

Structural and regulatory roles of muscle ankyrin repeat protein family in skeletal muscle

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Barash IA, Bang M-L, Mathew L, Greaser ML, Chen J, Lieber RL. Structural and regulatory roles of muscle ankyrin repeat protein family in skeletal muscle. *Am J Physiol Cell Physiol* 293: C218–C227, 2007. First published March 28, 2007; doi:10.1152/ajpcell.00055.2007.—The biological response of muscle to eccentric contractions (ECs) results in strengthening and protection from further injury. However, the cellular basis for this response remains unclear. Previous studies identified the muscle ankyrin repeat protein (MARP) family, consisting of cardiac ankyrin repeat protein (CARP), ankyrin repeat domain 2/ankyrin repeat protein with PEST and proline-rich region (*Ankrd2/Arpp*), and diabetes-associated ankyrin repeat protein (*DARP*), as rapidly and specifically upregulated in mice after a single bout of EC. To determine the role of these genes in skeletal muscle, a survey of skeletal muscle structural and functional characteristics was performed on mice lacking all three MARP family members (MKO). There was a slight trend toward MKO muscles having a slower fiber type distribution but no differences in muscle fiber size. Single MKO fibers were less stiff, tended to have longer resting sarcomere lengths, and expressed a longer isoform of titin than their wild-type counterparts, indicating that these proteins may play a role in the passive mechanical behavior of muscle. Finally, MKO mice showed a greater degree of torque loss after a bout of ECs compared with wild-type mice, although they recovered from the injury with the same or even improved time course. This recovery was associated with enhanced expression of the muscle regulatory genes *MyoD* and muscle LIM protein (*MLP*), suggesting that the MARP family may play both important structural and gene regulatory roles in skeletal muscle.

CARP; *Ankrd2*; *Arpp*; *DARP*; eccentric contractions; titin

ECCENTRIC CONTRACTIONS (ECs), in which an activated muscle is forced to lengthen, lead to muscle injury and delayed-onset muscle soreness. ECs are mechanically unique, because the muscle is forced to bear a load larger than it can actively create, and they have a unique influence on the gene expression program within a muscle. While it has been questioned whether ECs are absolutely necessary for strengthening and protection from injury (20, 22), it is clear that ECs do provide a potent stimulus for muscle growth, and repeated EC exercise leads to muscle strengthening and protection against further EC-induced injury (31, 37, 39). The biological basis of these observations remains the subject of much exploration.

Recent gene array studies provided clues into the types of genes that are upregulated after ECs and therefore may be important in muscle's response to this type of exercise. A

recent study (4) revealed that 36 genes were upregulated 48 h after a single bout of EC. Of these 36 genes, two were members of the three-member muscle ankyrin repeat protein (MARP) family: cardiac ankyrin repeat protein (CARP) and ankyrin repeat protein with PEST and proline-rich region/ankyrin repeat domain 2 (*Arpp/Ankrd2*; *Ankrd2*). The third member of the family, diabetes-associated ankyrin repeat protein, or *DARP* (15), was not present on the original chip, but all MARP family members share ~50% homology and similar intracellular distributions (26). The MARP family is a unique group of proteins that are found in differing abundance in the central I band of the sarcomere in both cardiac and skeletal muscles, where they bind the N2A region of titin (26) and the amino terminus of the nebulin anchoring protein myopalladin (3). They are found in both the nucleus and the cytoplasm of skeletal and cardiac tissue (17, 42, 48), and at least one family member, CARP, has been suggested to act as a transcription inhibitor that acts downstream of the homeobox gene *Nkx2.5* (19, 48) *Ankrd2* has also been shown to bind to YB-1, PML, and p53, suggesting a role in signaling (23).

