The Effect of *ACTN3* Gene Doping on Skeletal Muscle Performance

Fleur C. Garton,[1,2,3,13] Peter J. Houweling,[1,2] Damjan Vukcevic,[1,4,5,6] Lyra R. Meehan,[1] Fiona X.Z. Lee,[7,8] Monkol Lek,[7,9] Kelly N. Roeszler,[1,2] Marshall W. Hogarth,[7] Chrystal F. Tiong,[1] Diana Zannino,[1,2] Nan Yang,[7] Stephen Leslie,[1,4,5,6] Paul Gregorevic,[10] Stewart I. Head,[11,12,14] Jane T. Seto,[1,2] and Kathryn N. North[1,2,*]

Loss of expression of *ACTN3*, due to homozygosity of the common null polymorphism (p.Arg577X), is underrepresented in elite sprint/power athletes and has been associated with reduced muscle mass and strength in humans and mice. To investigate *ACTN3* gene dosage in performance and whether expression could enhance muscle force, we performed meta-analysis and expression studies. Our general meta-analysis using a Bayesian random effects model in elite sprint/power athlete cohorts demonstrated a consistent homozygous-group effect across studies (per allele OR = 1.4, 95% CI 1.3–1.6) but substantial heterogeneity in heterozygotes. In mouse muscle, rAAV-mediated gene transfer overexpressed and rescued α-actinin-3 expression. Contrary to expectation, *in vivo* "doping" of *ACTN3* at low to moderate doses demonstrated an absence of any change in function. At high doses, *ACTN3* is toxic and detrimental to force generation, to demonstrate gene doping with supposedly performance-enhancing isoforms of sarcomeric proteins can be detrimental for muscle function. Restoration of α-actinin-3 did not enhance muscle mass but highlighted the primary role of α-actinin-3 in modulating muscle metabolism with altered fatiguability. This is the first study to express a Z-disk protein in healthy skeletal muscle and measure the *in vivo* effect. The sensitive balance of the sarcomeric proteins and muscle function has relevant implications in areas of gene doping in performance and therapy for neuromuscular disease.

Introduction

The *ACTN3* gene encodes for α-actinin-3, an actin-binding protein that is specifically expressed in fast skeletal muscle fibers (all fast glycolytic type 2X fibers and 50% of fast oxidative type 2A fibers).[1] The common *ACTN3* polymorphism (Arg577X) (rs1815739) is one of the most highly replicated genetic associations in human muscle performance. Homozygosity of the 577X allele (577XX) occurs in 20% of the global population[2] and results in complete loss of function (absence) of α-actinin-3. Association studies in sprint/power performance have consistently demonstrated reduced 577X allele frequency in elite athletes, suggesting that the expression of α-actinin-3 (or, presence of 577R allele) plays an integral role for optimal generation of explosive muscle power. No other common variant or study has been reproducibly associated with elite sprint/power performance.[3]

The effect of *ACTN3* Arg577X on function has also been examined in various non-athlete populations. Absence of α-actinin-3 has been associated with loss of muscle power[4] and strength[5] reduction of bone and muscle mass[6,7] as well as susceptibility to injury.[8] Other studies have also

shown that the absence of α-actinin-3 is linked to increased risk of falls in the elderly,[9] earlier onset and poorer prognosis among individuals with Pompe disease (MIM: 232300),[10] and reduced muscle strength in boys with Duchenne muscular dystrophy (MIM: 310200).[11] Mechanistic studies on healthy human muscle reported a slower, more oxidative muscle profile in people carrying two copies of the null 577X allele, with some evidence for shifts in fiber-type proportions,[12,13] increased muscle glycogen,[14,15] calcineurin signaling,[16] and expression of structural and oxidative signaling proteins.[17] Collectively, these studies identify *ACTN3* as an important influence upon muscle attributes in the context of elite performance, healthy individuals, and among those contending with heritable and acquired conditions of compromised muscle function.

Phenotypic analyses of the *Actn3* knockout (KO) mouse model recapitulated the human 577XX phenotypes, with *Actn3* KO mice showing reductions in muscle strength, increased endurance capacity,[18] reduced fast fiber size, shifts in fast fiber metabolism toward oxidative metabolism,[14,18] enhanced calcineurin signaling, and increased response to endurance exercise training compared to wild-type (WT) mice.[16] Similar to 577XX human findings,

impacts on muscle function are life-long, with aged *Actn3* KO mice showed reduced muscle strength, muscle mass, and fast fiber size compared to WT mice.[19] The expression pattern of *Actn3* in the mouse muscle remains similar to the human (*Actn3* is expressed in fast 2B and 2X fibers but not type 2A or I), but the impact of *Actn3* deficiency is likely accentuated due to the higher proportion of fast fibers in a mouse compared to a human muscle.[1,20] For this reason, human association studies remain critical to ascertain the effect of *ACTN3* deficiency.

One of the most studied and replicated human association study results is the detrimental effect of α-actinin-3 deficiency (577XX) on elite sprint/power muscle performance. However, the nature of the genetic model that best explains the association—for example, an α-actinin-3 gene dosage effect (RR > RX > XX), a recessive effect (XX versus RR/RX), or a dominant effect (RR versus RX/XX)— has not been properly explored.[4] Physiological investigations (phenotypes and expression data) in the *Actn3*$^{+/-}$ heterozygous (HET) and homozygous null (KO) mice supported the use of an additive model when testing *ACTN3* genotype associations.[21] The additive model (KO > HET > WT) best explained relative muscle force (percentage of maximum) in response to fatigue, the reduction in 2B fiber size, and increases in calcineurin signaling and oxidative metabolism.[21] Expression of *Actn3* in HET mouse muscles was lower relative to WT and were compensated by upregulation of α-actinin-2 in a dose-dependent, genotype manner. Similarly, Z-disk proteins such as ZASP, mitochondrial proteins (COX IV, porin), and calcineurin signaling (as shown by downstream RCAN1-4 expression) were at intermediate levels in HET mice relative to WT and KO.[21]

Collectively, the results reported to date suggest that restoring α-actinin-3 expression may increase muscle mass and force-producing capacity in α-actinin-3-deficient skeletal muscle. We and others have previously shown that recombinant adeno-associated viral vectors (rAAV) can efficiently transfer genes to mature mouse muscles, resulting in long-term gene expression.[16,22] Given the *ACTN3* Arg577X association with human muscle performance and muscle traits throughout life, augmenting expression of α-actinin-3 in skeletal muscle could further enhance muscle power and sprint performance in individuals already expressing α-actinin-3 (RR, RX). Additionally, replacement of α-actinin-3 in muscles of *ACTN3* 577XX individuals could improve muscle power performance with beneficial consequences for prognosis in regards to muscle-wasting disorders and mitigating the risks of falling in the elderly.[23]

In this study, we examined the potential of *ACTN3* overexpression in mature skeletal muscle to enhance muscle strength, mass, and the susceptibility/resistance to fatigue in the course of repeated stimulation. A general meta-analysis was first performed using data from published, independent studies of *ACTN3* Arg577X to determine what type of genetic model best explains the association with elite sprint/power performance. Using the *Actn3* WT and KO mouse models, we further investigated the functional and morphological effects of post-natal manipulation of α-actinin-3 dosage by overexpressing sarcomeric α-actinin-3 in mature skeletal muscle via rAAV-mediated gene transfer. We hypothesized that altering skeletal muscle α-actinin-3 expression would modify muscle function in a dose-dependent fashion.

Material and Methods

Study Design
Regarding systematic meta-analysis, refer to Supplemental Material and Methods.

For mouse studies, sample sizes (n > 5) were selected based on previous *Actn3* KO and WT studies.[11,21,24] All experiments were replicated in independent groups of mice or using different muscles (TA and EDL). Functional testing was performed by investigators that were blinded to genotype and treatment. All data were collected from male mice and age-matched littermates.

Mice Housing Conditions
Mice were from C57BL/6 (N12-17) genetic backgrounds aged between 6 and 24 weeks. One group of WT mice were from a house colony of C57BL/6 (N70). Mice were housed under the same conditions and were fed meat-free mouse pellets and water *ad libitum* and were maintained in a 12:12 hr cycle of light and dark at ambient room temperature (~22°C). All experiments were approved by the Animal Care and Ethics Committee of the Children's Medical Research Institute (CMRI approval ID: K190) and the Murdoch Children's Research Institute (MCRI approval ID: A761).

rAAV Vector and Delivery
The generation of recombinant adeno-associated viral (rAAV6) vectors was previously described.[16] For intramuscular injections, mice were anesthetized (3.5% isoflurane in oxygen from a precision vaporizer) and given temgesic (0.05 mg/kg) for pain management. Hamilton syringes (32 gauge) (Hamilton) were used to deliver 30 μL of rAAV-*ACTN3* or rAAV-MCS (control empty vector) diluted with of HBSS to required dose (5E8-1E11 vector genomes). Vectors were injected into the anterior compartment of the lower limb, targeting the TA and EDL.

Muscle Physiology
Ex vivo EDL: Mice were sacrificed by cervical dislocation and the EDL was isolated to determine muscle force and recovery from fatigue. All experiments were carried out at room temperature (~22°C–24°C) using techniques previously described.[18,24] *In situ* TA: Force and fatigue was performed using the Aurora Scientific Dual Mode Lever System with supplied software (DMC5 4.5, DMA) and carried out at 37°C as previously described.[11] Notes on both protocols are supplied in the Supplemental Material and Methods.

Immunoblotting
Immunoblotting was performed as previously described.[11] Briefly, 10-μm frozen muscle sections were homogenized in 4% SDS lysis buffer and assessed for total protein concentration using the Pierce BCA protein assay kit (ThermoFisher Scientific). Proteins were separated by SDS-PAGE using precast minigels (Invitrogen) or

criterion gels (Biorad), then transferred to polyvinylidene fluoride membranes (PVDF, Millipore). These were blocked with 5% skim milk in 1× PBST, probed with probed antibodies (Supplemental Material and Methods) overnight and secondary antibodies at room temperature for 2 hr, then developed with ECL reagents (Amersham Biosciences). Membranes were washed, developed, and imaged by Image Quant (GE Healthcare). Densitometry was performed using ImageJ image processing software (NIH) and quantified using the area under the curve. This was normalized to myosin and actin expression, obtained from PVDF membranes stained with Coomassie brilliant blue (1610436, Sigma-Aldrich).

Histology and Immunofluorescence

Haematoxylin and eosin and SDH histological stains were performed on 8-μm muscle sections as previously described.[18] For immunofluorescence, 8-μm sections were blocked with 2% BSA or affinipure goat α-mouse Fab fragment (Jackson ImmunoResearch) before overnight primary incubation (antibodies are in Supplemental Material and Methods) at 4°C. Sections were washed with 1× PBS. Secondary incubation was performed in combination with Hoechst staining at room temperature for 1 hr. Fiber-typing was performed as previously described.[25] Sections were imaged on the Mirax slide scanner (ZEISS), V-Slide Scanner (MetaSystems), or the confocal microscope (Zeiss LSM 780).

Statistics for Experimental Studies

Data results (sample size) are presented as individual points, with the calculated group mean (line) and standard error of the mean (SEM) or 95% confidence interval error bars for each group. As group sizes consisted of <12, two-sided, unpaired t tests using nonparametric statistics, Mann-Whitney U-tests were applied using an alpha of 0.05 for all analyses. Analyses were performed in GraphPad Prism (V7.0c, GraphPad Software).

Results

Meta-analysis of *ACTN3* Arg577X Association Studies in Elite Sprint/Power Athletes Demonstrates a Consistent Homozygote Effect but a Heterogeneous *577RX* Effect

Previous meta-analyses have assumed the association between human Arg577X genotype and elite international athlete status fits a dominant model[26] and/or a recessive model[27] but did not test whether data may better fit alternative models, such as an additive model. We therefore systematically searched relevant articles (Figure S1) and performed a meta-analysis using raw data from 12 independent studies that met a strict inclusion criterion (Table S1). This criterion aimed to minimize heterogeneity of the genetic effect by restricting analyses to similar-caliber athletes across studies who competed in similar events; in particular, by including study genotyping results comprised of >50%–100% international level (elite) athletes who had competed in an individual sprint/power event (Table S2). Elite endurance performance, such as the marathon, was not considered for meta-analysis purposes due to the absence of any consistent effect of the *ACTN3* Arg577X variant.[28]

To analyze the effect of the *ACTN3* genotype on sprint performance, we used a general genetic model for analyses of these data. This allowed us to assess the extent of evidence across the full range of simpler models (which includes dominant, additive, and recessive as special cases). This approach can be described as a Bayesian random effects meta-analysis and decomposes the association into two parts: (1) a "homozygote" component, which models the per-allele effect when comparing the two homozygotes (analogous to the "additive" component in a pure additive model); and (2) a "dominance" component, which models the extent to which the effect of the heterozygotes deviates from a purely additive (or dosage) effect. In particular, this second component can take values consistent with the overall model being dominant, recessive, or additive (and all models in between, see Figure S2 for visualization).

