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Supplemental Data

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

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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:

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

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

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

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

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

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^{o}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).

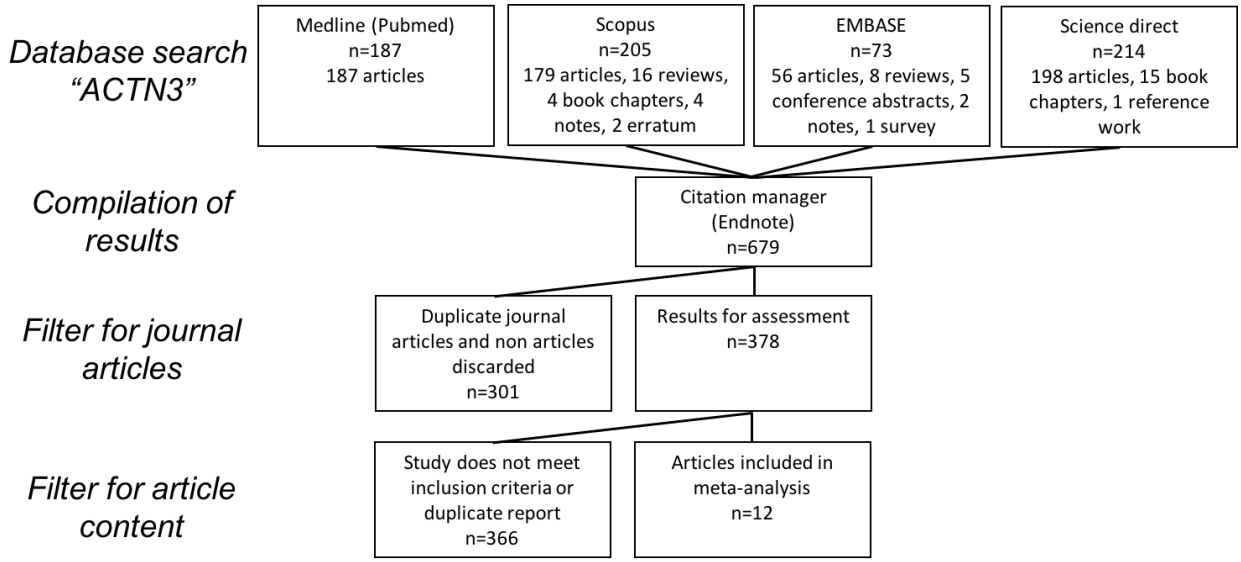

Figure S1. Search strategy for the Systematic Meta-analysis. Four databases were used to search for relevant articles using the search term "ACTN3." Results were compiled into a single citation manager and subsequently filtered for relevant journal articles, twelve of which were taken forward into the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

