

ACTN3 genotype is associated with increases in muscle strength in response to resistance training in women

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quantitative trait loci; muscle genetics; alpha-actinin

α -ACTININS COMPRISE A family of actin-binding proteins that are important in binding and anchoring actin filaments (13, 14).

Four genes for α -actinin are found in humans: ACTN1, ACTN2, ACTN3, and ACTN4. ACTN1 and ACTN4 are non-muscle proteins (10), whereas ACTN2 and ACTN3 are myofibrillar proteins localized at the Z disk. ACTN2 and ACTN3 are highly conserved through evolution, and ACTN3 is a fast-twitch-specific isoform, expressed only in type II myofibers (2, 13, 15). A common polymorphism of ACTN3 was identified in humans and results in a stop codon and lack of detectable protein in homozygous individuals [C \rightarrow T transversion at position 1,747 in exon 16, converting an arginine to a stop codon at residue 577 (R577X)] (14). The relative allele frequency of the 577X allele has been found to differ between populations, ranging from 0.16 in Africans to 0.52 in Asian Americans, with Caucasians having an allele frequency of 0.42 (13). Homozygotes for the 577X allele are unable to produce any ACTN3 protein in their muscle, and it is estimated that ~18% of the population are homozygous for this loss-of-function polymorphism (13).

The ACTN3 homozygous null genotype does not cause any discernible phenotype or muscle histological changes, suggesting that the presence of the protein is not critical to the function of myofibers. Consistent with this, the ACTN2 gene is expressed in both type I and type II myofibers, and it is thought that the ACTN2 and ACTN3 proteins are functionally redundant, where ACTN2 compensates for loss of ACTN3 in type II myofibers in 577X homozygotes. To the contrary, the ACTN3 gene is highly conserved through evolution, suggesting that ACTN3 is indeed functionally important to muscle structure and function (13). These observations led to the hypothesis that ACTN3 genotype may influence variation in muscle function in humans [quantitative trait locus (QTL)].

To test whether the ACTN3 genotype was associated with variation in muscle function in humans, Yang et al. (28) examined ACTN3 genotypes in 429 elite Australian athletes training at the Australian Institute of Sport and in 436 healthy Caucasian controls. Sprint athletes included 46 track athletes

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competing in events ≤ 800 m, 42 swimmers competing in events ≤ 200 m, 9 judo athletes, 7 short-distance track cyclists, and 3 speed skaters. Endurance athletes included 77 long-distance cyclists, 77 rowers, 18 swimmers competing over distances of ≥ 400 m, 15 track athletes competing in events $\geq 5,000$ m, and 7 cross-country skiers. Of the 194 male and 107 female athletes studied, 72 men and 35 women were classified as sprint athletes, and 122 men and 72 women were classified as endurance athletes. Comparisons by χ^2 analysis showed significant differences in allele frequencies of ACTN3 between sprint athletes and controls for both men and women ($P < 0.01$). Sixteen percent of male controls but only 8% of sprint athletes were homozygous for the ACTN3 577X genotype. For women, 20% of the controls but no sprint athletes were homozygous for the ACTN3 577X allele. From these data, it appeared that the presence of the ACTN3 protein in type II myofibers was associated with greater success in activities requiring sprint or power performance.

In contrast to the findings for power athletes, the data from the Australian cohort showed that the frequency of the ACTN3 577X allele was overrepresented in endurance athletes (28). Twenty percent of male endurance athletes vs. 16% of controls and 29% of female endurance athletes vs. 20% of controls in the Australian cohort were homozygous for the ACTN3 577X allele (28). These data appear to indicate that a lack of the ACTN3 protein was beneficial to the endurance athletes tested (28) or at least was not deleterious to endurance performance. Yang et al. (28) suggested that the 577X allele may be maintained in the population because it confers a selective advantage under different environmental conditions. This study was critical for defining ACTN3 genotype as a QTL for muscle function in humans. However, it is important to extend these data to other populations for the following reasons. First, athletes in this study represented a range of broadly categorized sport activities and thus did not have a “quantifiable phenotype” on which to judge the specific and quantitative effect of ACTN3 genotype. Second, the elite athletes tested were a highly selected group of individuals and were likely to differ with regard to many environmental and genetic factors relative to the population control group (Australians) to which they were compared. For example, given that the allele frequency of ACTN3 has been shown to differ between ethnic groups, it is difficult to control for ethnic differences in allele frequencies between the elite athletes studied and the control population to which they were compared. Moreover, there is a need to extend cross-sectional studies regarding the influence of gene variation at the R577X locus to training effects in longitudinal studies.

On the basis of the relationship of ACTN3 to exercise performance (28) and the known physiological function of ACTN3 in muscle fibers, we expected that the R577X genotype would be associated with baseline muscle strength and muscle cross-sectional area and the response of these variables to 12 wk of resistance training. Specifically, we hypothesized that the ACTN3 null mutation homozygotes (XX) would have lower baseline muscle strength compared with the wild-type homozygotes (RR). We also hypothesized that the XX genotype would have less strength gain and muscle mass gain in response to resistance training compared with the RR genotype. This study extends the previous Australian elite athlete genotype association studies (28) to a large population of normal volunteers. We used standardized methods to quantify

upper arm muscle size and strength phenotypes and the response of these phenotypes to 12 wk of unilateral progressive resistance training (25). The opportunity to quantify phenotypes with a continuous outcome measurement allowed us to use more powerful statistical methods to compare actual mean outcome values among ACTN3 genotypes rather than simply comparing genotype frequencies. These methods also enabled us to control for potentially confounding factors, such as age and body mass. Lastly, the continuous nature of the outcome phenotype allowed us to use multivariate linear regression models to estimate the amount of variation in outcomes attributable to ACTN3 genotype.

We report the association of ACTN3 genotypes to the strength and muscle size phenotypes of 602 subjects. When significant associations were found within the entire cohort in one sex, we then studied validation in ethnic subsets and quantitated the relative contribution of ACTN3 genotype to the observed total variation. We report significant associations of ACTN3 with muscle phenotypes in women and find that ACTN3 explains $\sim 2\%$ of all variation within the associated phenotypes.

METHODS

Study overview. This study is part of the Functional Polymorphisms Associated with Human Muscle Size and Strength (FAMuSS) study, a large multi-institutional cooperative effort designed to identify nonsynonymous single nucleotide polymorphisms with functional effects on muscle mass and strength in humans (25). The muscle phenotypes examined were baseline elbow flexor muscle size and strength and the response of these same phenotypes to 12 wk of progressive resistance exercise training (25). The study protocol and informed consent documents were approved by the appropriate institutional review board at each site.