We found there was evidence for a consistent homozygote effect (overall per-allele odds ratio [OR] = 1.4, 95% CI 1.3–1.6; between-study SD τ = 0.12, 95% CI 0.01–0.31) (Figure 1). However, there was substantial heterogeneity in the dominance effect (overall OR = 0.98, 95% CI 0.73–1.3; between-study SD τ = 0.40, 95% CI 0.18–0.72). The variability was large, to the extent that some of the individual studies were consistent with a dominant model[29] and some a recessive model[30] (Figure S3), and overall, they were centered on an additive model (i.e., dominance OR of ≈ 1). Comparisons of both the homozygote and dominance effects against potential explanatory variables (ancestry, sex) did not show any clear trends (Figure S4) that were worth pursuing with formal heterogeneity measures (Supplemental Material and Methods). Overall, the meta-analysis showed that the difference between the homozygotes was consistent across these studies but no single genetic model can consistently explain the association between *ACTN3* Arg577X and elite sprint/power performance due to the effect of heterozygotes being highly variable.

Overexpression of α-Actinin-3 Results in Reciprocal Downregulation of α-actinin-2 and Is Detrimental to WT Muscle Function

Given the *ACTN3* 577RR genotype-positive association with elite sprint/power performance, irrespective of the genotype model, we hypothesized that overexpression of *ACTN3* in WT mice ("doping") may still enhance muscle force generation and modify fatigue resistance. We have previously used rAAV-CMV-*ACTN3* to induce α-actinin-3 overexpression in mouse muscles by intramuscular injections[16] (Figure 2A); here we assess its effect on force and fatigue response at 5–6 weeks after injection. We examined the rAAV-CMV-*ACTN3* vector expression at various doses (5E8, 1E9, 1E10, 1E11 vg) in WT muscles (WT-*ACTN3*) compared to muscles injected with empty vector (WT-MCS) (Figure 2B). Flag expression was highest at 1E11 vg, but there was no appreciable increases in α-actinin-3 expression at any dose examined.

We further assessed the effect of α-actinin-3 overexpression in WT TA and EDL muscles at 1E11 vg. At this dose, ubiquitous rAAV-*ACTN3* expression (as shown by Flag staining) caused wide-spread muscle damage, with
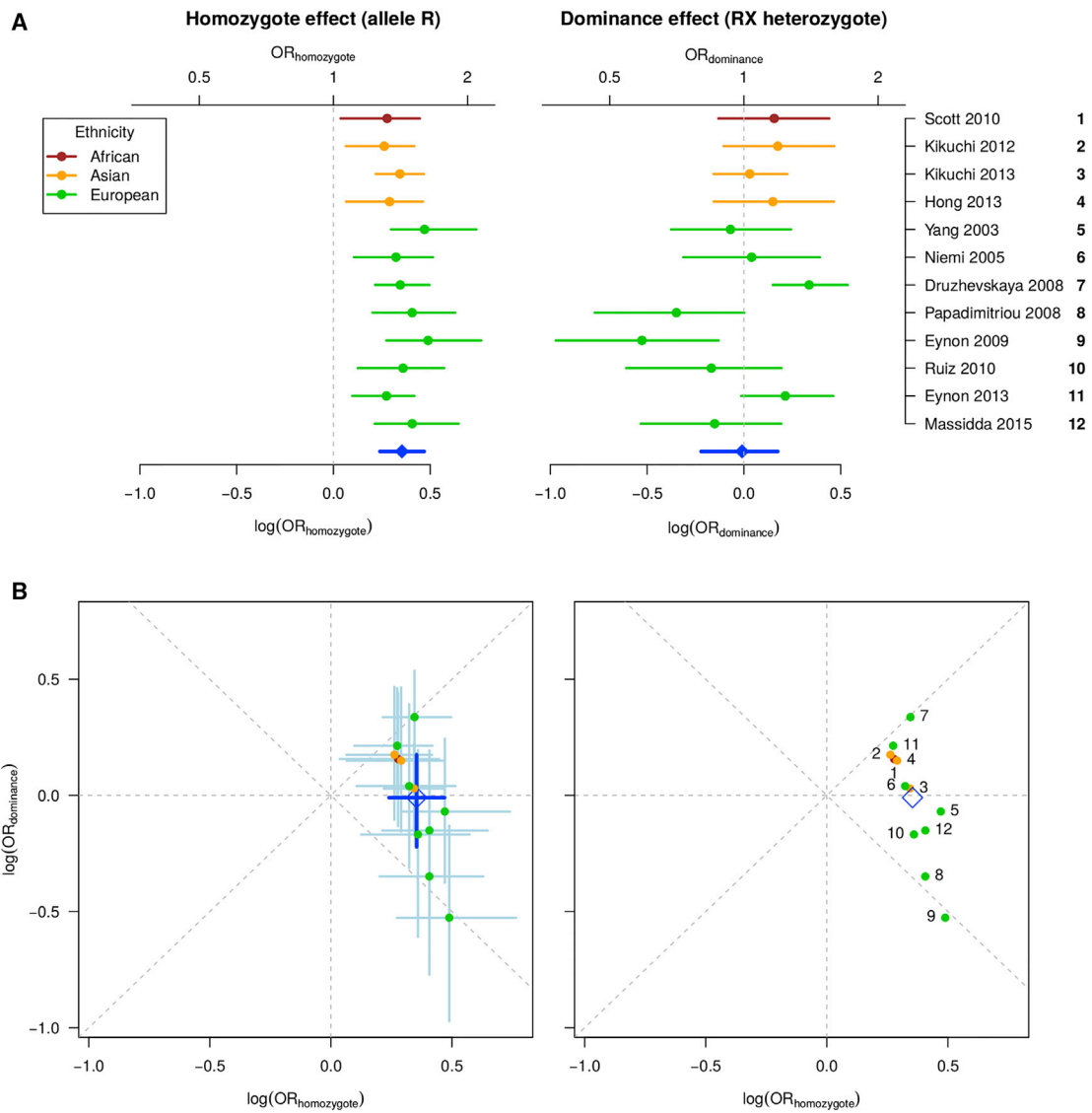
Figure 1. Meta-analysis of the *ACTN3* Arg577X Genotype Effect on Elite Power Performance
(A) Forrest plots with estimates of the posterior median and 90% credible interval of the odds ratios (OR) for each study are shown to demonstrate both the homozygote and dominance effects from the general meta-analysis model. Studies are grouped based on the ancestry of their participants, according to the key provided, and overall estimates are shown in blue.
(B) The same OR estimates are plotted jointly, with homozygote and dominance effects on the x and y axes, respectively. Diagonal gray lines correspond to dominant and recessive models. The point and interval estimates are identical to those in (A) and are numbered by study according to the key provided.

increased centralized nuclei and inflammatory infiltrates relative to WT-MCS (Figure 2C). Staining with wheat germ agglutinin[31] demonstrated increased fibrosis in WT-*ACTN3* TA muscles. Desmin, an intermediate filament protein associated with muscle remodeling, was also upregulated in WT-*ACTN3* TA muscles (Figure S5C). There was no overall increase in α-actinin-3 expression (Figure 3A), and no difference between WT-MCS and WT-*ACTN3* TA muscles in maximal force generation and response to fatigue (Figures 3B and 3C).

In contrast to TA muscles, the level of α-actinin-3 was ~50% greater in WT-*ACTN3* EDL muscles relative to WT-MCS EDL controls (p = 0.002). The increase in α-actinin-3 was accompanied by ~30% downregulation of

endogenous α-actinin-2, suggesting that expression of sarcomeric α-actinins (encoded by *ACTN2* and *ACTN3*) is reciprocally regulated (p = 0.003) (Figure 3D). Despite increased α-actinin-3 expression, the maximal force in the WT-*ACTN3* EDL was ~15% lower than WT-MCS muscle (p < 0.001) (Figure 3E). In response to fatigue stimulation, WT-*ACTN3* EDL muscles showed improved recovery from fatigue compared to the WT-MCS (p < 0.01) (Figure 3F). Overall, these results indicate that adult mammalian skeletal muscle fibers are sensitive to the levels of Actn3 expression and that supraphysiological overexpression of α-actinin-3 in mature skeletal muscle damages subcellular structure and is detrimental to muscle's force-generating capacity.
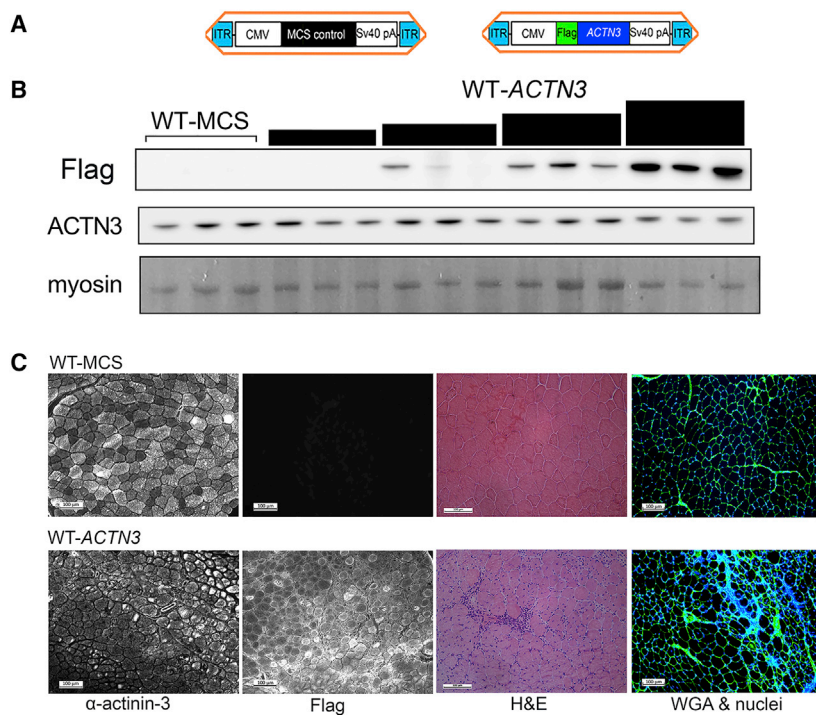
A

B

WT-MCS

WT-*ACTN3*

Flag

ACTN3

myosin

C

WT-MCS

WT-*ACTN3*

α-actinin-3 | Flag | H&E | WGA & nuclei

Figure 2. High Doses of rAAV-*ACTN3* Cause Muscle Damage

(A) Schematics of the rAAV6 vector constructs. Expression of FLAG-tagged human *ACTN3*, or empty vector (MCS control), is driven by a CMV promoter, flanked by a SV-40 poly(A) tail, and capped by short inverted terminal repeats (ITRs).

(B) rAAV-*ACTN3* vector expression, as shown by FLAG expression, increase with dose (5E8, 1E9, 1E10, and 1E11 vg) in WT (n = 3) muscles, indicative of efficient product expression. However, total ACTN3 protein did not increase with dose.

(C) Six weeks after high-dose injection (1E11 vg rAAV-*ACTN3*) into the WT TA muscles, cross-section shows positive flag staining accompanied by substantial centralized nuclei and cellular infiltration (H&E) and fibrosis (wheat germ agglutinin [WGA] staining).

Postnatal Replacement of α-Actinin-3 in *Actn3* KO Muscles Does Not Rescue Force or Enhance Muscle Mass or Fast Fiber Size

Since supraphysiological overexpression of α-actinin-3 in WT muscles is damaging to skeletal muscle and detrimental to muscle force generation, we explored whether post-natal replacement of α-actinin-3 expression in *Actn3* KO mice to WT levels could instead "rescue" the muscle force deficit in *Actn3* KO muscles and increase susceptibility to fatigue to levels similar to that of WT muscles. We again performed a dose escalation assay with rAAV-CMV-*ACTN3* (Figure S6) and determined that a dose of 1E9 vg was sufficient to replace α-actinin-3 expression in KO muscles to WT levels 6 weeks after injection.