CARP is also known as *C-193* and was originally discovered as a cytokine-inducible gene in fibroblasts (11) before it was recognized as differentially regulated between embryonic and adult heart (7, 19, 24, 48). As its name suggests, in adult animals CARP is primarily expressed in cardiac tissue, where it is thought to be a marker for cardiac hypertrophy (1, 24). In skeletal muscle, CARP is upregulated in muscle from patients suffering from Duchenne muscular dystrophy (2) as well as other dystrophies (29, 30) and in response to acute resistance exercise (10) or work-overload hypertrophy (9). *Ankrd2* is similar to CARP, although it is more highly expressed in skeletal than cardiac muscle, preferentially in type 1 fibers (21, 42). Its expression in skeletal muscle is increased with denervation (42), with immobilization in a stretched position, with differentiation in culture (21), and in tumors (18). *DARP* is expressed in both heart and skeletal muscle (in addition to brown fat) and was identified by its upregulation in Type 2 diabetes and insulin-resistant animals, suggesting a potential role in energy metabolism (15).

Despite the evidence suggesting that the MARP family is important for the structure and function of striated muscle, the precise role that it plays in muscle remains unclear. The consistent and robust increase in expression of these genes after various physiological stimuli, including EC-induced mus-

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cle injury, suggests that these genes may play an important role in the response of muscle to these stimuli, and perhaps in the normal structure of muscle. Thus the three members of the MARP family are excellent candidates for proteins that both serve a structural role and act as mechanically sensitive signal transducers within striated muscle.

Our hypothesis is that the MARP family members may be key players in either the structural stability of skeletal muscle, particularly in regard to either muscle morphology and phenotype or its ability to withstand ECs, or the gene expression response and remodeling after injury. We present the results of a detailed analysis of the skeletal muscle phenotype of mice lacking each MARP family member individually, double knockouts lacking either *CARP* and *DARP* or *Ankrd2* and *DARP*, and triple MARP knockout (MKO) mice, lacking all three members of the MARP family. Our results suggest that while the MARP family is not essential for the basal functioning of skeletal muscle, with only mild effects on fiber size or fiber type distribution, it plays a role in the passive mechanical behavior and the structural stability of the tissue after ECs. In addition, gene expression results suggest that the MARP family may serve a modulatory role in the regenerative response in muscle, such that MKO mice showed an enhanced regenerative response to EC-induced muscle injury. Our results also suggest that these three proteins are not only structurally but also functionally homologous, with the necessity for all three genes being removed to see a significant effect on the performance of the muscle.

METHODS

Experimental subjects. Three- to four-month-old male and female mice were housed four to a cage at 20–23°C with a 12:12-h light-dark cycle. Single, double, and triple knockout (KO) mice for *CARP*, *Ankrd2*, and *DARP* were obtained (Bang M-L, Gu Y, Dalton N, Petersson K, Knowlton KU, Chen J, and Chien KR, unpublished), and their genotypes were confirmed by PCR analysis. Control animals were age-, weight-, and sex-matched Black Swiss mice (Taconic Farms, Germantown, NY). Where indicated, single and double KO and heterozygote animals were also used as controls for the MKO mice (Table 1). All procedures were approved by the Department of Veterans Affairs and University of California at San Diego Animal Subjects Committees.

Histology/histochemistry. Tibialis anterior (TA), extensor digitorum longus (EDL), and soleus muscles from unexercised male mice were removed and frozen in isopentane cooled by liquid nitrogen. Ten-micrometer sections were processed for routine hematoxylin and eosin (H&E) staining, and myosin ATPase histochemistry was performed with standard methods using an acid pH of 4.3 to label type 1 fibers and a basic pH of 10.2 to label type 2 fibers. Type 1 and type 2 fiber size were determined by using Metamorph (Universal Imaging,

Downington, PA) to record the area stained dark in either the acidic or the basic staining conditions at ×10 magnification and dividing this area by the number of stained fibers in each section. One section per muscle was analyzed.