The experimental design of this parent study has been described (25). In brief, after informed consent was obtained from all individuals, isometric [maximal voluntary contraction (MVC)] and dynamic strength [one repetition maximum (1 RM)] of the forearm flexors and cross-sectional area (CSA) of the upper arm musculature were measured before and after 12 wk of forearm flexor and extensor resistance training in 355 women and 247 men aged 18 to 40 yr. To ensure that the investigators were trained, all investigators received on-site training at least once per year, and an instructional video describing the procedure in detail was made for all sites to follow.

Study population, exercise training, and testing (strength, size). Men and women were excluded if they used medications known to affect skeletal muscle such as corticosteroids; had any restriction of activity; had chronic medical conditions such as diabetes; had metal implants in their body that would prohibit MRI testing; had performed strength training or employment requiring repetitive use of the arms within the prior 12 mo; consumed on average >2 alcoholic drinks daily; or had used dietary supplements reported to build muscle size and strength or to cause body mass gain such as protein supplements, creatine, or androgenic precursors, and those who were restricting calories or dieting.

Isometric elbow flexion strength testing. MVC of the elbow flexor muscles of each arm was determined before and after 12 wk of strength training by use of a specially constructed, modified preacher bench and strain gauge (model 32628CTL, Lafayette Instruments, Lafayette, IN). Baseline measures of MVC strength were assessed on 3 separate days spaced no more than 48 h apart (25). The average of the results obtained on the second and third testing days was used as the baseline criterion measurement. Three maximal contractions were assessed. Each contraction lasted 3 s, and 1 min was allowed between

contractions. An average of the peak force produced during the three contractions was used as the criterion score.

1-RM biceps strength testing. The dynamic strength of the elbow flexor muscles of each arm was assessed by determining the 1-RM test on the standard preacher curl exercise (25). The 1-RM test protocol modified from Baechle et al. (1) was used, and investigators were carefully trained to carry out the test. At each site, one experienced investigator typically supervised all 1-RM tests both before and after training.

1 RM was assessed once at baseline for each arm and repeated after 12 wk of training. Details on subject position were recorded at baseline to ensure proper position during posttraining testing. Each subject performed two warm-up sets with increasing weight. During the test, subjects were instructed to go through a full range of motion starting from 180° to full flexion. Care was taken to ensure that the subject completed the full range of motion. After warm-up, weights were increased, and each subject attempted to perform one full contraction. If the subject successfully completed one contraction, weights were raised slightly, and the subject again attempted to complete one repetition. Weights were chosen so that the 1 RM could be determined in three to five attempts, although more attempts were completed when necessary. Further details can be found in Thompson et al. (25).

Measurement of muscle CSA. Magnetic resonance imaging (MRI) was performed before and after exercise training to assess changes in the biceps brachii CSA (25). Posttraining MRI was performed 48–96 h after the final training session, ensuring that temporary exercise effects, such as water shifts, were avoided, while also avoiding any reduction of muscle size from detraining. Before MRI, the point of measure of both the nondominant and dominant arms was determined in the following manner: subjects stood with shoulder abducted at 90°, palm supinated and open, with the elbow flexed at 90°, and were instructed to flex the biceps. Maximum biceps circumference or the point of measure was visually determined, the skin was marked, and the top of a radiographic bead (Beekley Spots, Beekley, Bristol, CT) was aligned and placed on the marked skin to standardize pre- and posttraining MRI localization. To standardize MRI scaling, the bead's measured CSA was compared with that of the MRI-determined CSA. The MRI involved imaging a 24-cm length of the upper arm beginning at the superior portion of the arm and proceeding distally toward the elbow joint. With the maximum biceps circumference as the point of measure, 15 spoiled gradient images were generated (echo time = 1.9 s, repetition time = 200 ms, flow artifact suppression, 30° flip angle). Each individual image slice was 16 mm thick with a 0-mm interslice gap, 256 × 192 matrix resolution, 22 cm × 22 cm field of view, number of experiments = 6. All investigational sites submitted MRI data to the central imaging facility at Hartford Hospital via Magneto Optical Disk or CD-ROM for further analysis. The same investigator analyzed magnetic resonance (MR) images using a custom-designed program created to function within MATLAB (The MathWorks, Natick, MA). To address the potential for training effect on the location of the landmark to analyze pre- vs. posttraining images, the analyst first located the landmark and then observed the anatomy displayed in the image. If the anatomy matched (i.e., the observer could identify similar anatomical landmarks pre- vs. posttraining), the analysis proceeded. If the analyst observed differing anatomy at the landmarked slices (pre- vs. posttraining), the analyst moved to the adjacent slices to look for an anatomical match. For the vast majority of subjects, the ninth axial slice was measured for maximum biceps CSA. Intraobserver reliability for the entire process of image acquisition and analysis of biceps CSA was $r = 0.99$. Interobserver variability for upper arm CSA was $\pm 3.5\%$. MR image analysis was blinded for which arm was trained (i.e., which arm was nondominant and which was dominant); however, it was primarily unblinded for baseline vs. posttraining. A small percentage of the MRI data was blinded and reanalyzed (~50 subjects), demonstrating no

significant difference between the unblinded analysis and the blinded analysis.

Exercise training program. Subjects underwent gradually progressive, supervised strength training of their nondominant arm at one of the collaborating facilities. Exercises were performed with the nondominant arm only. The exercises consisted of the biceps preacher curl, biceps concentration curl, standing biceps curl, overhead triceps extension, and triceps kickback. For every contraction, both the concentric and eccentric phases were emphasized such that the motion was timed and controlled at 2 s up (concentric) and 2 s down (eccentric). Subjects were not allowed to “drop” the weight during the eccentric phase of the movement. All training sessions were supervised and lasted ~45–60 min. The exercise progression used the following weekly training protocol: *weeks 1–4*: 3 sets with 12 repetitions of the 12-repetition maximum weight; *weeks 5–9*: 3 sets with 8 repetitions of the 8-repetition maximum weight; *weeks 10–12*: 3 sets with 6 repetitions of the 6-repetition maximum weight. The primary interest was to train the elbow flexors, but we also trained the elbow extensors to balance muscle strength across the joint.

Dietary control procedures. Subjects were instructed to maintain their habitual dietary intake over the course of the study so that significant body mass loss or gain was avoided. Individuals who had supplemented their diet with additional protein or taken any dietary supplement reported to build muscle or to cause body mass gain (dietary supplements containing protein, creatine, or androgenic precursors) were not included.

Standardization between sites. Adaptations to resistance training are highly specific to the training protocol. To control for any difference among training sites, each site used an identical training protocol and identical exercise equipment purchased from the same manufacturers. The techniques for MRI, strength and anthropometrical measurements, and exercise training were videotaped, and research personnel from each study site reviewed the videotape before the start of each training group. In addition, meetings were held several times per year among group members to maximize compliance to the standard protocol, including some hands-on training sessions.