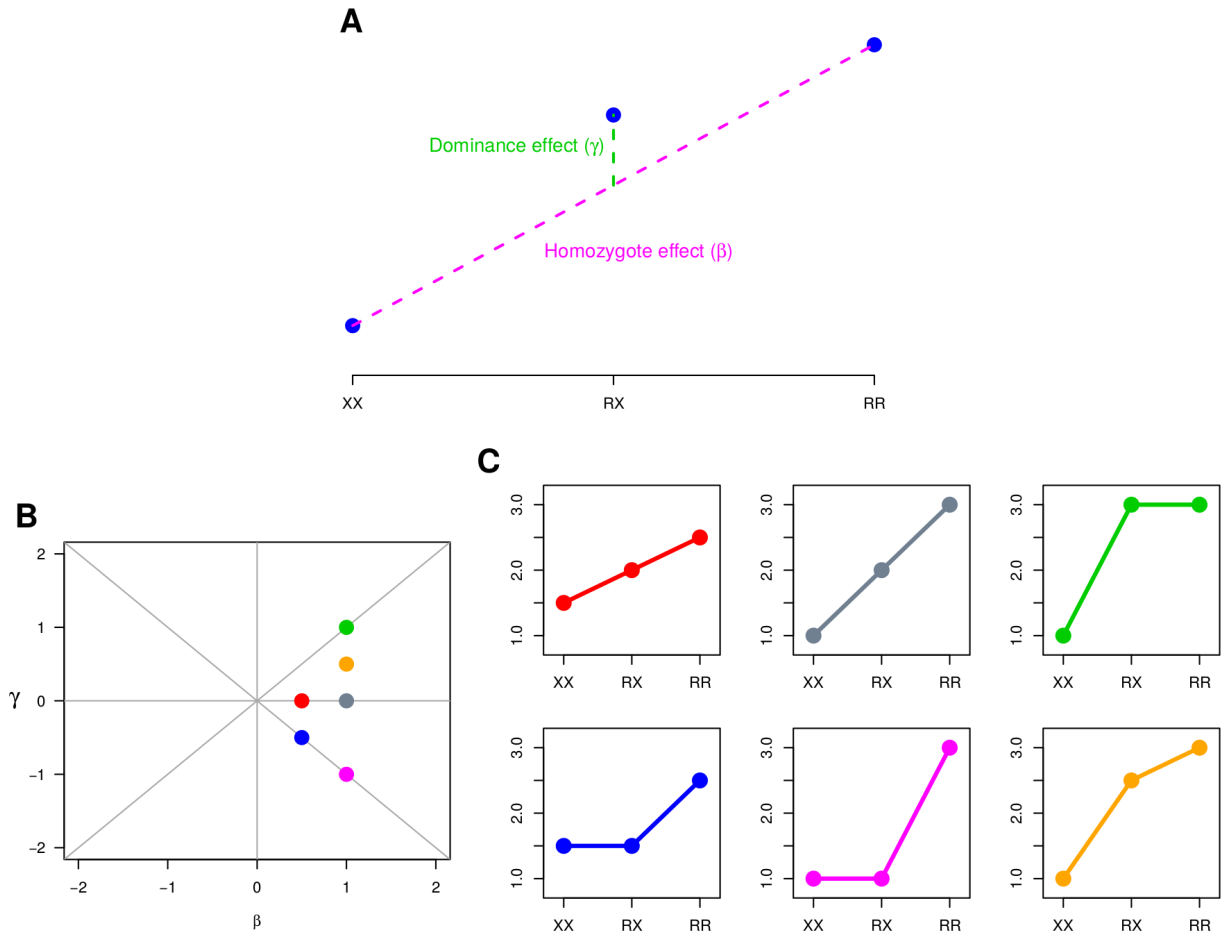

Figure S2. Explanation of the general genetic model.

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.