Despite ubiquitous expression of α-actinin-3 in all muscle fiber types, localization to the Z-disk (Figure 4A), and reciprocal downregulation of α-actinin-2 similar to WT-MCS muscles (Figure 4B), KO-*ACTN3* TA (Figures 4C and 4D) and EDL (Figures 4E and 4F) muscles failed to reach the maximal forces elicited from WT-MCS muscles (Figures 4C and 4E). There was a small increase (5%) in absolute force in KO-*ACTN3* EDL muscles relative to KO-MCS, but WT-MCS EDL absolute force was still 9% greater (p < 0.001) (Figure 4C). Similarly, KO-*ACTN3* TA muscles demonstrated no improvement in muscle strength relative to KO-MCS, maintaining 18% lower absolute force compared to WT-MCS TA muscles (p < 0.05) (Figure 4E). There was also no effect on muscle mass in KO-*ACTN3* EDL (Figure 4D) and TA (Figure 4F) muscles relative to KO-MCS. Mean fast 2B fiber size of KO-*ACTN3* TA muscles was similar to KO-MCS and 20% smaller relative to WT-MCS (p < 0.0001) (Figures 4G and 4H). How-

ever, KO-*ACTN3* 2X fibers demonstrated a small reduction in size relative to KO-MCS (KO-*ACTN3* 1,000 ± 78 μm², KO-MCS 1,037 ± 62 μm² versus WT-MCS 951 ± 97 μm²) (p = 0.02) (Figure 4G), likely caused by increased proportions of smaller 2X fibers (Figure 4I). There was no change in total fiber count between groups (Figure 4J), but KO-*ACTN3* muscles showed a small (6%) increase in 2B fiber proportions (p = 0.03) and a corresponding decrease in 2X fiber proportions relative to KO-MCS (p = 0.03) (Figure 4K).

Postnatal α-Actinin-3 Replacement in *Actn3* KO Muscles Does Not Alter Calcineurin Activity or Expression of Other Z-disk Proteins

We further assessed RCAN1-4 expression as a marker of calcineurin activity to determine whether this could explain the shift in fiber type toward a fast-glycolytic phenotype. Calcineurin activity is calcium/calmodulin dependent and is inhibited by binding with calsarcin-2.[32] However, in the absence of α-actinin-3, α-actinin-2 is upregulated and competes with calcineurin for binding with calsarcin-2, resulting in release of calcineurin from the inhibitory effect of calsarcin-2 and elevating calcineurin activity.[16] We assessed RCAN1-4 expression by western blot, which is a downstream marker of calcineurin activity. Interestingly, despite a 28% downregulation of α-actinin-2 in KO-*ACTN3* muscles relative to KO-MCS, RCAN1-4 expression was not significantly altered in TA muscles (Figure 5A). Calsarcin-2 (Figure 5B) and calcium signaling proteins SERCA1 and sarcalumenin (Figure 5A) were also not different between KO-*ACTN3* and KO-MCS muscles.

Other Z-disk proteins, ZASP and myotilin and the intermediate filament protein desmin, upregulated in *Actn3* KO muscles compared to WT,[33] also remained unchanged in KO-*ACTN3* muscles relative to KO-MCS (Figure 5B).
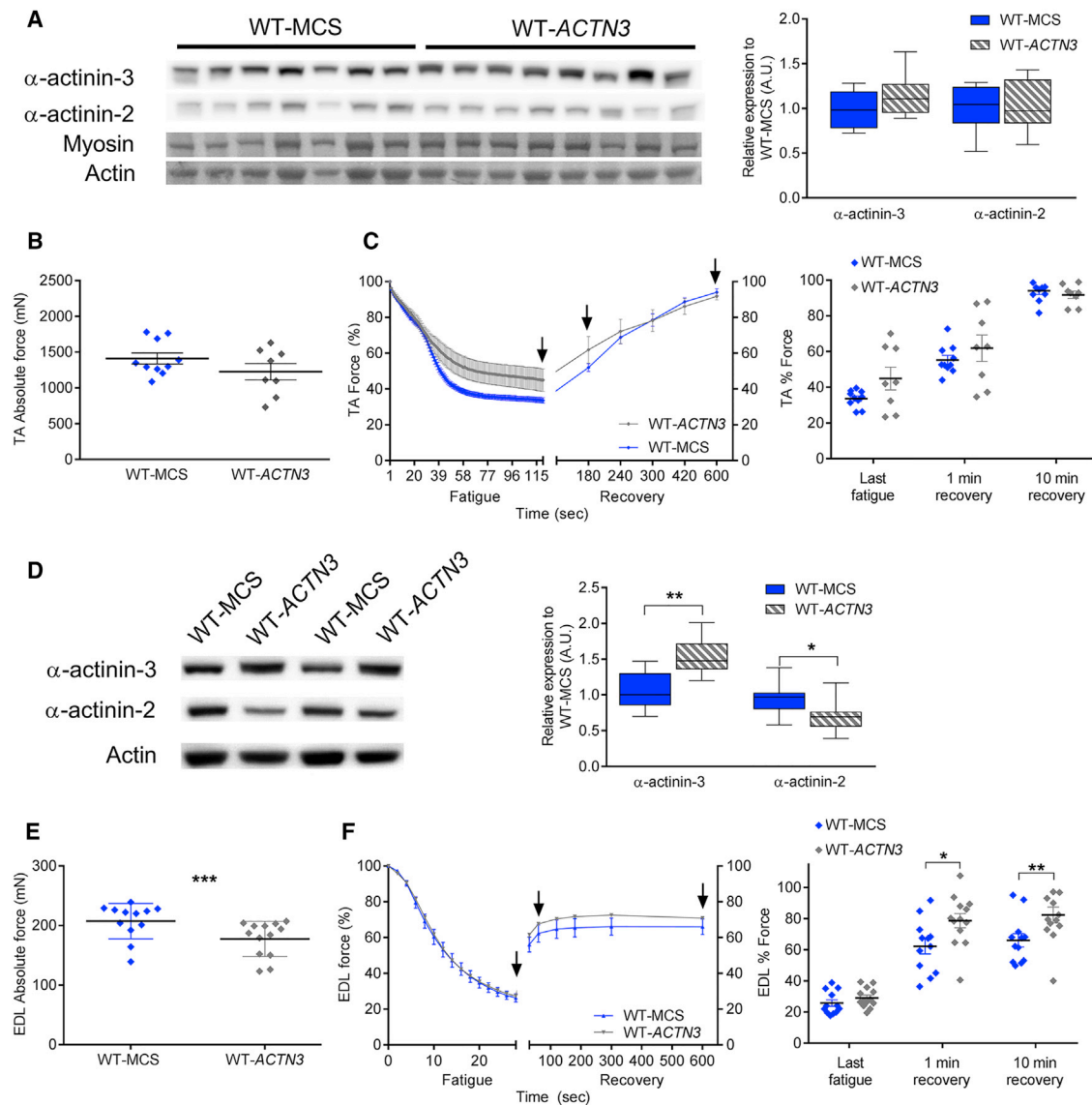
Figure 3. High Doses of rAAV-*ACTN3* Intramuscular Injection (1E11 vg) Did Not Enhance Muscle Force in WT-*ACTN3* Muscles

(A) α-Atinin-3 levels varied across injections in WT-*ACTN3* TA (n = 8) compared to WT-MCS (n = 8), with no mean changes in α-actinin-3 or 2.

(B) No change in WT-*ACTN3* TA force (1,227 ± 225 mN, n = 8) compared to WT-MCS (1,412 ± 152 mN, n = 10) 5 weeks after injection.

(C) Fatigue response and force recovery at 1 and 10 min (arrows) was also not different.

(D) α-Actinin-3 was increased in WT-*ACTN3* EDL muscles compared to WT-MCS at 1E11 vg while α-actinin-2 is decreased.

(E) EDL force was lower in WT-*ACTN3* (177 ± 16 mN, n = 13) compared to WT-MCS (207 ± 17 mN, n = 23) (p = 0.001).

(F) WT-*ACTN3* EDL muscles showed improved recovery at 1 min and 10 min after fatigue compared to WT-MCS.

Boxplots represent the median, whiskers; min-max. Mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 Mann Whitney U test.

Postnatal Replacement of α-Actinin-3 in *Actn3* KO Muscles Increases Muscle Fatigability and Reduces Force Recovery after Fatigue

Given the baseline effects of α-actinin-3 deficiency in mice leading to enhanced recovery from fatigue,[18] we recorded force recovery (super maximally stimulated) in WT-MCS, KO-MCS, and KO-*ACTN3* muscles to determine whether overexpression of α-actinin-3 altered fatigue susceptibility. KO-A*CTN3* muscles demonstrated alterations in fatigue resistance and force recovery relative to KO controls (Figure 6). KO-*ACTN3* EDL muscles showed a trend for reduced force recovery compared to KO-MCS EDL muscle, but this did not reach statistical significance, with force recovery remaining higher relative to WT-MCS (p < 0.05) (Figures 6A and 6C). KO-*ACTN3* TA muscles showed a fatigue profile that was more similar to WT-MCS TA muscles, with significantly higher fatigue and slower force recovery relative to KO-MCS TA muscles 1 min after fatigue (p < 0.0001) (Figures 6B and 6D). These results indicate that although post-natal restoration of α-actinin-3 in *Actn3* KO TA muscles is unable to rescue force, there is a strong detrimental effect on the ability to recover from muscle fatigue.
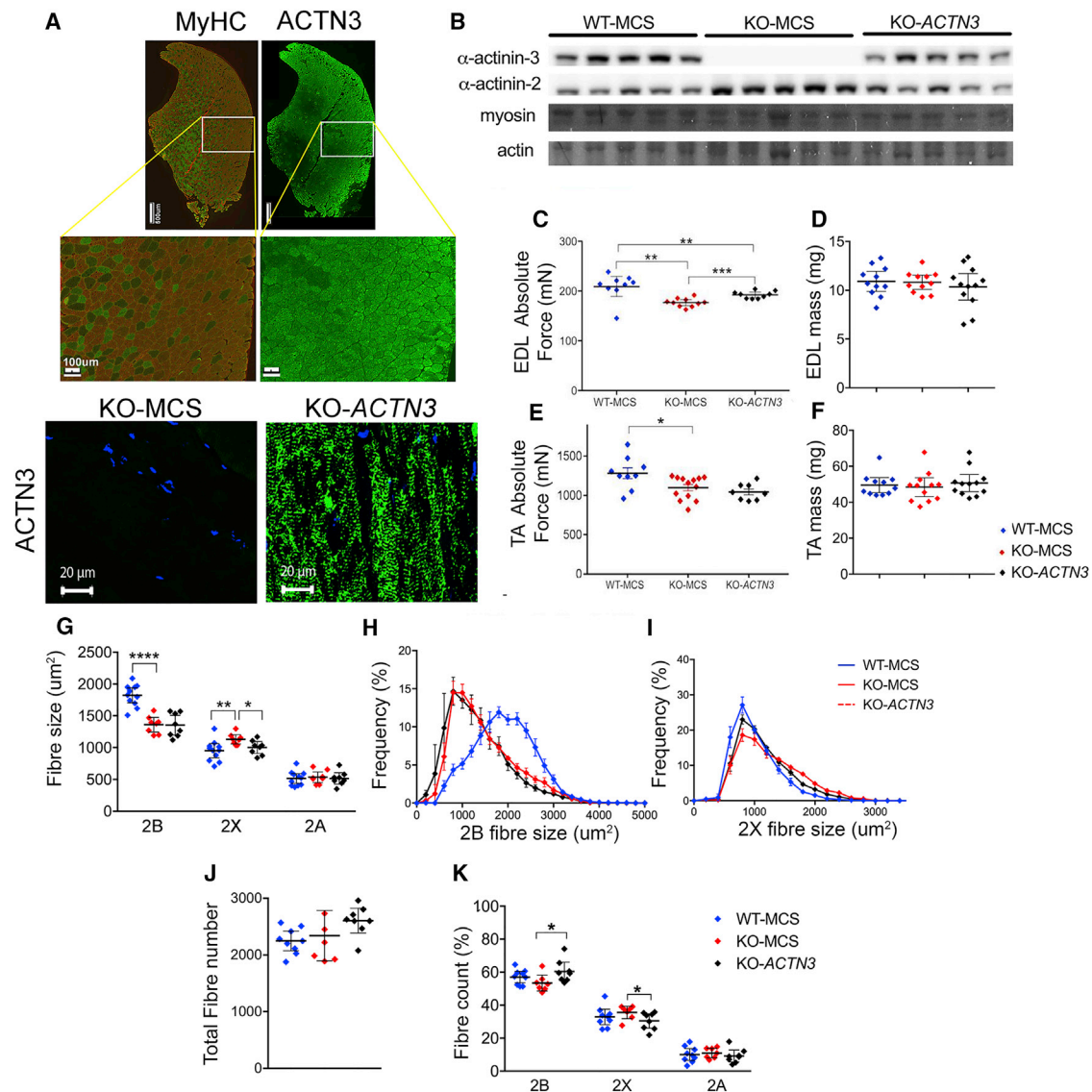
Figure 4. Postnatal Replacement of α-Actinin-3 in *Actn3* KO Muscles Does Not Enhance Force, Muscle Mass, or Fast Fiber Size

(A) Intramuscular delivery of *ACTN3* resulted in robust expression of α-actinin-3 in all fiber types. KO-*ACTN3* TA muscle is immuno-labeled for myosin heavy chain 2B (red) and 2A (green) to denote fiber types. A longitudinally cut KO-*ACTN3* muscle section stained for α-actinin-3 antibody (green), nuclei (blue) demonstrates localization to the Z-disk.