Genotyping. Blood samples were obtained from all individuals in EDTA anti-coagulant and sent to the coordinating site in Washington, DC without any subject identification, and DNA was isolated by use of Qiagen kits. Genotyping was done by using a novel TaqMan allele discrimination assay that employs the 5' nuclease activity of Taq polymerase to detect a fluorescent reporter signal generated during PCR reactions. Both alleles were detected simultaneously by using allele-specific oligonucleotides labeled with different fluorophores, and genotypes were determined by the ratio of the two fluorophores used. The allele-specific PCR reactions for the ACTN3 R577X variant contained 20 ng DNA, 900 nM PCR primers (forward: 5'-ACGAT-CAGTTCAAGGCAACACT-3' and reverse: 5'-ACCTGGATGCCATGATG-3'), 200-nM fluorescent allele discrimination probes (R allele: FAM-5'-CGCTCTCGGTCAGC-3'; X allele: VIC-5'-TCGCTCTCAGTCAGC-3'), and TaqMan Universal PCR master mix, No AmpErase UNG (Applied Biosystems, Foster City, CA) in a final volume of 25 μ l. The PCR was done using 10 min at 95°C (denaturation) and 44 cycles of 15 s at 92°C and 1 min at an annealing temperature of 60°C. Reactions were set up using a MWG robot, and fluorescence ratios and allele calling were done using an Applied Biosystems (ABI) 7700.

To validate the accuracy of our TaqMan assay for the ACTN3 R577X locus, we genotyped 96 individuals using both the TaqMan assay and the previously reported restriction enzyme assay. The restriction enzyme assay has been previously validated through DNA sequencing (23). Briefly, a 489-bp fragment of ACTN3, consisting of exons 15 and 16, was amplified by PCR using primers ACTN3-E15F (5'-CGCCCTTCAACAACACTGGCTGGA-3') and ACTN3-E16R (5'-GGGTGATGTAGGGATTGGTGGAG-3'). PCR was performed by 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 3 min, and a final extension step of 7 min

at 72°C. The amplified fragment subsequently underwent digestion by *DdeI* (New England Biolabs, Beverly, MA) in a condition recommended by the supplier. The digested products were then electrophoresed in a 3% agarose gel. We found 100% concordance between genotypes detected by the TaqMan assay compared with the previously validated *DdeI* enzyme assay.

Statistical analysis. Individuals who dropped out of the study were not included in any analyses. Subjects who completed the study but had missing data fields were included in the statistical analyses. The number of individuals tested for each of the nine variables ranged from 551 to 602. The physical characteristics of the subjects are presented in Table 1.

Hardy-Weinberg equilibrium was determined for ACTN3 by using a χ^2 test to compare the observed genotype frequencies to those expected under Hardy-Weinberg equilibrium.

Nine muscle size and strength measurements were analyzed as continuous quantitative traits (baseline measures of biceps CSA, 1-RM strength, and MVC strength; difference in biceps CSA, 1-RM strength, and MVC from baseline to postexercise; and the percent change in biceps CSA, 1-RM strength, and MVC from baseline to postexercise). Normality of each quantitative trait was confirmed by the Shapiro-Wilk normality test.

Bivariate correlation analyses of each quantitative measurement showed several significant correlations with age, baseline mass, and biceps brachii CSA; therefore, associations between ACTN3 and muscle size and strength measurements were assessed by analysis of covariance (ANCOVA) methods. Because of large gender differences in baseline values and the response to training, all analyses were performed separately for men and women, and all ANCOVAs included either age and baseline body mass or age and CSA as covariates.

All significant associations from the main ANCOVA model were subjected to pairwise statistical tests among each of the three genotype groups (RR, RX, XX). Linear tests were performed between each of the genotype groups to determine which genotype groups were significantly different from one another. The resulting *P* values from these linear tests were adjusted for multiple comparisons by using the Sidak post hoc multiple-comparison test. Any trait showing statistical significance across the entire male or female cohort was then stratified with statistical analyses on individual ethnic groups (African Americans, Asians, Caucasians, Hispanics, and Others). Linear regression analysis, including likelihood ratio tests between full (containing

genotype, age, and weight) and constrained (containing age and weight only) models, were performed to estimate the proportion of variance in muscle size and strength measurements attributable to ACTN3 genotype.

RESULTS

Measures of muscle size, strength, and response to training in the FAMuSS cohort. The strength and training variables were baseline strength (MVC and 1 RM) and upper arm size (muscle CSA), and the response of these same variables after 12 wk of supervised progressive resistance training (see Ref. 25). Training effects were expressed as both an absolute effect and a relative effect (percentage increase relative to baseline MVC or 1 RM).

Tests for accuracy of measurements and variation within the studied population will be published in more detail in other reports. Briefly, the measurement errors associated with the phenotypic variables were as follows. For biceps muscle size, the error was 3–4%, calculated as the mean interinvestigator difference in CSA from MRI records of 50 subjects randomly selected and measured independently by two experienced investigators. For MVC strength, the error was $7.4 \pm 6.0\%$, determined as the mean within-subject coefficient of variation over 3 separate days of pretraining testing for all subjects. Repeated baseline tests were not performed for dynamic strength.

Within all subjects, muscle size changes ranged from -2% to $+59\%$ (-0.4 to $+13.6$ cm²), dynamic strength (1 RM) gains ranged from 0% to $+250\%$ (0 to $+10.2$ kg), and MVC strength changes ranged from -32% to $+149\%$ (-15.9 to $+52.6$ kg). It is interesting to note that, although most subjects increased muscle size and strength, some did not. We are at a loss to explain the sizable loss in strength for one subject (-15.9 kg); however, this subject also lost 10% CSA in the untrained arm and 2.2 kg in total body weight. Coefficients of variation for absolute change from baseline were determined to be 0.48 and 0.51 for CSA ($P = 0.44$), 1.07 and 0.89 ($P < 0.01$) for MVC, and 0.55 and 0.59 ($P < 0.01$) for 1 RM in men and women, respectively. Gender differences in variation were assessed with Levene's test for homogeneity of the variances. Men experienced $\sim 2.5\%$ greater gains for CSA ($P < 0.01$) compared with women. Despite greater absolute gains in men, relative increases in both strength measures were greater in women vs. men ($P < 0.05$); this was attributable to the significantly lower baseline strength in women. Men demonstrated significantly larger baseline biceps muscle size, higher baseline 1 RM and MVC strength, and greater absolute gains in muscle size and strength compared with women ($P < 0.01$). These data showed that women gain significantly more relative isometric strength (MVC) than men (22 vs. 16%) and significantly more relative dynamic 1-RM strength than men (64 vs. 40%) with resistance training. These increases for dynamic strength are similar to those found by Cureton et al. (5) (36.2% for men and 59.2% for women). Also, O'Hagan et al. (16) found a 116% increase in dynamic strength for women and 46% for men, the greater increases possibly reflecting that the subjects trained three times per week for 20 wk with near-maximal contractions.