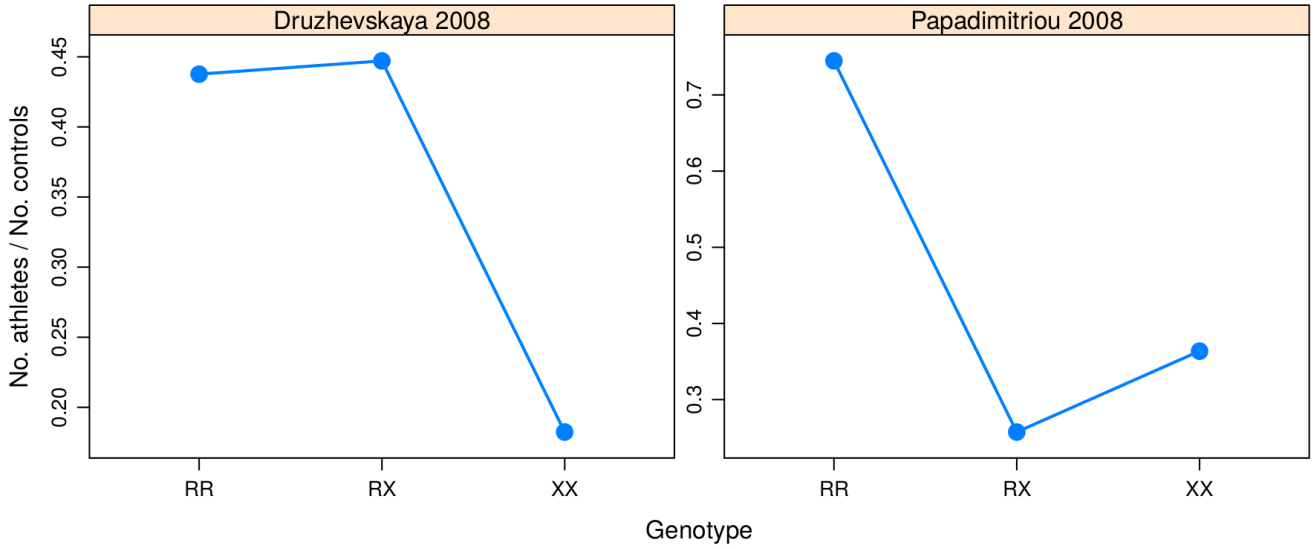

Figure S3. Examples of diverse genetic effects. The ratio of athletes to controls for each genotype for two of the studies included in the meta-analysis. These were chosen to illustrate the diversity in the observed genetic effects: the data from e.g. Druzhevskaya et al. [16] are consistent with a recessive model for allele X, whereas those from Papadimitriou et al. [13] are more consistent with a dominant model.

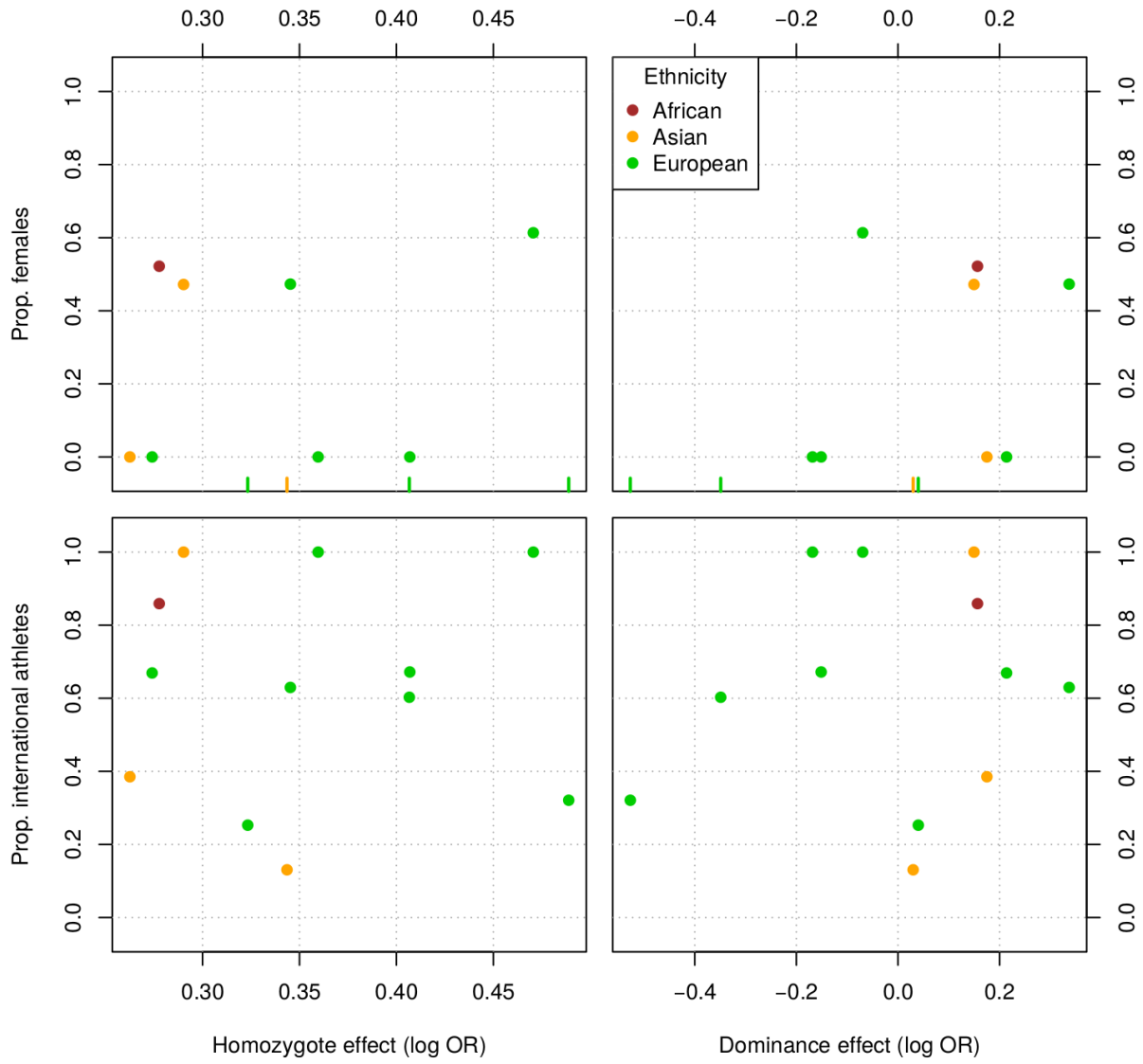

Figure S4. Comparison of genetic effects against potential covariates. Scatter plots of the estimated genetic effects from the general meta-analysis model (x-axis) compared against values of various attributes of each study (y-axis). Each point corresponds to a single study, coloured by the ethnicity 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).

