilin (*MYOT*), Porin (*VDAC1*), COX IV (*COX4*), ZASP (*LDB3*), SERCA1 (*ATP2A1*), Sarcalumenin (*SRL*) and RCAN1.4 (*RCAN1*). Dataset was GTEx pilot phase data with RNA-seq from 138 skeletal muscle samples from autopsy donors. Briefly, these tissues were extracted from deceased human donors aged between 21 and 70 years. Consent was obtained at site of collection from next of kin. Exclusion criteria were human immunodeficiency virus infection or high-risk behaviours, viral hepatitis, metastatic cancer, chemotherapy or radiation therapy for any condition within the past 2 years, whole-blood transfusion in the past 48 h or body mass index of >35 or <18.5.

For the genome wide scans, the *P*-value for a significant association was considered to be $P < 1.6 \times 10^{-6}$ using a Bonferroni correction (0.05/30106). Co-variates including sex, ethnicity, genotype principal components and probabilistic estimation of expression residuals factors were used in the analysis. For significant associations, both additive (RR > RX > XX) and recessive (RR + RX > XX) models were tested.

To further investigate findings from the GTEx dataset, we analysed protein expression by western blot in a separate cohort of human muscle biopsies obtained from healthy, consenting adults. Samples from 21 adult females were used in this analysis; 6 RR (mean age: 43 ± 18 years), 8 RX (43 ± 15 years) and 7 XX (45 ± 20 years). Sections were cut from frozen biopsies, homogenized in 4% sodium dodecyl sulphate (SDS) buffer and western blots were performed according to the protocol described below.

Immunoblotting

Sample preparation for western blots were performed as described in (17). Western blots were performed on mouse quadriceps samples and human vastus medius or lateralis samples. Forty (mouse) and 80–90 (human) × 8 μm cryosections were homogenized and sonicated in Laemmli buffer containing 4% SDS in 62.5 mm Tris (pH 6.8) with protease inhibitor (Sigma-Aldrich). Sample protein was quantified using a bicinchoninic acid (BCA) assay kit (BCA assay kit) and prepared at concentrations of 1 or 1.5 μg/μl with the addition of 10% glycerol, 50 mm dithiothreitol and bromophenol blue. Twenty micrograms of the protein lysate were loaded onto 4–12% Bis–Tris pre-cast mini-gels (Life Technologies), separated by SDS–polyacrylamide gel electrophoresis, then transferred to polyvinylidene fluoride membranes (Millipore), probed with antibodies and developed with enhanced chemiluminescence (ECL) or ECL Prime chemiluminescent reagents (Amersham Biosciences). Antibodies used included; α-actinin-3 (1:20000 5B3 kindly provided by A. Beggs), α-actinin-2 (1:800000 4B3 kindly provided by A. Beggs), porin (20B12; 1:5000; Molecular Probes), COX IV (20E8; 1:2000; Molecular Probes), desmin (1:800, NCL-DER11; Leica), myotilin (1:800 NCL-MYOTILIN; Leica), ZASP (1:200000 LDB3 Proteintech), αβ-crystallin (1:2000, NCL-ABCrys-512; Leica) and RCAN1 (D6694; Sigma-Aldrich). α-Sarcomeric actin (5C5 1:2000 Sigma-Aldrich) and total myosin were used as loading controls and densitometry was performed using ImageJ.

EDL physiology

Muscle preparation, force, fatigue and twitch characteristics were carried as previously described (8,15). Briefly, the EDL was dissected from the lower hindlimb, then attached at the proximal tendon to a force transducer (Fort 10, World Precision Instruments) and a linear tissue puller (University of New South Wales) at the distal tendon using silk suture (Deknatel 6.0). The isolated muscle was placed in a bath continuously perfused with Krebs solution and was continuously bubbled with carbogen to maintain pH at 7.4. The muscle was stimulated by delivering a supramaximal current between two parallel electrodes in the bath using an electrical pulse generator (A-M Systems). The optimal length (L_O) was determined as the muscle length at which maximum twitch force was generated prior to experimentation and set as the baseline. At the start of the experiment, the muscle was set to the L_O that produced maximum twitch force. All experiments were conducted at room temperature (~22–24°C). After testing, the EDL was removed from the bath, the tendons were trimmed and the wet weight of the muscle recorded. An estimate of the cross-sectional area was obtained by dividing the muscle mass by the product of its optimum length and the density of mammalian muscle (1.06 mg/mm^3). This was divided by force to give specific force calculation.

Statistical analysis

In each of the mouse experiments, we tested for a genotype effect by one-way analysis of variance. Where a *P*-value of <0.05 was returned we performed comparisons between the genotype groups using Fisher's least significant difference test. Histogram figures show mean values with standard error of the mean (SEM) unless otherwise stated.

Supplementary Material

Supplementary Material is available at *HMG* online.

Conflict of Interest statement. None declared.

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