We tested the complete data set for evidence of covariants with muscle strength and response to training. Height, biceps brachii CSA, body mass, and age were found to be significant covariants in both men and women, although height and body

Table 1. Physical characteristics and ACTN3 R577X genotypes of subjects

	Genotype		
	RR	RX	XX
Total group			
Age, yr	25.5 \pm 6.3	24.0 \pm 6.0*	24.3 \pm 5.5
Height, cm	169.4 \pm 9.1	169.9 \pm 9.5	169.7 \pm 9.2
Weight, kg	70.7 \pm 15.3	71.2 \pm 15.9	69.6 \pm 16.0
Baseline biceps CSA, cm ²	16.90 \pm 6.06	16.82 \pm 5.70	17.00 \pm 5.63
Men			
Age, yr	24.71 \pm 5.40	25.29 \pm 6.19	24.71 \pm 4.89
Height, cm	177.3 \pm 7.4	176.3 \pm 7.4	176.0 \pm 7.9
Weight, kg	78.7 \pm 15.4	78.6 \pm 16.3	76.9 \pm 17.1
Baseline biceps CSA, cm ²	21.79 \pm 6.26	21.06 \pm 5.72	21.71 \pm 5.18
Women			
Age, yr	25.94 \pm 6.71	22.96 \pm 5.58*	24.06 \pm 5.80
Height, cm	164.9 \pm 6.6	164.8 \pm 7.4	165.1 \pm 6.9
Weight, kg	65.5 \pm 12.9	65.5 \pm 13.0	64.9 \pm 13.3
Baseline biceps CSA, cm ²	13.77 \pm 3.21	13.74 \pm 3.13	13.81 \pm 3.14

Values are means \pm SD. CSA, cross-sectional area; RR, wild-type homozygote; RX, heterozygote; XX, ACTN3 577 mutant homozygote. *Significantly different mean ages between RX and RR genotypes ($P < 0.05$).

mass were highly correlated with each other. We therefore statistically adjusted all data with either age and body mass or with age and CSA as covariates.

Genotyping in the FAMuSS cohort. Genotyping for the ACTN3 R577X locus was performed and tested for Hardy-Weinberg equilibrium as a means of ensuring genotyping accuracy. ACTN3 was shown to be in Hardy-Weinberg equilibrium ($\chi^2 = 5.204$; $P = 0.074$). The genotype distributions of our Caucasian population ($N = 469$; $\chi^2 = 5.00$; $P = 0.082$), our Asian population ($N = 55$; $\chi^2 = 2.39$; $P = 0.30$), and our Hispanic population ($N = 25$; $\chi^2 = 0.27$; $P = 0.874$) (Table 2) did not significantly differ from a previously genotyped group of 107 white Europeans, 28 Asians, and 32 Hispanics (14). Our African-American population ($N = 28$; $\chi^2 = 16.86$; $P < 0.001$), however, did significantly differ from a previously reported population of 45 African-Americans (13).

To verify our genotyping procedures, we examined another ethnically diverse population of 96 individuals. In that independent population, we found no significant differences in genotype distributions for African-Americans ($N = 29$; $\chi^2 = 2.70$; $P = 0.32$), Asians ($N = 6$; $\chi^2 = 0.02$; $P = 0.98$), Caucasians ($N = 36$; $\chi^2 = 1.40$; $P = 0.50$), or Hispanics ($N = 25$; $\chi^2 = 2.47$; $P = 0.29$) compared with the FAMuSS cohort.

Associations of genotype with anthropomorphic variables are presented in Table 3. Genotype frequencies were similar for men and women when examined across the total population and when examined by age, height, and mass. The only exception was for age in the entire cohort where the RR

homozygote was significantly older than the heterozygote group, although this effect was small (Table 1).

Genetic associations of ACTN3 genotype with muscle strength and size. Body mass-to-age or CSA-to-age-adjusted data ratios for all subjects were then used and tested for significant associations with ACTN3 genotypes. Data were analyzed for men and women separately against the nine phenotypes. For any significant main effect in the ANCOVA analyses across the entire male or female cohort, we then tested for genotype effects by pairwise comparisons of each genotype group for the studied trait. When statistical significance was found between genotype groups, we then tested for validation in ethnic subgroups (Caucasian, Asian, and African-American).

For men, there was no significant difference among genotypes for any of the nine phenotypes across the entire cohort. For women, there were no significant differences among genotypes for muscle size, baseline 1 RM, or change in size and MVC strength in response to training. However, for women we found three phenotypes significantly associated with ACTN3 genotype: baseline MVC, absolute difference in strength (1 RM) after training, and percentage change in 1 RM after training (Table 3).

In concordance with our hypothesis, there was a significant association of the ACTN3 R577X genotype with baseline MVC strength. Results of pairwise comparisons between genotype groups showed that baseline MVC strength was significantly greater in the heterozygotes (RX) compared with the homozygous mutants (XX) in the women ($P < 0.05$). Homozygous wild-type individuals (RR) were not significantly different from either of the two other groups, but their mean baseline MVC strength was very near that of the heterozygotes. When the baseline MVC values were adjusted by baseline biceps CSA, RX was significantly greater than XX ($P < 0.01$), and RR was significantly greater than XX ($P < 0.05$). These data show that lower baseline MVC strength in women is associated with the XX genotype. We were able to validate this relationship within the Caucasian ethnic group, such that, when covaried for age and CSA, RX was significantly greater than XX ($P < 0.01$), and RR was significantly greater than XX ($P < 0.01$).