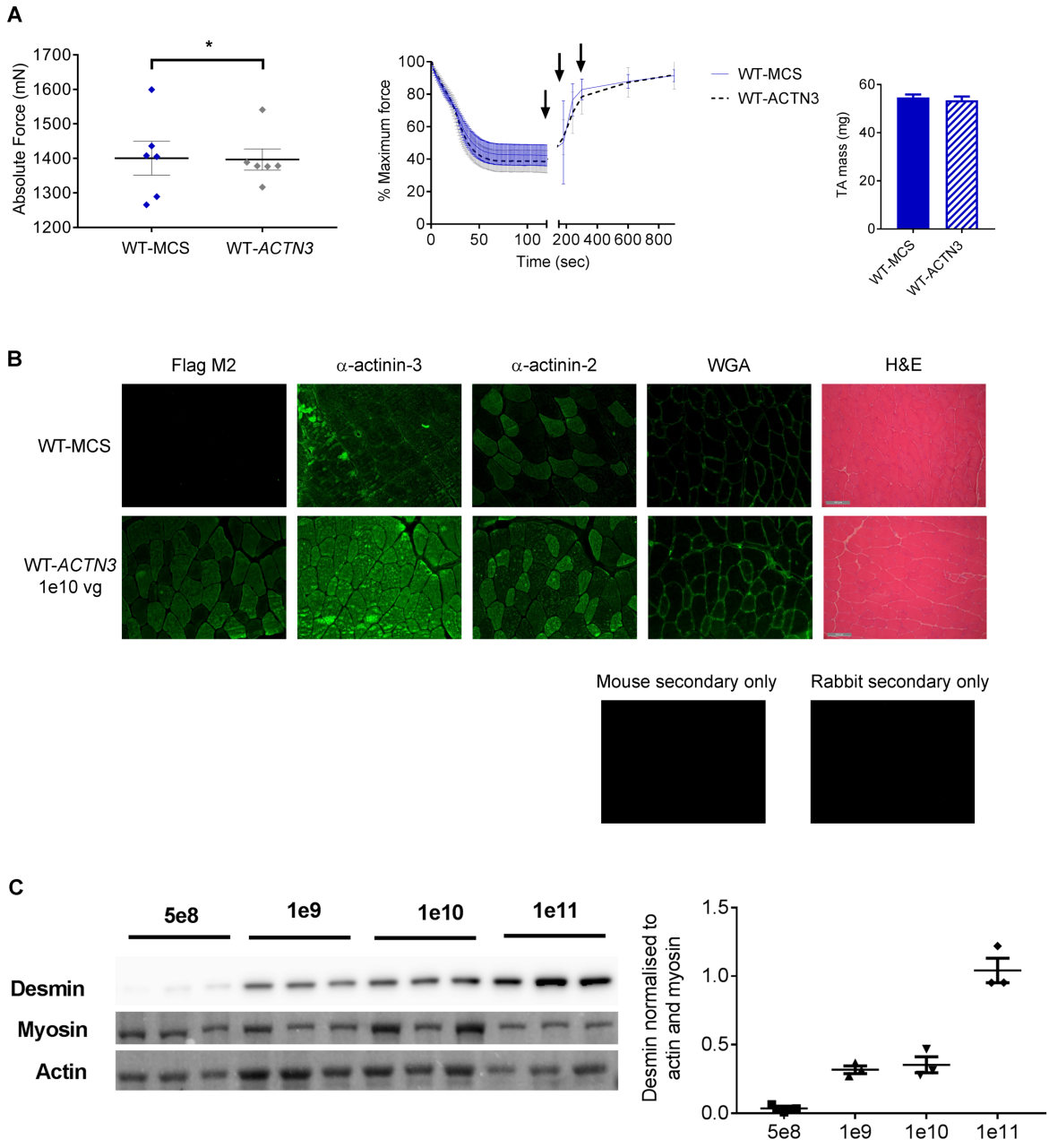

Figure S5. High (1E10 vg) expression of ACTN3 in WT muscle does not alter muscle force. A. rAAV-*ACTN3* at 1E10 vg in WT TA muscles did not enhance muscle mass, absolute force, or alter response to fatigue. B. There were no overt signs of muscle damage 6 weeks post injection. Immunostaining of WT-*ACTN3 (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.