(B) KO-*ACTN3* muscles show α-actinin-3 protein expression and reciprocal downregulation of α-actinin-2. Myosin and actin are loading controls.

(C) EDL force was increased in KO-*ACTN3* (192 ± 5 mN) versus KO-MCS (177 ± 5 mN) but was lower than WT-MCS (210 ± 17 mN).

(D) EDL mass was not different between groups.

(E) KO-*ACTN3* TA force is unchanged (1,044 ± 72 mN) compared to KO-MCS (1,099 ± 78 mN) and 18% lower than WT-MCS.

(F) TA mass.

(G–I) Mean 2B fiber size of KO-MCS and KO-*ACTN3* TA muscles was not different, but 2X fiber size was reduced in KO-*ACTN3* compared to KO-MCS, likely due to increased numbers of smaller 2X fibers.

(J) Total fiber number.

(K) KO-*ACTN3* TA muscles have increased 2B and decreased 2X fiber proportions relative to KO-MCS.

N = 7–13 for all groups. Mean ± 95%CI. *p < 0.05, **p < 0.01, ****p < 0.0001 Mann Whitney U.

Post-natal Replacement of α-Actinin-3 in *Actn3* KO Muscle Results in a Shift to an Anaerobic Metabolic Profile

To determine the cause for the changes in muscle fatigue with post-natal expression of α-actinin-3 in KO muscle, we investigated the effect of skeletal muscle metabolism in KO-*ACTN3* TA muscles. Succinate dehydrogenase activ-ity (SDH; complex II) was assessed semiquantitatively by histochemical reaction staining on muscle cross-sections (Figures 7A and 7B). Cross-sectional areas positive for rAAV-*ACTN3* were analyzed (n = 6 per group). These pri-marily consisted of 2B/2X fibers (the TA is 60% 2B/2X, Figure 4K) and demonstrated that KO-*ACTN3* muscles stained less intensely for SDH than the KO-MCS control
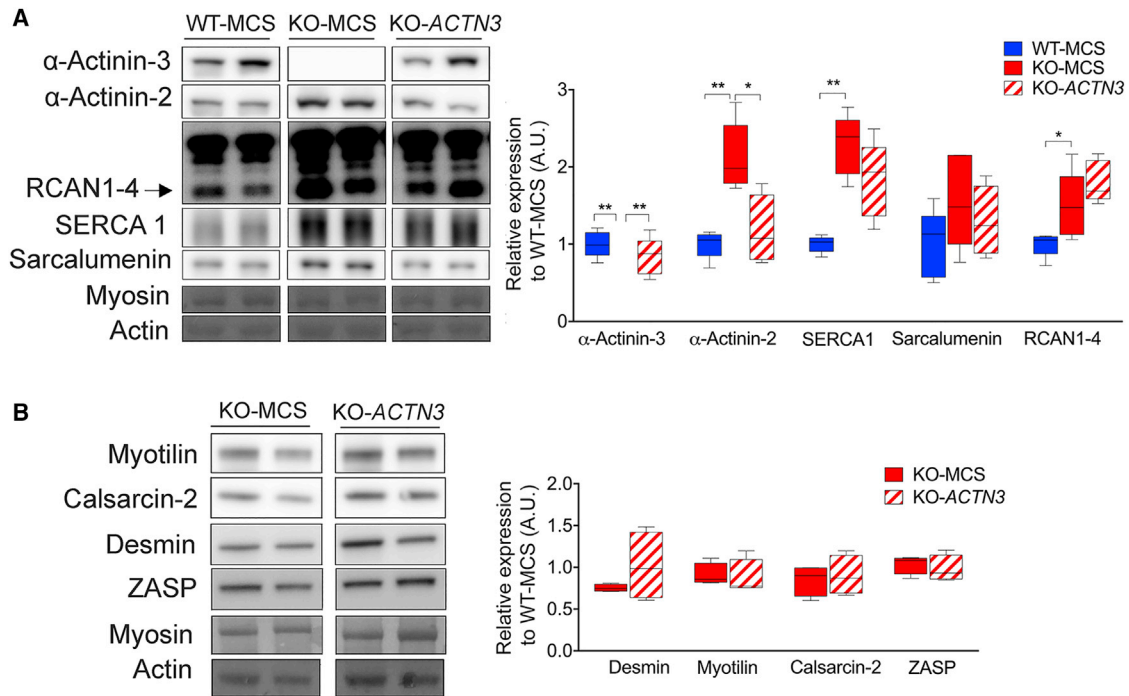
Figure 5. Postnatal α-Actinin-3 Replacement in *Actn3* KO Muscles Did Not Alter Calcineurin Activity or Expression of Other Z-disk Proteins

(A) Calcineurin activity (as shown by RCAN1-4 expression) was unaltered in KO-*ACTN3* muscles, despite reciprocal downregulation of α-actinin-2. Similarly, calcium handling proteins SERCA1 and sarcalumenin were unchanged in KO-*ACTN3* muscles relative to KO-MCS.
(B) Expression of the Z-disk proteins. Myotilin, ZASP, calsarcin-2, and the remodeling protein, Desmin, was not altered in KO-*ACTN3* muscles.
Mean ± 95%CI, N = 7–13 for all groups. Boxplots represent the median, whiskers; min-max. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Mann Whitney U.

but remained significantly different compared to WT-MCS, suggesting partial shift of the muscle oxidative metabolism. We further assessed all five mitochondrial complexes in the TA by western blot (Figures 7C and S8) (n = 5 per group). At baseline, complexes II and IV were increased in KO-MCS muscles compared to WT-MCS; these correspond to SDH and the cytochrome-c oxidase subunit I, respectively. We have previously shown that NADH, SDH, and CIV activity is upregulated in KO muscles.[34] Interestingly, replacement of α-actinin-3 in KO TA muscles resulted in significant decreases in all complexes (I, II, III, IV, and V) relative to KO-MCS, to be equal or below WT-MCS control levels. These results suggest that α-actinin-3 influences mitochondrial activity via specific effects on complexes on the electron transport chain.

Discussion

α-Actinin-3 Overexpression (WT-*ACTN3*) Damages Skeletal Muscle: The Case against Gene Doping

Results from our meta-analysis confirmed that complete absence of α-actinin-3 (*ACTN3* 577XX) is detrimental for elite sprint performance compared to 577RR homozygotes. Heterozygotes were intermediate (RR > RX > XX) but there was substantial variation across studies. Larger sample

sizes, expression levels, and genome-wide testing in elite-international cohorts may help understand these population-specific complexities noted here in our general model meta-analysis. The consistent positive effect associated with *ACTN3* 577RR homozygotes in human sprint/power performance supported the hypothesis that rAAV gene delivery of *ACTN3* would enhance muscle performance. Here we have tested multiple doses of rAAV-*ACTN3* in our wild-type (WT) mouse line, in two different muscles over 6 weeks, and demonstrated that overexpression of α-actinin-3 in mature skeletal muscle had no immediate effect on force. Furthermore, at high dose levels, *ACTN3* overexpression was detrimental to muscle cytoarchitecture and function, with muscles showing reduced force generation. Force generation and recovery after fatigue was incrementally altered in WT mice with rAAV-*ACTN3* injected into the EDL muscles but this was not replicated in TA muscles.

The presence of fibrosis and centralized nuclei in the WT muscles injected with rAAV-*ACTN3* indicates that supraphysiological *ACTN3* overexpression for 6 weeks triggered recurrent muscle damage and remodelling. The responses in the EDL may be an artifact related to reduced work performed by damaged/regenerating muscles.[35] This is unlikely to be due to the presentation of foreign antigens as a result of species differences in mouse and human α-actinin-3, as they share 97% identity. Furthermore, expression
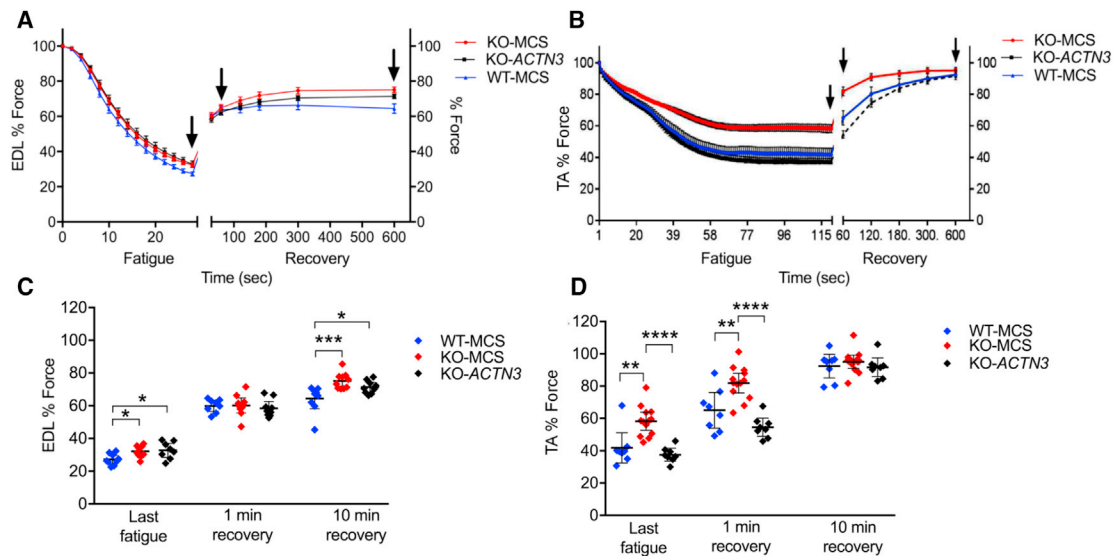
Figure 6. Postnatal Replacement of α-Actinin-3 in *Actn3* KO Muscles Increases Muscle Fatigability and Reduces Force Recovery after Fatigue

(A and B) Force is reduced with repeated muscle stimulation to fatigue, and force recovery is assessed at 1, 2, 3, 5, and 10 min post-stimulation in EDL (A) and TA (B) muscles (mean ± SEM). Arrows indicate time points of cohort comparisons in the corresponding column graphs.

(C) KO-*ACTN3* EDL demonstrated reduced force recovery at 10 min compared to the KO-MCS.

(D) KO-*ACTN3* TA fatigued more rapidly than KO-MCS and were slower to recover after 1 min, similar to the WT-MCS.