ACTN3 genotypes were also significantly associated with the 1-RM response to training. However, contrary to our initial hypothesis, the ACTN3 RR homozygotes showed smaller increases in relative and absolute 1 RM compared with the XX homozygotes ($P < 0.05$). When the analysis was stratified into ethnic groups, a greater increase in 1-RM performance of the XX over the RR was found in both Caucasian women ($P < 0.01$) and Asian women ($P < 0.05$). When adjusted for age and baseline biceps CSA, the association of absolute increase in 1 RM and genotype became more significant for all women combined ($P = 0.01$) and for the Caucasian ($P < 0.01$) and Asian ($P < 0.01$) women (data not shown). We then adjusted 1) the absolute increase in 1 RM with absolute change in CSA and 2) the relative increase in 1 RM with the relative change in CSA. For the adjusted absolute increase in 1 RM, the results were unchanged or more significant, suggesting that the absolute gains are independent of muscle size increase. For adjusted relative increase in 1 RM, the Asian group no longer showed a significant association (Table 3). Thus the relative 1-RM gains are independent of muscle size in Caucasians but not Asians. In general, there was a trend for a dosage effect with genotype

Table 2. ACTN3 R577X genotype distributions of the sample population

	Genotype			Allele Frequency	
	RR	RX	XX	R	X
All subjects ($N = 602$)	176 (29.3%)	262 (43.5%)	164 (27.2%)	0.511	0.489
Caucasians ($N = 469$)	128 (27.3%)	216 (46.1%)	125 (26.6%)	0.503	0.497
Asians ($N = 55$)	20 (36.4%)	18 (32.7%)	17 (30.9%)	0.527	0.473
Hispanics ($N = 25$)	5 (20.0%)	11 (44.0%)	9 (36.0%)	0.420	0.580
African-American ($N = 28$)	16 (57.1%)	6 (21.4%)	6 (21.4%)	0.679	0.321
All men ($N = 247$)	68 (27.5%)	114 (46.2%)	65 (26.3%)	0.506	0.493
Caucasian ($N = 182$)	46 (25.3%)	88 (48.3%)	48 (26.4%)	0.495	0.505
Asian ($N = 32$)	11 (34.4%)	13 (40.6%)	8 (25.0%)	0.547	0.453
Hispanic ($N = 13$)	2 (15.4%)	8 (61.5%)	3 (23.1%)	0.462	0.538
African-American ($N = 6$)	3 (50.0%)	1 (16.7%)	2 (33.3%)	0.583	0.417
All women ($N = 355$)	108 (30.4%)	148 (41.7%)	99 (27.9%)	0.513	0.487
Caucasian ($N = 287$)	82 (28.6%)	128 (44.6%)	77 (26.8%)	0.509	0.491
Asian ($N = 23$)	9 (39.1%)	5 (21.7%)	9 (39.1%)	0.500	0.500
Hispanic ($N = 12$)	3 (25.0%)	3 (25.0%)	6 (50.0%)	0.375	0.625
African-American ($N = 22$)	13 (59.1%)	5 (22.7%)	4 (18.2%)	0.705	0.295

Table 3. Muscle size and function measures by ACTN3 R577X genotype

	Genotype ^a			Genotype ^b		
	RR	RX	XX	RR	RX	XX
All women (N = 352)						
Baseline MVC strength, kg	67.9±2.1	69.7±1.8 ^c	61.5±2.2	69.8±2.3	70.6±1.9 ^d	61.5±2.3 ^e
Absolute change in MVC, kg	14.0±1.2	13.5±1.0	12.2±1.3	14.5±1.3	13.8±1.1	12.1±1.3
Relative change in MVC, %	23.4±2.1	21.9±1.8	22.3±2.2	23.7±2.1	22.0±1.7	21.0±2.2
Baseline CSA, cm ²	13.8±0.3	13.7±0.2	13.9±0.3	N/A	N/A	N/A
Absolute change in CSA, cm ²	2.2±0.1	2.5±0.1	2.4±0.1	N/A	N/A	N/A
Relative change in CSA, %	16.6±0.9	19.0±0.8	17.9±0.9	N/A	N/A	N/A
Baseline 1-RM strength, kg	14.0±0.3	13.6±0.3	14.2±0.4	13.7±0.3	13.7±0.3	14.1±0.3
Absolute change in 1 RM, kg	7.2±0.4	8.0±0.3	8.7±0.4 ^e	7.3±0.4	8.0±0.3	8.8±0.4 ^e
Relative change in 1 RM, %	55.5±3.5	63.9±2.9	68.5±3.6 ^e	57.9±3.6	63.1±3.0	70.4±3.7
Caucasian (N = 286)						
Baseline MVC strength, kg	67.7±2.5	69.8±1.9 ^c	59.9±2.5	70.5±2.6	70.7±2.0 ^d	59.0±2.6 ^f
Absolute change in MVC, kg	13.6±1.5	13.4±1.1	12.8±1.5	14.1±1.6	13.6±1.2	12.5±1.6
Relative change in MVC, %	23.5±2.5	21.7±1.9	23.9±2.6	23.6±2.5	21.6±1.9	22.3±2.5
Baseline CSA, cm ²	13.5±0.3	13.9±0.2	14.2±0.3	N/A	N/A	N/A
Absolute change in CSA, cm ²	2.1±0.1	2.5±0.1	2.4±0.1	N/A	N/A	N/A
Relative change in CSA, %	16.2±1.0	18.9±0.8	17.7±1.0	N/A	N/A	N/A
Baseline 1-RM strength, kg	14.3±0.4	13.9±0.3	14.3±0.4	14.3±0.4	13.8±0.3	14.0±0.4
Absolute change in 1 RM, kg	7.6±0.4	8.1±0.3	9.3±0.4 ^e	7.6±0.4	8.1±0.3	9.4±0.4 ^f
Relative change in 1 RM, %	57.6±4.1	64.8±3.2	73.6±4.2 ^e	60.0±4.2	63.3±3.3	75.7±4.3 ^e
Asian (N = 23)						
Baseline MVC strength, kg	60.3±9.2	71.2±11.3	49.5±7.6	63.2±10.6	57.5±11.0	66.0±7.3
Absolute change in MVC, kg	12.1±3.9	6.0±4.8	7.3±3.2	15.7±4.9	4.2±5.1	7.9±3.4
Relative change in MVC, %	23.6±6.7	8.3±8.2	15.8±5.5	28.7±7.9	8.4±8.3	12.1±5.5
Baseline CSA, cm ²	13.2±1.5	13.7±1.9	11.7±1.5	N/A	N/A	N/A
Absolute change in CSA, cm ²	1.7±0.9	2.0±1.0	2.9±0.9	N/A	N/A	N/A
Relative change in CSA, %	15.2±7.9	17.0±9.7	25.9±7.9	N/A	N/A	N/A
Baseline 1-RM strength, kg	12.3±1.4	12.0±1.6	12.9±1.4	11.5±1.1	11.9±1.5	15.1±1.0
Absolute change in 1 RM, kg	3.7±0.7	5.4±0.8	7.4±0.7 ^e	4.0±0.6	6.2±0.8	7.7±0.6 ^f
Relative change in 1 RM, %	30.8±6.9	48.1±8.2	61.2±6.9 ^e	37.5±8.0	57.4±10.6	53.40±7.4

Values are means ± SE. MVC, maximal voluntary contraction; 1 RM, 1 repetition maximum. ^aAll means adjusted for baseline body mass and age by analysis of covariance (ANCOVA). ^bBaseline means adjusted for biceps CSA and age by ANCOVA; absolute and relative change means adjusted by absolute change in CSA and age and relative change in CSA and age by ANCOVA, respectively. ^c*P* < 0.05, RX significantly different from XX; ^d*P* < 0.01, RX significantly different from XX; ^e*P* < 0.05, XX significantly different from RR; ^f*P* < 0.01, XX significantly different from RR.

such that XX > RX > RR for increases in 1-RM strength in response to training, suggesting that ACTN3 acts as a QTL associated with strength response to resistance training in women.