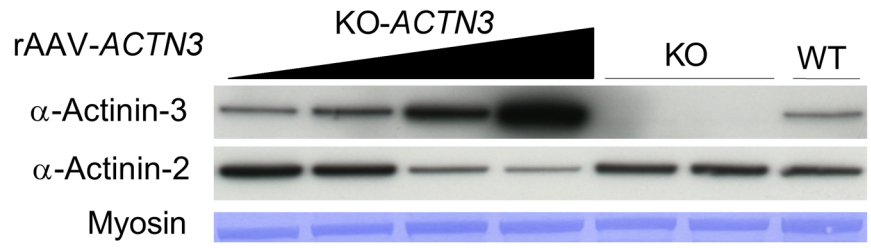

Figure S6. Expression of ACTN3 in KO muscle results in a reciprocal downregulation of α-actinin-2. Increasing doses of rAAV-ACTN3 results in expression of α-actinin-3 in KO TA muscles and reciprocal downregulation of α-actinin-2.

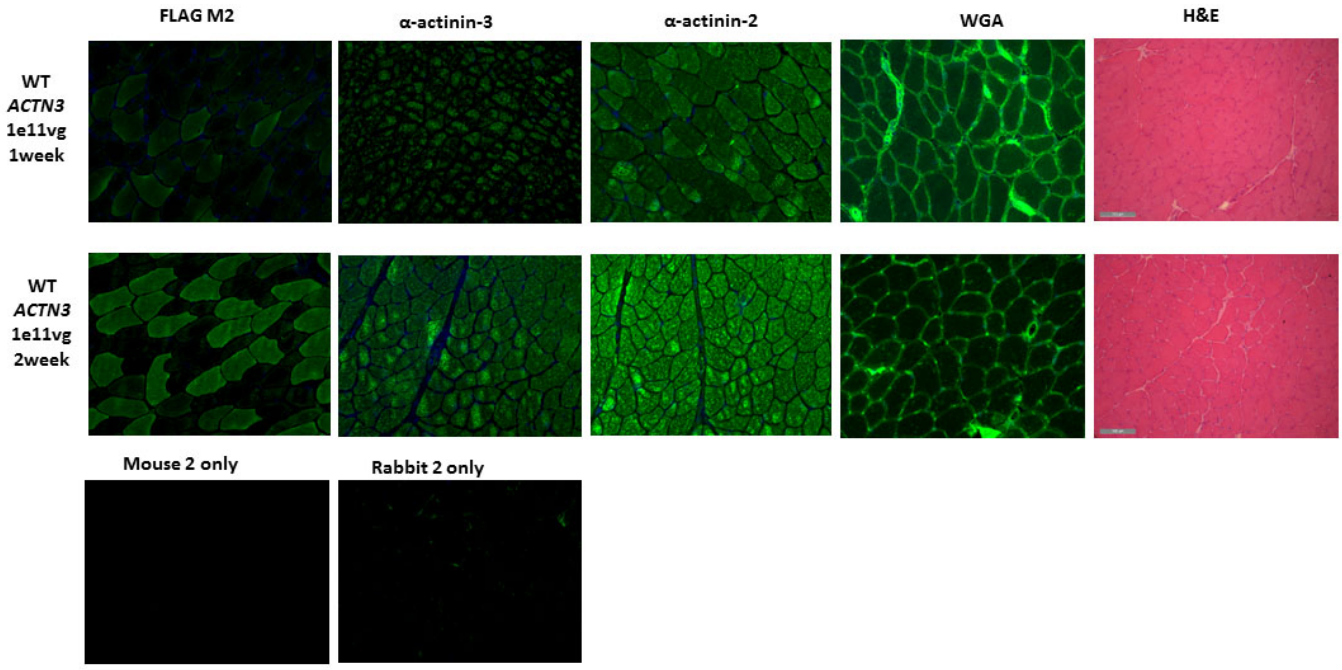

Figure S7. Initial muscle damage is not seen at 1-2 weeks with high over-expression of α-actinin-3. Intramuscular injection of rAAV-*ACTN3* at 1E11 vg does not cause in muscle damage at 1 and 2 weeks post injection. Immunostaining of WT-*ACTN3* muscle cross-section from left to right showing positive 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.