Mean ± 95% CI *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, Mann Whitney U, N = 6–13 for all groups.

of *ACTN3* in *Actn3* knock-out (KO) muscles, which were devoid of α-actinin-3 from birth, and low doses (5E8–1E10) of rAAV-*ACTN3* in WT mice did not result in any consistent muscle damage. One explanation for the presence of recurring muscle damage may be associated with the level of α-actinin overexpression and the length of vector expression. We note that no consistent muscle damage was observed in WT muscles injected with ≤1E10 vector genomes (vg) of rAAV-*ACTN3* for 6 weeks, or in muscles injected with 1E11 vg of rAAV-*ACTN3* for 1 and 2 weeks (Figures S5 and S7). This suggests that muscle fibers can incorporate additional sarcomeric α-actinin up to a certain threshold, consistent with normal 2- to 3-fold variation in Z-line layers between fiber types.[36,37] Excessive α-actinin-3 expression, however, whether this is dispersed across the whole muscle or localized to a certain region due to variation in delivery, has a "toxic" effect on skeletal muscles. This sensitive balance has previously been shown with other contractile proteins.[38–40] For example, moderate overexpression of titin (the Z-band region) localized normally at the Z-line in contracting myofibrils and normal function was observed. However, high levels caused myofibril disassembly as the fragments accumulated.[41] High expression of desmin has also been found to form a reticular intermediate filament meshwork and non-filamentous aggregates.[42]

In WT quadriceps muscles at baseline (with a large proportion of fast fibers 2B fibers), sarcomeric α-actinin expression in skeletal muscle is maintained at a ratio of 1:1 for α-actinin-3 and α-actinin-2.[32] Therefore, the muscle damage observed in WT-*ACTN3* may be associated with

loss of the optimal ratio of relative expression of the two sarcomeric α-actinins. Overexpression of α-actinin-3 in WT muscles was not accompanied by an equivalent level of α-actinin-2 downregulation, regardless of rAAV dose (Figure 2). It is possible that high, non-toxic, levels of α-actinin-3 may be achieved only if there is parallel α-actinin-2 knockdown that we did not investigate. We note that maintenance of α-actinin-2 may reflect an essential role in normal muscle function. Knockdown of α-actinin-2 in zebrafish results in sarcomeric disorganization in skeletal muscles and is not compensated by increased endogenous α-actinin-3.[43] This contrasts with α-actinin-3 deficiency, which does demonstrate partial compensation by endogenous upregulation of *ACTN2* in muscles of mice[18,21] and humans (controlled for fiber type)[44] and results in normal, highly organized, and functional sarcomeres. Moreover, in zebrafish, replacement with *actn3a*, *actn3b*, or human *ACTN3* fails to ameliorate the developmental defects associated with α-actinin-2 deficiency,[43] highlighting the functional differences between α-actinin-2 and α-actinin-3.

α-Actinin-3 Rescue (KO-*ACTN3*) Does Not Restore Muscle Mass and Strength to Wild-Type Levels and Is Unlikely to Confer a Therapeutic Benefit in Muscle Wasting Diseases

Contrary to expectations, postnatal replacement of α-actinin-3 in *Actn3* KO muscles failed to restore force production, muscle mass, and fast fiber size to WT levels, despite 6 weeks of gene expression and reintroduction of α-actinin-3 to ~64% of WT levels (Figure 4). At a dose of 1E9 vg, α-actinin-2 was also reciprocally downregulated
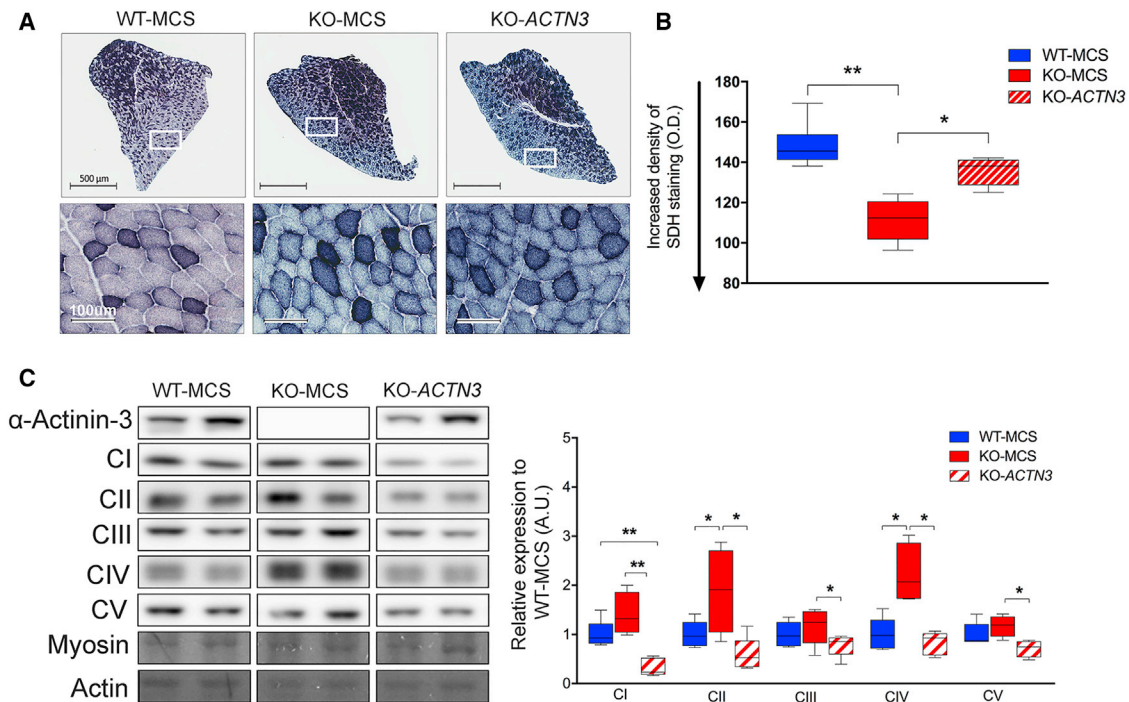
Figure 7. α-Actinin-3 Replacement in KO TA Muscles Reduces Mitochondrial Oxidative Metabolism
(A) Succinate dehydrogenase activity staining in WT-MCS, KO-MCS, and KO-*ACTN3* TA muscle.
(B) Intensity of positive blue staining is reduced in KO-*ACTN3* relative to KO-MCS (indicative of lower SDH activity) but remains different compared to the WT-MCS, N = 6 all groups.
(C) KO-*ACTN3* TA muscle demonstrated decreases in mitochondrial complexes I, II, III, IV, and V to WT levels in response to α-actinin-3 expression. N = 5 for all groups.
Boxplots represent the median, whiskers; min-max. *p < 0.05, **p < 0.01, ***p < 0.001.

in KO-*ACTN3* muscles by approximately equal proportions relative to KO controls. This mimics the normal developmental regulation of total α-actinin levels in the WT muscles (where α-actinin-3 is detectable from 2 weeks of age and is associated with downregulation of α-actinin-2[14]) and the dose-dependent upregulation of α-actinin-2 in the *Actn3* KO and HET mice.[18,21] However, the reincorporation of α-actinin-3 in *Actn3* KO muscle Z-disks was not accompanied by a change in Z-disk structural protein composition. Despite a ∼56% reduction of α-actinin-2, myotilin, calsarcin-2, and ZASP levels were unchanged in KO-*ACTN3* muscles, whereas we would have predicted a decrease in levels (Figure 5). Contrary to expectations, RCAN1-4 expression was also unchanged. We have previously shown that upregulation of α-actinin-2 results in increased activity of calcineurin activity through its release from the inhibitory effects of calsarcin-2,[16] so a reduction in RCAN1-4 was expected. It is possible that the reduction in α-actinin-2 in KO-*ACTN3* muscles was insufficient to significantly influence calcineurin activity, since these muscles still express 44% higher levels of α-actinin-2 compared to WT. This is supported by the presence of only minor (6%–10%) fiber type shift toward fast 2B (Figure 4K). The lack of change in RCAN1-4 and Z-disk structural protein expression in KO-*ACTN3* muscles is also consistent with a lack of muscle damage and regeneration.

The absence of any meaningful effect on muscle mass and force with postnatal α-actinin-3 replacement in *Actn3* KO muscles indicates that this model is not recapitulating muscle phenotypes of *Actn3* WT and heterozygous mice, which express α-actinin-3 from birth. These results also suggest that the pivotal effects of α-actinin-3 on muscle traits begin during skeletal muscle development. α-Actinin-3 is detectable in mouse skeletal muscle at E14.5,[1] a time point that coincides with the end of primary myogenesis, during which myotubes lengthen as new nuclei fuse with primary myotubes.[45] Therefore, α-actinin-3 may, in fact, play a role in regulating fiber size and muscle growth during embryonic muscle development. Calcineurin signaling is also known to govern the formation, growth, and maturation of the musculature through specific, temporal expression of NFATc2[46] and NFATc3.[47] In the absence of α-actinin-3, calcineurin activity is elevated in mature muscles.[16] This raises the possibility that *ACTN3* genotype influences fiber size and muscle mass via calcineurin signaling during muscle development.

Despite the absence of an effect on muscle mass and strength, replacement of α-actinin-3 in *Actn3* KO muscles did significantly alter skeletal muscle response to fatigue. KO-*ACTN3* muscles showed a fatigue profile that was more similar to WT muscles, producing 20% lower maximum relative force compared to KO-MCS after 2 min of repeated electrical stimulation (Figure 6D). The shift

toward increased fatigability was expected, since α-actinin-3 deficiency is associated with enhanced force recovery after fatigue, increased endurance capacity, and response to exercise training.[16,18] Analysis of mitochondrial enzyme activity showed that increased fatigability of KO-*ACTN3* muscles was associated with marked decreases in expression of mitochondrial complexes I-V, which include NADH dehydrogenase, SDH, and the ubiquinol-cytochrome *c* reductase complex (Figures 7 and S8). Intriguingly, the expression of some of these complexes in KO-*ACTN3* muscles are on par with levels found in WT muscles, suggesting that the primary role of α-actinin-3 in skeletal muscle is the regulation of muscle metabolism, specifically, mitochondrial enzyme activity. Moreover, the lack of change in other measured phenotypes in KO-*ACTN3* muscles suggest that the effect of α-actinin-3 on oxidative metabolism in skeletal muscle occurs prior to, or independent of, its effects on Z-disk protein composition, fast fiber size, and calcium/calcineurin signaling.[16,18]

Our present study thus confirms that a primary function of α-actinin-3 in skeletal muscle is to regulate muscle metabolism, and this precedes or occurs independently of its other functions in skeletal muscle. This supports the shift in muscle metabolism as a key driver for the positive selection of the *ACTN3* 577X allele during recent human evolution and the functional specialization of *ACTN2* and *ACTN3* genes following divergence.[34,48] We suggest, however, that these metabolic effects may be specific to muscle. The prevalence of metabolic disease is highly influenced by genetic factors[49] and while a number of studies have investigated *ACTN3* Arg577X as a modifier of these complex traits,[17,50,51] no *ACTN3*-specific loci effect has been identified genome-wide. Further expression studies focused on the skeletal adaptation effects, such as denervation or exercise training seen in both human and mouse studies,[16,52–54] may provide greater insight into the effect of *ACTN3*. Specifically, our results demonstrate that the metabolic impacts of *ACTN3* may not be limited to the calcineurin pathway and other mechanisms may also be at play. In the context of gene-doping risks and gene therapy, our findings demonstrate the sensitive balance of sarcomeric α-actinin expression at the Z-line. Both *in vivo* rescue and overexpression of *ACTN3* reveals insight into its metabolic role in skeletal muscle, but this is innately linked to the level of expression which can be easily disrupted causing detrimental functional effects, reminiscent of disease.

Supplemental Data

Supplemental Data include Supplemental Material and Methods, eight figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.ajhg.2018.03.009.

Acknowledgments

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Supplemental Data

The Effect of *ACTN3* Gene Doping

on Skeletal Muscle Performance

Fleur C. Garton, Peter J. Houweling, Damjan Vukcevic, Lyra R. Meehan, Fiona X.Z. Lee, Monkol Lek, Kelly N. Roeszler, Marshall W. Hogarth, Chrystal F. Tiong, Diana Zannino, Nan Yang, Stephen Leslie, Paul Gregorevic, Stewart I. Head, Jane T. Seto, and Kathryn N. North

Methods

Systematic review and meta-analysis

A systematic search and review was initiated following PRISMA-P guidelines [1]. The aim defined in the meta-analysis was whether the *ACTN3* genotype frequency in elite athletes (majority competing internationally) in sprint/power events demonstrated an additive (RR>RX>XX), recessive (RR/RX vs. XX) or dominant effect (RR vs. RX/XX). Studies were considered eligible if they assessed the association between *ACTN3* R577X genotype in elite athletes (competing at an international level in sprint/power events) and controls. At least 50% of the athletes needed to be competing in an individual (i.e. not team sports) event such as athletics (<800m, jumps, throws), swimming (<200m) and weightlifting. Data was from published studies written in English, with cross-sectional study designs, published between April 1st 1999-September 30th 2016. Data was not included if references were made to previously published cohort results, likely to have overlapping individuals. The search strategy included four electronic databases (Medline, EMBASE, Scopus, Science direct) using the search term "*ACTN3*". Data collection involved two investigators. A single investigator performed the search and filtering before defining studies that met the inclusion criteria. The numbers of male and female athlete and control genotypes were recorded in a spreadsheet alongside study details, country of origin/ethnicity, athlete event, percentage of team athletes, percentage of international athletes. These data items were predefined by three investigators as those that may contribute to study heterogeneity and could be defined as relevant covariates and/or sub-group assessment in the analysis (i.e. single ethnic group only). If only genotype percentage frequencies were reported these were extrapolated into raw counts. A second investigator checked all genotype counts were consistent with totals, removed any redundancy in the data with any discrepancies reassessed. Individuals were only represented once in

datasets to avoid duplication of controls and sub-groups. If there were missing data the corresponding author of the study was contacted and results updated.