Proportion of variance attributable to ACTN3 genotype in women. Our sample size was sufficiently large to perform a statistical test for the proportion of all variance that could be attributable to ACTN3 genotype. This analysis compares the variance without genotype effects to the variance when ACTN3 genotype is factored in. As shown in Table 4, 2.1% of the variability in the absolute difference in 1-RM strength, 1.8% of the variability in relative difference in 1 RM, and 2.2% in baseline MVC strength are attributable to ACTN3 genotype.

DISCUSSION

The ACTN3 R577X polymorphism has been shown to result in loss of the ACTN3 protein in XX homozygotes (15). This common polymorphism has been associated with muscle per-

formance in elite Australian athletes, in whom the R allele was more common in sprint and power athletes and the X allele more common in endurance athletes. Here, we tested whether the R577X genotype was associated with baseline muscle size, strength, and response to resistance training in a large untrained volunteer population and quantified any observed association. Elbow flexor muscle strength (isometric and dynamic) and CSA of the biceps brachii were assessed before and after 24 supervised resistance training sessions (2 sessions per week for 12 wk) (25). The nine traits studied in our population were baseline muscle size, 1-RM baseline strength, MVC baseline strength, absolute change in size after training, absolute change in 1-RM strength, absolute change in MVC strength, percentage (relative) change in muscle size, percentage change in 1 RM, and percentage change in MVC. We found that men and women were statistically different for each of the nine variables, and thus men and women were analyzed

Table 4. Variability in strength of women attributable to ACTN3 R577X genotype

Muscle Strength Measurement	<i>r</i> ² Full Model	<i>r</i> ² Constrained Model	Variability Attributable to Genotype Effect	Likelihood Ratio Test <i>P</i> Value*
Absolute change in 1-RM strength	0.1277	0.1062	2.1%	0.014
%Change in 1-RM strength	0.0868	0.0683	1.8%	0.029
Baseline MVC strength	0.1861	0.1639	2.2%	0.011

*Likelihood ratio test comparing full model (with genotype, age, body mass) to constrained model (age and body mass only).

separately. These results extend pioneering work that has established associations of other genotypes with muscle phenotypes related to size and performance (e.g., Refs. 9, 18, 19, 21, 24, 27).

Study population genotype. The relative allele frequency of the 577X allele for our total study population (0.49) and for Caucasians (0.50) was similar to that previously reported for Caucasians of 0.44 (28) and 0.42 (13). The population frequency for XX homozygotes, however, was significantly higher than that previously reported for Caucasians ($\chi^2 = 10.2115$; $P = 0.006$); we found 26.6% for our study population to be XX homozygotes compared with 18% in Australian Caucasians (28). This difference could be explained by different subsets of "Caucasians" being studied (Australians vs. Americans and Irish). Our 577X allele frequency for Asians (0.47) and African-Americans (0.32) was similar to a previous report (0.50 and 0.27, respectively) (13). In our population, there was no difference in the frequency of the 577X allele between men and women.

We then segregated our study population into three genotype groups (RR, RX, XX) and performed ANCOVA analyses to determine whether there were significant differences among genotype groups for any of the nine phenotypes tested. It is important to point out that our statistical methods are quite different from those reported previously; we used the nine phenotypes or traits as continuous quantitative traits across genotype groups. The single previous study used a log-linear modeling approach, where the study population was segregated into two groups (sprint/power, endurance) and allele frequencies (not genotype groups) were compared (28).

Validation of genotype-phenotype associations. From the ANCOVA analyses, we found that three of the nine phenotypes showed significant differences among genotype groups in women only: baseline MVC, absolute increase in 1 RM after training, and relative increase in 1 RM after training. In support of our initial hypothesis, we found that the XX genotype was associated with the lowest baseline MVC strength for all women, and this association was validated in the Caucasian subpopulation. Contrary to our hypothesis, the XX genotype was associated with significantly greater relative and absolute 1-RM strength gain compared with RR. We observed a trend for a dosage-response effect, where 1-RM strength increases for XX were greater than RX, and RX greater than RR (Fig. 1). These data suggest that absence of ACTN3 protein results in greater response to training compared with a presence of ACTN3. We did not observe a difference among genotypes for baseline 1 RM. Thomis et al. (24), through elegant twin studies, noted that the genetic factors that served to explain part of the variance in increased 1-RM strength in response to resistance training differed from those that explained variation in pretraining phenotypes. The posttraining genotype-1-RM associations were validated in both the Caucasian and Asian subpopulations.

Genetic association studies are subject to many confounding variables, such as the appropriateness of control populations, questions regarding homogeneity of traits under study, the sensitivity and specificity of tests used to measure such traits in large populations, and questions regarding the accuracy of genotyping methods (17). These problems make it difficult to replicate reports of statistically significant gene association studies. As such, it is increasingly recognized that "validation"

data sets are critical for the interpretation of genetic association data (17). Here, we show internal validation data sets that serve to increase the confidence of our findings of the robustness of association of ACTN3 genotypes with strength in women. First, significant associations observed for both relative and absolute 1-RM strength increases in the entire female cohort, and, second, these relationships were validated in two ethnic subgroups (Caucasians and Asians) (Table 4). Also, there appears to be a dosage effect of the ACTN3 X allele in both the entire female cohort and the ethnic subpopulations for increases in 1-RM strength (Fig. 1). In light of these complementary types of internal validation, we consider our data for 1-RM strength gain to be particularly robust with regard to the effects of ACTN3 and strength gain in women. We also consider the association of ACTN3 genotype with MVC baseline strength strong because of the validation within one ethnic subpopulation and the high level of significance between XX and RX when covaried for age and CSA ($P < 0.01$), in addition to the difference between XX and RR ($P < 0.05$).