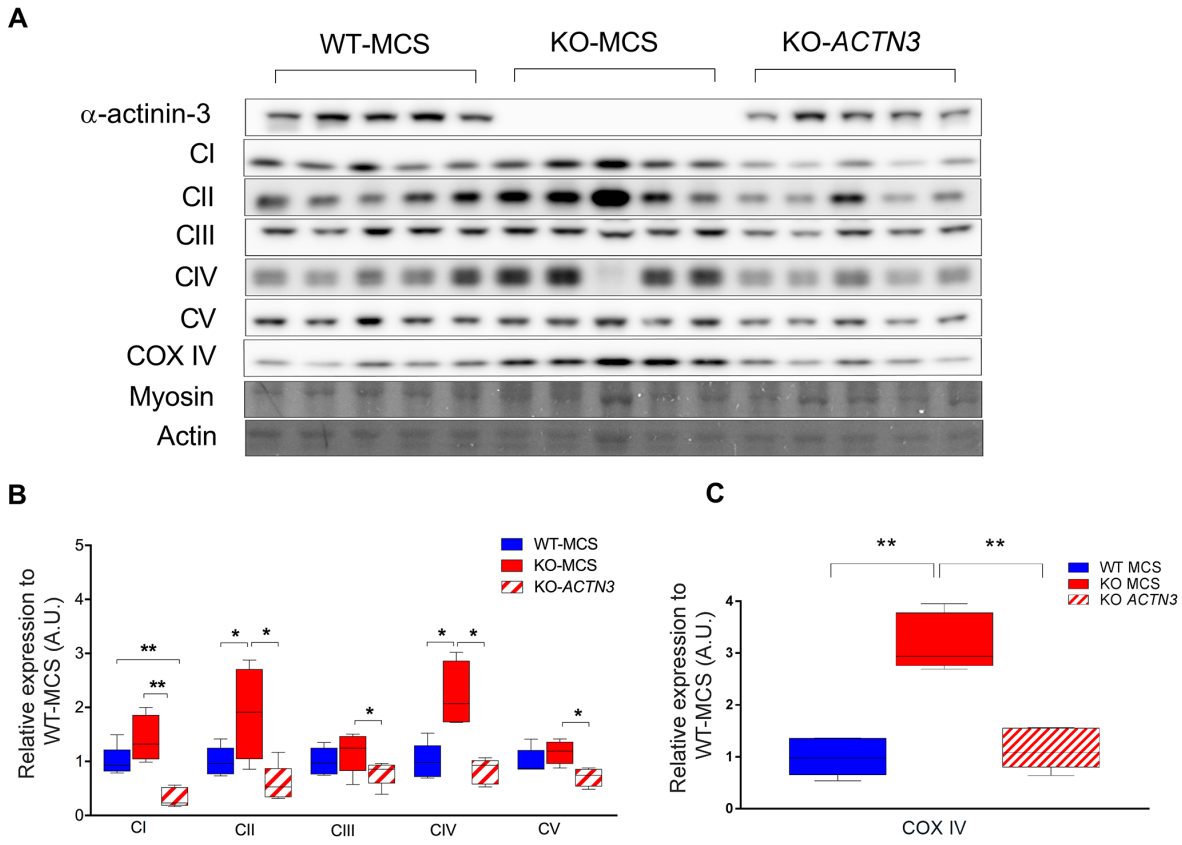

Figure S8. Post-natal replacement of α-actinin-3 in *Actn3* KO muscle results in a shift to an anaerobic metabolic profile. A-B) KO-*ACTN3* TA muscle demonstrated decreases in mitochondrial complexes I, II, III, IV and V to WT levels in response to α-actinin-3 expression (full blot for Figure 7) C) Rescued 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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