Notes on study inclusion/exclusion: Three studies included overlapping cohorts and results [2-5]. The Eynon et al. [6] data included the Russian and Polish athletes alongside Polish controls, while the Spanish cohort was excluded. The Spanish data was obtained from Ruiz et al. [3] which did not include team athletes. Kikuchi et al. [5] included an expanded cohort from Mikami et al. [4]. A number of studies were included in previous meta-analyses that were not included here due to low percentage of international athletes or sport event definitions.

We retrieved the data for a number of variables to investigate them as potential explanatory factors for the heterogeneity we observed. While most studies reported male and female genotype counts, some did not, and the breakdown of sex in international athletes were not reported. Moreover, most of the covariates were only meaningfully available as a summary on a per-study level (e.g. proportion of international athletes) rather than a per-genotype group (or per-individual) level on which we do the modelling, meaning that incorporating the limited available co-variate data into a formal measure, such as I^2 (which has its own drawbacks) in this meta-analysis would not be useful or a valid method to interpret the true heterogeneity [7]. For visual reference, the effect of covariates were compared against the parameter estimates for each study. Given the low proportion of the *ACTN3* 577X allele frequency in African ancestry (<10%) compared to Asian and European ancestry (40-50%) a separate meta-analysis was initially planned. However, due to small sample size (one African study) in the analysis (<10% of the total number of individuals) – no additional conclusions would have been possible.

Similar to other association studies and meta-analyses, it is possible that publication bias exists for positive association studies. Eligibility was not based on association results, but rather homogenous performance specialties and defined qualification levels (international standards). To ensure all data was consistent with previous meta-analysis [8], the analysis was repeated by an independent operator to replicate results.

Muscle physiology

Isolated EDL muscle physiology

Animals aged eight weeks were injected with rAAV-MCS into the control leg and rAAV-ACTN3 into the contralateral leg. Four or six weeks post injection, mice were sacrificed by cervical dislocation before measures of the EDL force and recovery from fatigue were carried at room temperature (22–24°C) using techniques as previously described [9; 10]. Briefly, after dissecting the EDL, silk (Deknatel 6.0) suture was tied to the proximal tendon to attach a force transducer (Fort 10, World Precision Instruments) to a linear tissue puller (University of New South Wales). The isolated muscle was placed in a bath continuously perfused with Krebs solution and bubbled with carbogen to maintain pH (7.4). Two parallel platinum electrodes in the bath controlled by an electrical pulse generator (A-M Systems) were attached to a current amplifier to ensure that sufficient current density is used to maximally stimulate the muscle. Throughout the experiment, the resting length of the muscle was its optimum length (L_0). This is the length at which the muscle produces its maximum isometric force. At the start of the experiment, the muscle was set to this length by stretching it in increments of 0.1 mm until maximum twitch force was produced. This length was then measured as the distance between the musculotendinous junctions, using a scale imprinted into a microscope eyepiece. In this study, the muscle was stimulated at frequencies of 5.0, 15.0, 25.0, 37.5, 50.0, 62.5, 75.0, 87.5,100 and 120Hz. The muscle was stimulated for 500 ms at each frequency, using electrical pulses of 1 ms duration. A 1 minute interval was allowed between

stimulation at each frequency. The forces produced at each frequency were plotted as a sigmoidal dose-response type curve. The curve had the equation:

$$P = P_{min} + \frac{P_{max} - P_{min}}{1 + \left(\dfrac{K_f}{f}\right)^h}$$

The following contractile properties of the muscle were then derived from the fitted parameters of its force-frequency curve:

$$Maximum\ tetanic\ force\ =\ P_{max}$$

$$Twitch\ to\ tetanus\ ratio\ =\ \frac{P_{min}}{P_{max}}$$

$$Half\text{-}frequency\ =\ K_f$$

Five minutes after the force analysis, the EDL muscles were then examined for their responses to a fatigue protocol consisting of fifteen contractions at 150Hz over 30 secs. The muscle force recovery was monitored by 6 brief (250 ms) stimulations over 10 mins. The EDL was then removed, tendons were trimmed and the wet weight recorded.

TA muscle physiology

In situ TA muscle force and fatigue was determined using the Aurora Scientific Dual Mode Lever System (1300A whole mouse test system and 701C stimulator) with supplied software (DMC5 4.5, DMA). Precision weights (Masscal Precision weighing equipment, Australia) were used to calibrate the force transducer as per manufactures instructions. Mice were kept anaesthetised throughout the procedure using isoflurane. The proximal TA tendon was released from the ankle and 5.0 silk suture was used to tie the tendon to the force transducer lever arm. The knee was clamped in place to stabilize the leg. Electrodes were placed above and below the sciatic nerve. The TA muscle was set to its optimal length (L_O) before assessing the force-frequency response: muscle was stimulated for 250 ms every 2 mins at incrementing frequencies of 5, 10, 20, 30, 40, 50, 75, 100, 150, 200, and 250 Hz and force was recorded.

After five minutes recovery, the TA was maximally stimulated at 150 Hz (1 second on, 1 second off) over 3 minutes to induce muscle fatigue. Force recovery was measured at 1, 2, 3, 5 and 10 mins post fatigue. Following completion of muscle physiology tests, mice were euthanized by cervical dislocation the TA removed and the wet weight recorded. Collected muscles were stored at -80°C.

Antibodies

Primary antibodies used included: actin (A2172, Sigma Aldrich), RCAN/DSCR1 (D6694; Sigma-Aldrich); α-actinin-2 (4A3; gift from A. Beggs, Children's Hospital Boston, Boston Massachusetts, USA); and α-actinin-3 (ab68204, Abcam), COX IV (Ab14744, Abcam); MitoProfile Total OXPHOS antibody cocktail (ab110413, Abcam), desmin (NCL-L-DES-DERII, Novocastra); ZASP (11004-1-AP, Proteintech); and porin (ab17734, abcam).
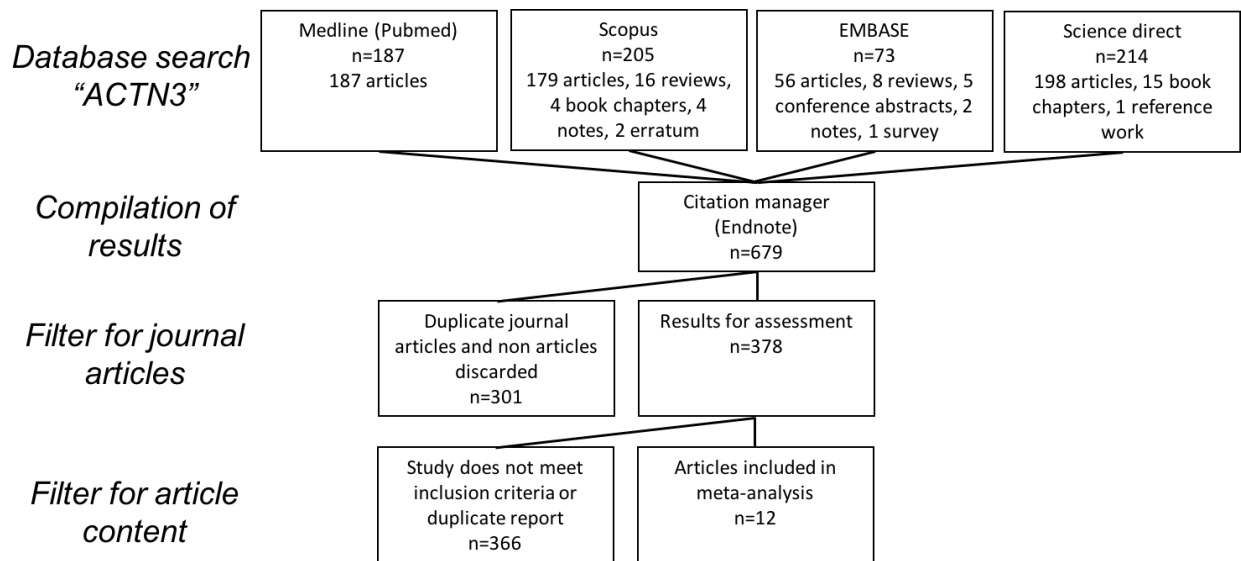
| Database search "ACTN3" | Medline (Pubmed)
n=187
187 articles | Scopus
n=205
179 articles, 16 reviews,
4 book chapters, 4
notes, 2 erratum | EMBASE
n=73
56 articles, 8 reviews, 5
conference abstracts, 2
notes, 1 survey | Science direct
n=214
198 articles, 15 book
chapters, 1 reference
work |

Database search "ACTN3"

Compilation of results

Citation manager (Endnote) n=679

Filter for journal articles

Duplicate journal articles and non articles discarded n=301

Results for assessment n=378

Filter for article content

Study does not meet inclusion criteria or duplicate report n=366

Articles included in meta-analysis n=12

Figure S1. Search strategy for the Systematic Meta-analysis. Four databases were used to search for relevant articles using the search term "ACTN3." Results were compiled into a single citation manager and subsequently filtered for relevant journal articles, twelve of which were taken forward into the meta-analysis.

Table S1. Study details included in the meta-analysis

Study reference	Country of origin	Sex	Athletes (number, % international)
European			
Yang et al. [11]	Australia	M&F	Track and field athletes (≤800m) (n=46), swimmers (≤200m) (n=42), judo athletes (n=9), short-distance track cyclists (n=7), and speed skaters (n=3). (n= 107, 100%)
Niemi & Majamaa [12]	Finland	M&F	Sprinters (100-400m) & field athletes (n= 23, international, n=68 national level^)
Papadimitriou et al. [13]	Greece	M&F	Sprinters (100- 400m), jumpers, throwers and decathletes (international n=44, n=29 national)
Eynon et al. [14]	Israel	M&F	Sprinters (100 to 200m) (n= 26, international, n=55 national)
Massidda et al. [15]	Italy	M	Sprinters (n=16), swimmer (n=1), wrestlers (n=17), power lifters (n=11), artistic gymnasts (n=19) (n=64, 67% international)
Eynon et al. [6]	Poland, Russia	M&F	Poland; weightlifters (n=43), Track (≤200 m, n=48), wrestlers (n=72) jumpers (n=11), volleyball players (n=4) (n=118 international, n=60 national) Russian; speed skaters ≤1000 m (n=17), boxers (n=34), wrestlers (n=10), swimmers ≤200 m (n=8), weightlifters (n=6), figure skaters (n=6), one strongman, (n=56 international, n=26 national)
Ruiz et al. [3]	Spain	M	Sprinters n=40, jumpers n=13 (n=53, 100%)
Druzhevskaya et al. [16]	Russia	M&F	Alpine skiing (n=29), artistic gymnastics (n=44), bodybuilding (n=23), figure skating (n=10), ice hockey (n=34), jumping events (n=8), powerlifting (n=9), running 100–400 m (n=70), ski jumping (n=18), soccer (n=4), speed skating (n=90), swimming 50–100 m (n=10), throwing events (n=15), volleyball (n=9), weightlifting (n=55) and wrestling (n=58). (n=306 international, n=180 regional)
Asian			
Kikuchi et al. [17]	Japan	M	Wrestlers (international, n=52, national, n=83)
Kikuchi et al. [5]	Japan	M&F	Sprinters ≤400 m (n=235), jumpers (n=57), throwers (n=36), and decathletes (n=9) (n=44 international, n=293 national)
Hong et al. [18]	Korean	M&F	Artistic gymnastics, sprint, short distance speed skating, weightlifting, taekwondo, and throwing athletes (n=84 international)
African			
Scott et al. [19]	USA, Jamaica	M&F	Jamaican: Sprinters (≤400m n=71), jumpers and throwers (n=10) (n=86 international, n=28 national); African American: Sprinters (≤400m, n=90), jumpers and throwers (n=24) (n=109 international, n=5 national)