In analyzing MRI data to determine biceps CSA, the potential for coregistration errors (mismatched pre- vs. posttraining image slices) must be considered as a potential limitation. The best way to avoid these errors is through the identification of bony landmarks through which images from different data sets can be compared. In this study, MR image slices were taken in the belly of the muscle, which corresponds to the shaft of the humerus. Unfortunately, identification of bony landmarks in this region of the humerus is not feasible. So comparison of pre- and posttraining slices had to be made on the basis of soft tissue landmarks, which differed based on each subject's anatomy. Assigning pre- vs. posttraining slices based solely on placement of the positioning marker proved reliable in ~85% of subjects (i.e., similar soft tissue anatomy with matching slice numbers). In the remainder of subjects, the MRI analyst needed to judge slice selection on the basis of the soft tissue anatomy of adjacent image slices. Between-group comparison of the 85% with correct positioning vs. the 15% with improper positioning ($n > 600$) revealed no significant differences in training response (mean %increase in CSA: 85% group: $19.3 \pm 8.9\%$, 15% group: $18.7 \pm 8.8\%$, $P = 0.5368$, via unpaired *t*-test). Another method for monitoring potential coregistration errors is by comparison of trained (nondominant) arm CSA increases with CSA changes in the untrained (dominant) arm. Coregistration errors in the untrained arm would be expected to produce different pre- vs. posttraining numbers, which were not observed. The best way to avoid coregistration errors due to mismatched slices is by volumetric analysis. However, this type of analysis must be performed manually to analyze individual muscles. Although volumetric analysis is the most accurate, it is time consuming and costly (26). To compare volumetric and CSA data, we performed volumetric analysis on a subset of ~80 subjects. In this small population, the percent CSA increase was $15.12 \pm 2.13\%$ and the percent volume increase was $15.73 \pm 2.06\%$, and there was no significant difference between the two measures ($P = 0.1319$, via paired *t*-test). More importantly, the range of subject training responses was similar between the two analyses (i.e., subjects with the greatest change based on CSA also had the greatest change based on volume).

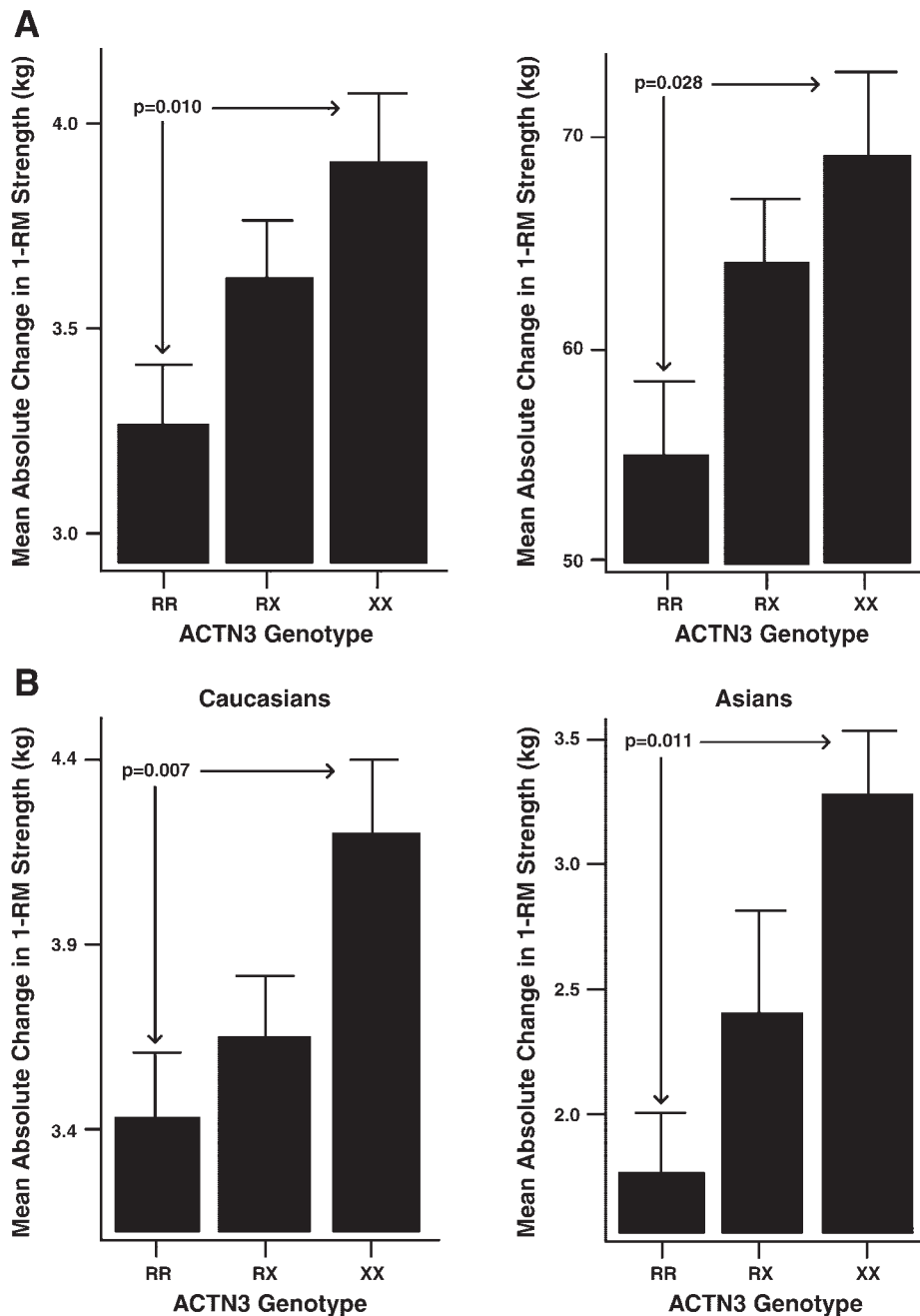


Fig. 1. Significant relationship of ACTN3 R577X genotypes with 1-repetition maximum (1-RM) strength gain in women. *A*: entire study population of women. *B*: ethnic subpopulations. 1 RM was corrected for age and body weight. RR, wild-type homozygote; RX, heterozygote; XX, ACTN3 577 mutant homozygote.

Significant associations of muscle strength and ACTN3 genotypes. Previous studies reported that ACTN3 null allele homozygotes (577XX) were underrepresented in athletes who specialize in high-force, high-intensity exercise (28). Our results regarding lower MVC strength for the null allele homozygote (XX) genotype are in accordance with these data and support our initial hypothesis. It appears that the XX genotype is inherently weaker. Isometric strength is a better representation of the muscle's capacity to produce maximal force compared with the 1-RM strength measure, which is demonstrated in the amount of force that each strength measure can generate (e.g., ~30 kg for MVC and ~6 kg for 1 RM). The force-velocity relationship in skeletal muscle states that there is an inverse relationship between force and velocity of shortening

(8). Thus the maximal force-generating capability of muscle at a shortening velocity of 0° per second (as during MVC assessment) is greater than that at ~60° per second (the testing speed of the 1-RM assessment). The ACTN3 577R allele appears to be advantageous in generating maximal force. Yang et al. (28) suggested that the ACTN3 protein may confer a greater capacity for the absorption or transmission of force at the Z line during rapid contraction. Given that ACTN3 is only present in type II fibers and type II fibers are related to force, contractile speed, and power production, a strength test more related to muscle power or high-speed contraction might show an even larger genotype effect.