^=had one pair of twins, 4 siblings

Table S2. Cohort details for studies included in the meta-analysis

| Specialist event	Caucasian n (%) 8 studies	Asia n (%) 3 studies	Africa n (%) 1 studies
Track and field ≤800m	387 (40.1%)	90 (45.5%)	114 (100%)
Swimmers≤200m	61 (6.3%)		
Judo	9 (0.9%)		
Short distance track cyclist	7 (0.7%)		
Speed skaters≤1000m	110 (11.4%)	5 (2.5%)	
Artistic gymnasts	70 (7.2%)	13 (6.6%)	
Weightlifters	110 (11.4%)	27 (13.6%)	
Volleyball	13 (1.3%)		
Boxers	34 (3.5%)		
Wrestlers	85 (8.8%)	52 (26.3%)	
Figure skaters	13 (1.3%)		
Strongman	1 (0.1%)		
Taekwondo	1 (0.1%)	11 (5.6%)	
Ski jumping	18 (1.8%)		
Bodybuilding	23 (2.4%)		
Powerlifting	20 (2.0%)		
Soccer	4 (0.4%)		
TOTAL	966	198	114
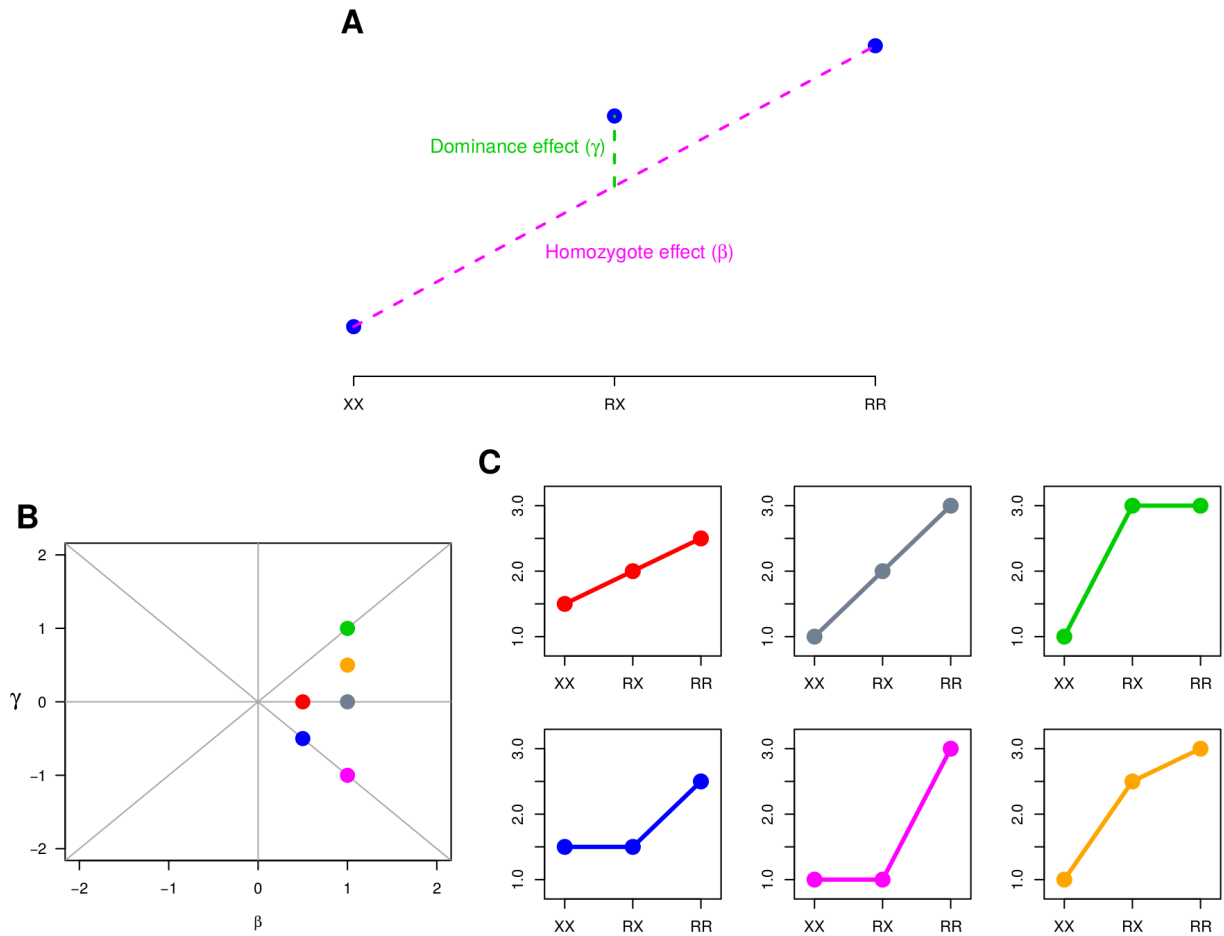
Figure S2. Explanation of the general genetic model.

A. A visual depiction of the parameterisation of the general model used in the meta-analysis. The y-scale can refer to any measure of the phenotype; no y-axis is pictured to emphasise this fact. **B.** The space of possible values for the two parameters in the general model. The specific points drawn on this plot are highlighted as examples shown in more detail in panel C. Some of the grey lines refer to special cases of simpler models: the horizontal line ($\gamma = 0$) consists of pure additive models, the diagonals at the top refer to dominant models, and the diagonals at the bottom refer to recessive models. **C.** Examples of possible models, corresponding to points in panel B of the same colour.
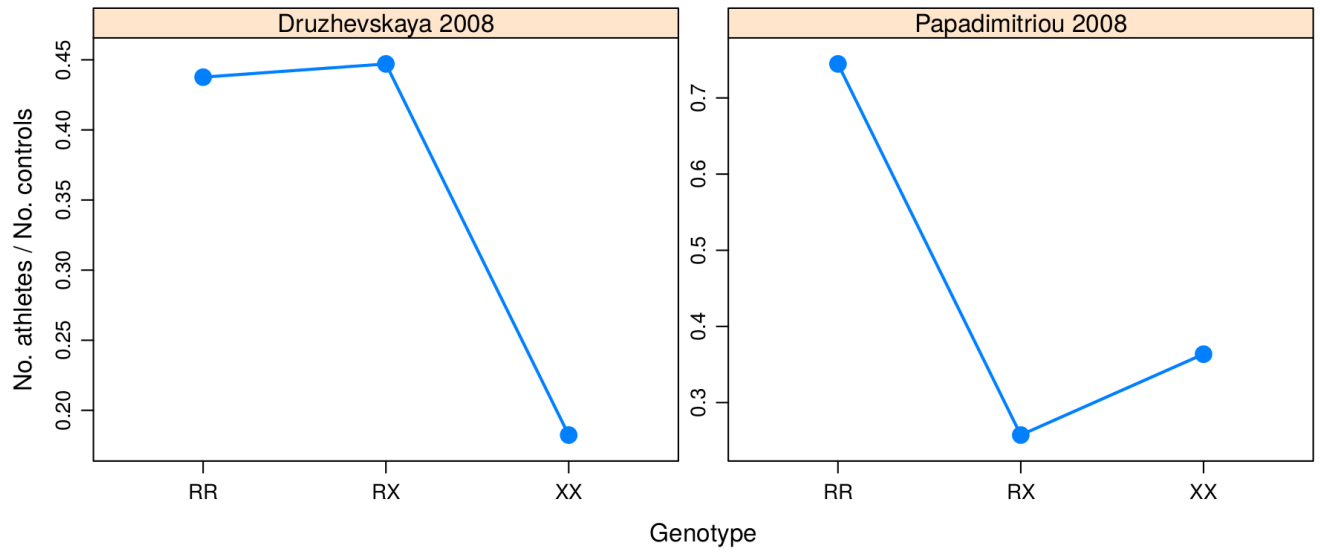
Figure S3. Examples of diverse genetic effects. The ratio of athletes to controls for each genotype for two of the studies included in the meta-analysis. These were chosen to illustrate the diversity in the observed genetic effects: the data from e.g. Druzhevskaya et al. [16] are consistent with a recessive model for allele X, whereas those from Papadimitriou et al. [13] are more consistent with a dominant model.
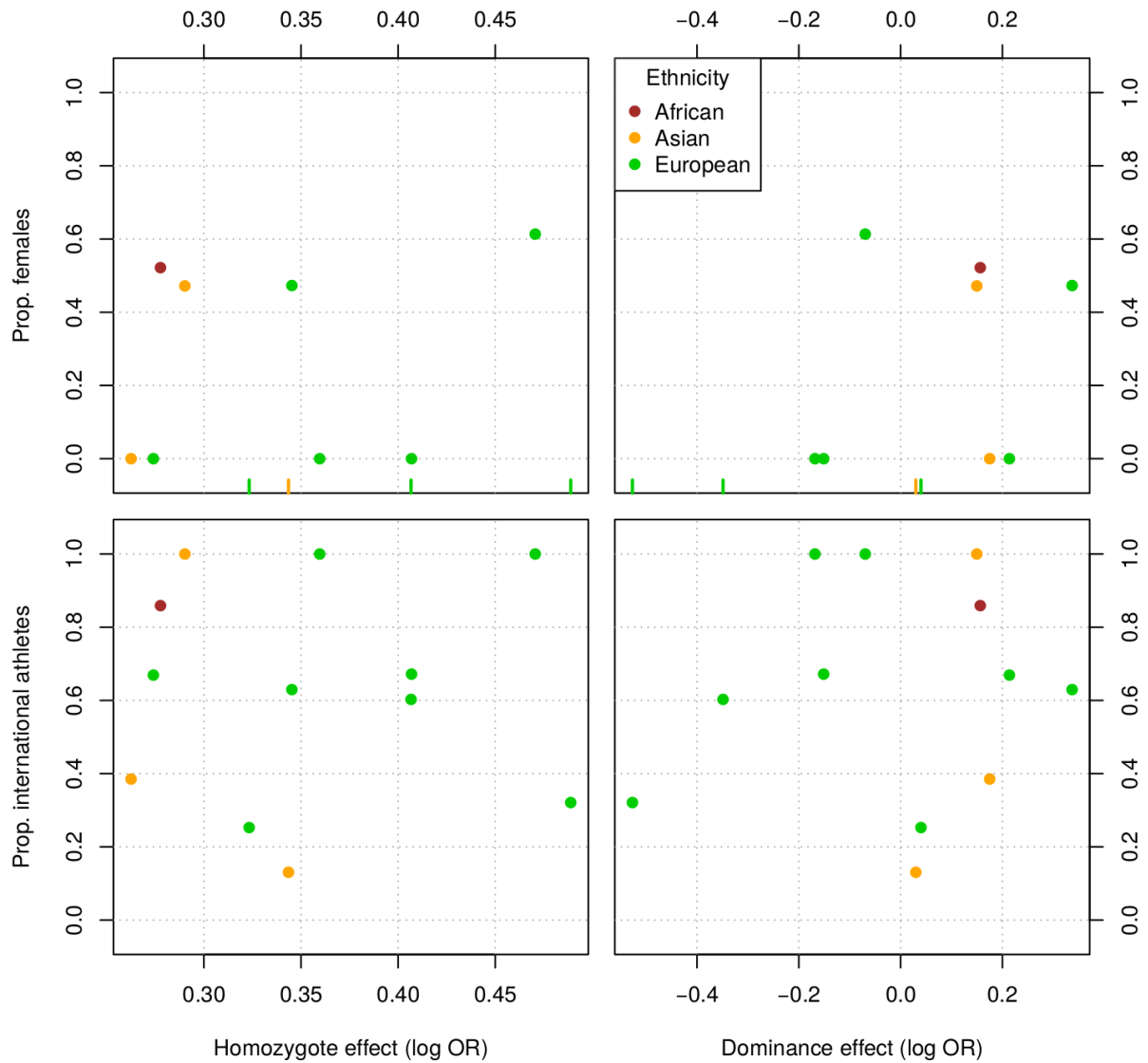
Figure S4. Comparison of genetic effects against potential covariates. Scatter plots of the estimated genetic effects from the general meta-analysis model (x-axis) compared against values of various attributes of each study (y-axis). Each point corresponds to a single study, coloured by the ethnicity of the individuals in that study according to the key provided. For some studies, the sex information of the athletes was not available; these studies are shown as dashes along the x-axes in the top row of plots. Studies with <50% of international athletes, had a subset of genotyping results that were used in the analyses (Table S1).
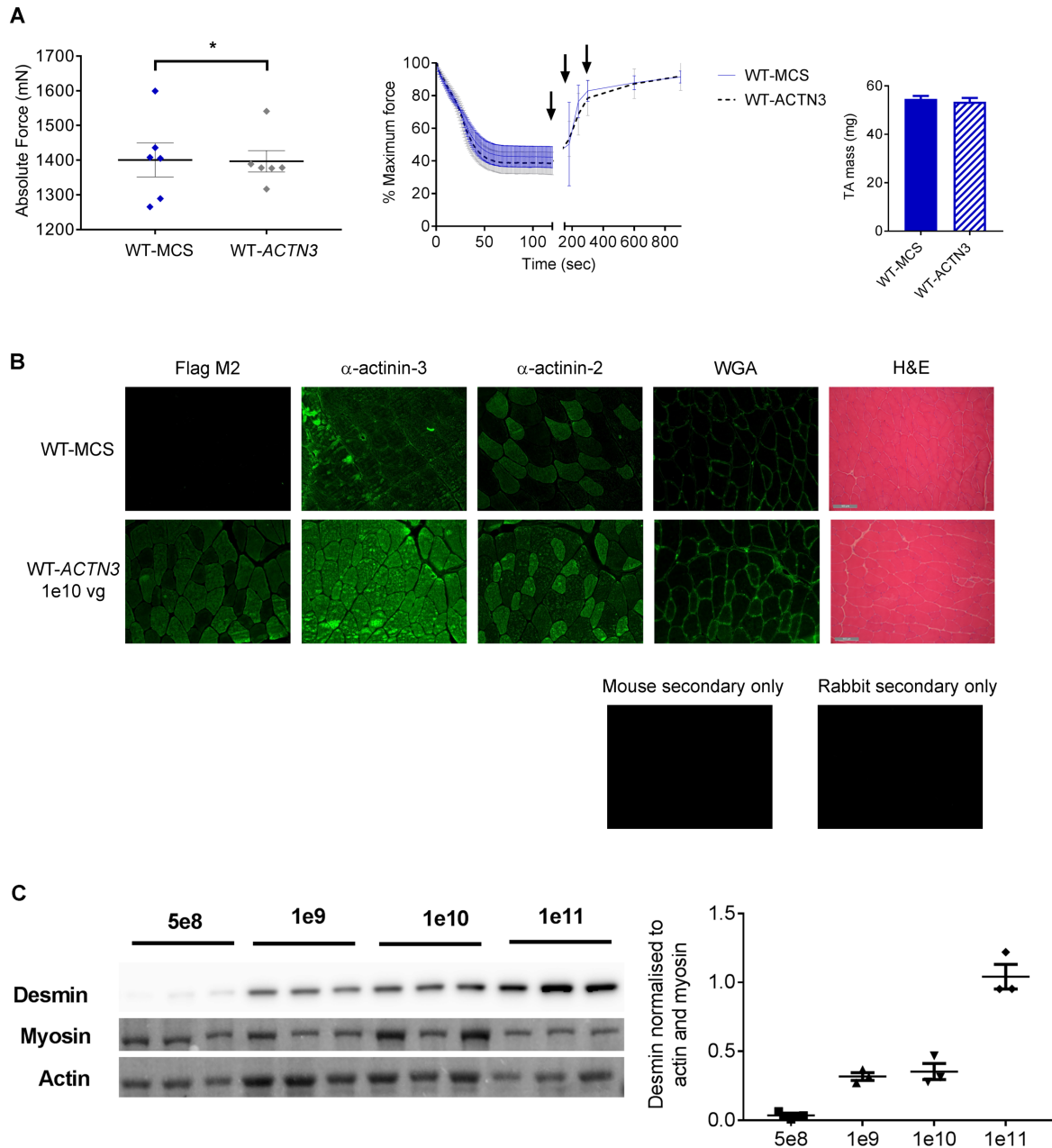
Figure S5. High (1E10 vg) expression of ACTN3 in WT muscle does not alter muscle force. A. rAAV-*ACTN3* at 1E10 vg in WT TA muscles did not enhance muscle mass, absolute force, or alter response to fatigue. B. There were no overt signs of muscle damage 6 weeks post injection. Immunostaining of WT-*ACTN3 (top panel)* and WT-MCS (bottom panel) muscle cross-sections. Positive Flag M2 in WT-*ACTN3* TA muscles, as well as increased α-actinin-3. α-Actinin-2, wheat germ agglutinin (WGA) and H&E shows normal muscle cytoarchitecture.

C. Desmin is increased in WT-*ACTN3* TA muscles 6 weeks post rAAV-*ACTN3* injection at
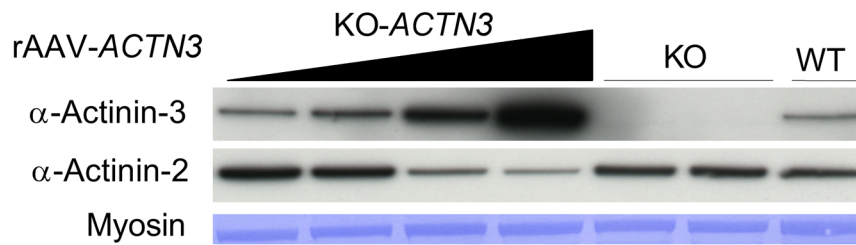
1E11 vg, consistent with increased muscle damage.

Figure S6. Expression of ACTN3 in KO muscle results in a reciprocal downregulation of

α-actinin-2. Increasing doses of rAAV-ACTN3 results in expression of α-actinin-3 in KO TA

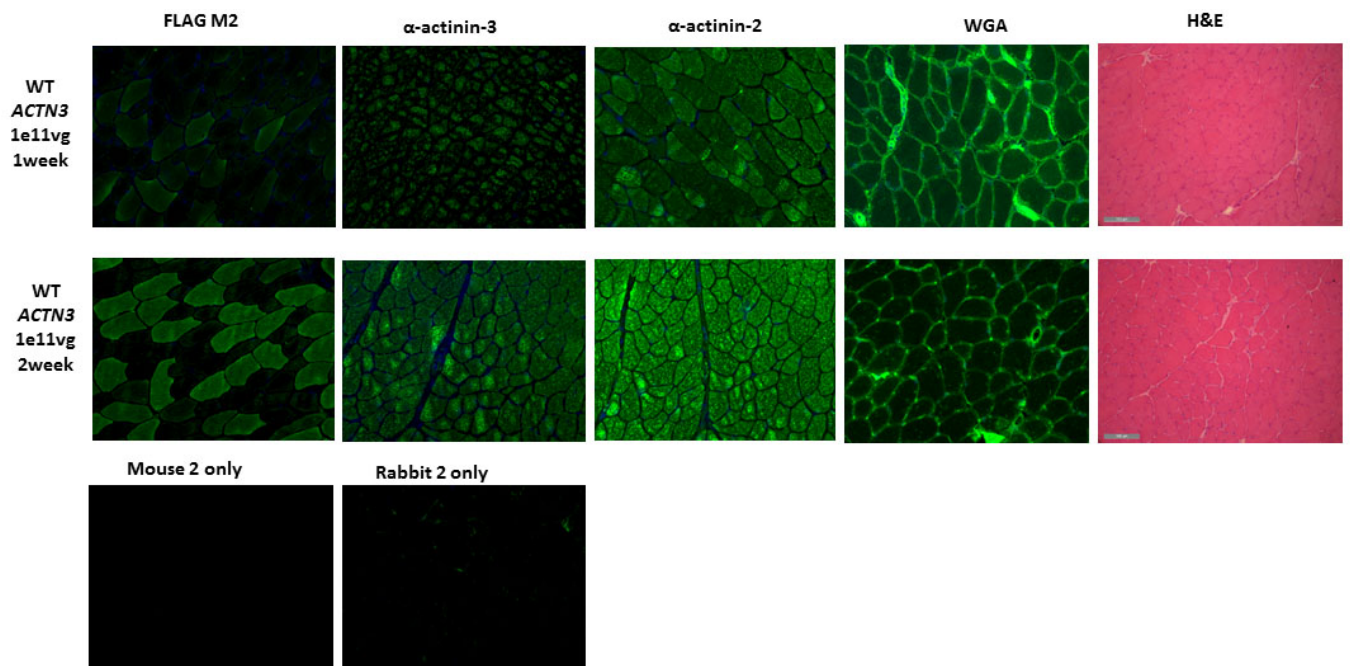muscles and reciprocal downregulation of α-actinin-2.

Figure S7. Initial muscle damage is not seen at 1-2 weeks with high over-expression of α-actinin-3. Intramuscular injection of rAAV-*ACTN3* at 1E11 vg does not cause in muscle damage at 1 and 2 weeks post injection. Immunostaining of WT-*ACTN3* muscle cross-section from left to right showing positive Flag M2, α-actinin-3, α-actinin-2, wheat germ agglutinin (WGA), H&E, alongside secondary only controls (mouse and rabbit). Imaging demonstrates normal muscle cytoarchitecture at both the 1 week (top panel) and 2 week (bottom panel) timepoints.
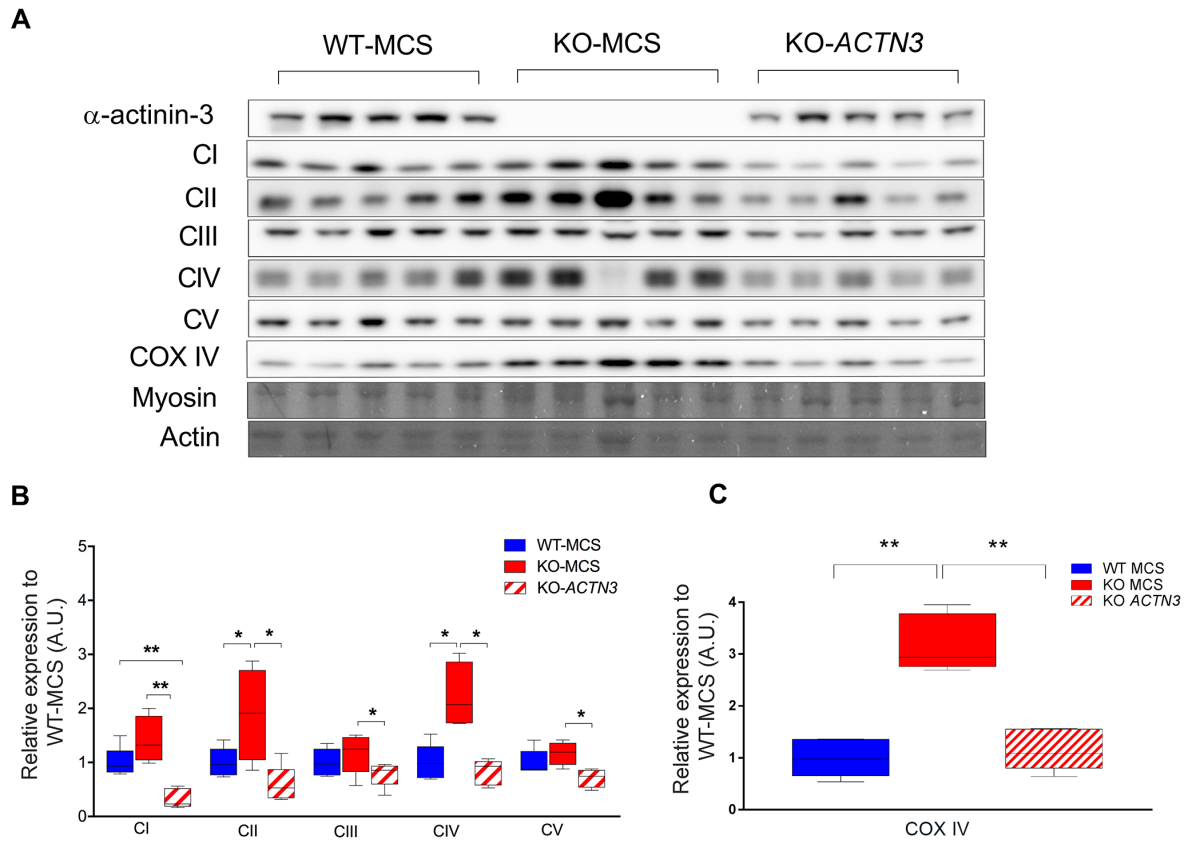
Figure S8. Post-natal replacement of α-actinin-3 in *Actn3* KO muscle results in a shift to an anaerobic metabolic profile. A-B) KO-*ACTN3* TA muscle demonstrated decreases in mitochondrial complexes I, II, III, IV and V to WT levels in response to α-actinin-3 expression (full blot for Figure 7) C) Rescued changes in KO-*ACTN3* were replicated using a different CIV antibody (COX IV, ab14744). *N*=5 for all groups. Box plots represent the median, whiskers; min-max. *$P< 0.05$, **$P<0.01$, ***$P< 0.001$.

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