Although there was a strong association of the ACTN3 577R allele with baseline MVC, there was no significant association

with MVC strength gain after training. Because of the principle of resistance training specificity, increases in performance are most evident when the testing mode is the same as the training mode. Indeed, we observed a greater increase in relative 1-RM strength (~60%) than in MVC strength (~20%). Rutherford and Jones (20) reported that 12 wk of resistance exercise nearly tripled the training weights, whereas MVC strength only marginally increased (15%). The relationship of ACTN3 with the increase in 1-RM strength, and not with the increase in MVC strength, may result from the greater gain in 1 RM because of specificity of the exercise and testing mode. Thus we may have greater sensitivity for genotype effects in 1 RM because of the greater dynamic range for increases in this strength measure.

Contrary to our hypothesis, the mutant genotype displayed the greatest 1-RM response to training. Because the mutant genotype had the lowest baseline MVC, perhaps the "principle of initial values" may explain the greater increase in 1 RM for the XX genotype. However, there was no significant association of baseline 1-RM strength with genotype, and the mean baseline 1-RM strength was nearly identical among the groups, differing by ≤ 0.6 kg (Table 3). Thus initial 1-RM strength could not explain the increases in 1 RM we observed for the XX genotype.

The resistance exercise protocol used in this study incorporated both concentric and eccentric contractions. Although we did not assess muscle damage in the present study, training that involves eccentric contractions is known to cause strain on the myofibril-cytoskeletal network that results in myofibrillar damage, particularly Z-line streaming (7). α -Actinin is localized to the Z line and considered to be involved in maintaining the integrity of the sarcomere. The lack of ACTN3, as occurs in the null homozygote, may impair the stability of sarcomeres, making them more susceptible to damage. Damage is a potent stimulus for muscle adaptive responses (3, 12). Yu et al. (30) proposed that strain-dependent processes acting on the Z disk during forceful contractions may lead to a release of α -actinin and other cytoskeletal proteins like nebulin and titin. Furthermore, Yu et al. (29, 30) proposed that the release of α -actinin from the Z disk in response to contraction-induced damage would allow the α -actinin to initiate formation of additional sarcomeres elsewhere along the myofibril. Observation of supernumerary sarcomeres after the eccentric exercise protocol supports this contention (29). Thus α -actinin may play an important role in muscle remodeling in response to contraction-induced damage (29, 30). We speculate that those individuals with a deficiency of ACTN3 may incur greater muscle damage in response to resistance exercise. Damage is a potent stimulator for increased muscle adaptation, and exercise regimens that include eccentric contractions result in greater strength gain than concentric-only exercise (4, 6). Those individuals with a deficiency of ACTN3 may be better able to adapt to training and thereby produce greater strength gain.

Although the mutant genotype can develop greater dynamic strength, they still could be at a disadvantage in producing maximal muscle power. Indeed, Stone et al. (22) reported that isometric force was strongly related to peak power, and in our study, MVC strength of the heterozygote genotype was associated with the highest strength. Yang et al. (28) proposed that the 577X allele may confer some advantage because it has been conserved through evolution and that mutant genotypes were

well represented in endurance athletes. We speculate that type II fibers without ACTN3 are able to adapt to a stressful condition, but they are not able to develop power to the extent that persons with the 577R allele would have.

It is conceivable that differences in the amount of exercise training affected the different responses of the genotypes to training. We examined the total training volume (calculated as the total amount of weight lifted over the course of the training program) among genotypes for a subsample of 35 women. Women with XX, RX, and RR genotypes trained with 147, 132, and 149 kg over the 12 wk. Thus women with the RR genotype developed less strength, despite training volumes similar to the other genotypes.

Sex differences in genotype-phenotype associations. There is no obvious explanation for the sex difference for the relationship of ACTN3 and strength in our data. Yang et al. (28) also reported a sex relationship in ACTN3 genotypes associated with athletic performance; they found an 8% frequency of the XX homozygote in men power athletes ($N = 72$), but no women power athletes ($N = 35$) had this null genotype. Given the 20% frequency of the XX homozygote in the control sample of women in that report, it would be predicted that approximately seven of the women power athletes would be XX homozygotes. Particularly for women, there appears to be a selection against the XX homozygous condition in this type of athletic performance, which is in accordance with our data, in which the XX homozygous women had the lowest MVC strength.

Regarding strength gain, only the women demonstrated a significant relationship with the ACTN3 577 genotype. Women have significantly lower baseline strength, and this appears to lead to considerably greater relative increases in strength in women (~64%) compared with men (~40%). We may simply have greater sensitivity for detecting genotype effects because of the greater dynamic range for increases in strength in women. Given that our data indicate that ACTN3 genotype is responsible for ~2% of the variation in strength gains in women, a decrease in our statistical power in men could lead to an inability to statistically detect genotype effects in men. An alternative explanation is that ACTN3 genotype has a proportionally smaller effect in men than women. For example, the effects of steroid hormones could mask the "structural" effects of ACTN3 genotype. MacArthur and North (11) suggested that lower average levels of testosterone in women athletes may make the variation in other parameters, such as ACTN3 genotype, more important in determining athletic performance.

In summary, we found no association between ACTN3 R577X genotype and muscle strength in men, but we did find a significant association between this genotype and strength in women, and this was validated in ethnic subpopulations. Women homozygous for the ACTN3 mutation (XX) had significantly lower baseline MVC (isometric) strength compared with the homozygous wild-type (RR) and the heterozygote (RX) groups when adjusted for baseline CSA. However, women homozygous for the null mutation (XX) demonstrated the greatest gain in 1-RM (dynamic) strength. Our data in a large sample size show that women with the nonsense allele in ACTN3 are at a disadvantage in the ability to produce high-force isometric strength, but they are at an advantage in developing dynamic muscular strength in response to a pro-

gressive resistance training program. Furthermore, 2.1% of the variability in the absolute difference in 1-RM strength, 1.8% of the variability in relative difference in 1 RM, and 2.2% of the variability in baseline MVC strength are attributable to ACTN3 genotype. These data suggest that ACTN3 is one of many genes and other factors that contribute to observed variance in muscle performance and response to resistance training.

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