SDS-PAGE. Fiber type analysis was performed on the TA, EDL, and soleus of wild-type and KO animals with a modified version of the SDS-PAGE method developed by Talmadge and Roy (41). Either a 55-μm-thick section of the muscle (for muscle fiber type percentage analysis) or a single-fiber segment (for determination of fiber type after passive mechanics) was dissolved in sample buffer [100 mM dithiothreitol, 2% SDS, 80 mM Tris base pH 6.8, 10% glycerol, and 1.2% (wt/vol) bromophenol blue], boiled for 2 min, and then either frozen at –80°C or run immediately on the gel. Stacking and resolving gels, with a total gel size of 16 cm × 22 cm, were 4% and 8% acrylamide, respectively, and 0.75 mm thick. They were run at a constant voltage of 275 V for ~21 h at 4°C and then silver stained according to the manufacturer’s protocol (Bio-Rad, Hercules, CA). Fiber type percentage analysis was performed in duplicate or triplicate and analyzed with NIH Image (version 1.62). Single-fiber analysis was performed once, simply to determine the fiber type of the mechanically tested fiber.

Single-Fiber Mechanics. Passive mechanical experiments were performed on cells from either the fifth toe muscle of the EDL or the soleus muscle. These two muscles were chosen based on the fiber type and length homogeneity observed within each muscle (8) and the dramatically different fiber type profiles between muscles. Immediately after the animals were euthanized by cervical dislocation under general anesthesia, hindlimbs were transected proximal to the knee and placed into a mammalian Ringer solution (mM: 137 NaCl, 5 KCl, 24 NaH₂PO₄, 2 CaCl₂, 1 MgSO₄, and 11 glucose, with 10 mg/l curare). Occasionally, the contralateral limb from an exercised animal was used for mechanical experiments; in this case, hindlimbs were transected after the TA muscles were removed and frozen for RNA analysis (described below).

EDL and soleus muscles were quickly removed from the hindlimb and placed in a relaxing solution containing (mM) 59.4 imidazole, 86 KCH₄O₃S, 0.13 Ca(KCH₄O₃S)₂, 10.8 Mg(KCH₄O₃S)₂, 5.5 K₃EGTA, 1 KH₂PO₄, and 5.1 Na₂ATP, with 50 μM leupeptin (40), for at least 60 min. While in relaxing solution the fifth toe muscle of the EDL was dissected under magnification, and both this and the soleus were separated into two bundles for storage. These muscles were then pinned to Parafilm-coated cork and stored in a storage solution of (mM) 170 K propionate, 5 K₃EGTA, 5.3 MgCl₂, 10 imidazole, 21.2 Na₂ATP, 1 NaN₃, and 2.5 glutathione, with 50 μM leupeptin and 50% (vol/vol) glycerol (13), at –20°C for up to 3 wk.

At the time of the experiment, bundles were removed from storage solution and placed in relaxing solution. Single-fiber segments (2–3 mm in length) were carefully dissected and transferred to the mechanics chamber, where they were tied with 10-0 nylon suture to titanium wires (26601-9, Aldrich Chemical, Milwaukee, WI) secured to a force transducer (405A, Aurora Scientific) and a motor (318B, Aurora Scientific). The motor was connected to a micromanipulator such that fiber stretching could be controlled by either the motor or the micro-

Table 1. MARP family members and knockout combinations

No. of Genes Knocked Out	Genotype	Genes Deleted	Accession No.
Single	CARPKO	<i>CARP</i>	AF041847
Single	ArppKO	<i>Ankrd2/Arpp</i>	AJ011118
Single	DARPKO	<i>DARP</i>	AF492400
Double	CARP/DARPKO	<i>CARP</i> and <i>DARP</i>	AF041847 and AF492400
Double	Arpp/DARPKO	<i>Arpp</i> and <i>DARP</i>	AJ011118 and AF492400
Triple	MKO	<i>CARP</i> , <i>Arpp</i> , and <i>DARP</i>	AF041847, AJ011118, and AF492400

MARP, muscle ankyrin repeat protein; *CARP*, cardiac ankyrin repeat protein; *Arpp*, ankyrin repeat protein with PEST and proline-rich region; *Ankrd2*, ankyrin repeat domain 2; *DARP*, diabetes-associated ankyrin repeat protein; KO, knockout; MKO, triple MARK family KO.

manipulator and fiber orientation could be adjusted. Slack length was determined by a combination of visually orienting the fiber such that it did not have a curved appearance and maintaining the tension within 0.002 mV (<0.1 mg) of its background level. This method has been shown to be repeatable (14). Under $\times 10$ magnification, the fiber length and diameter in three places were measured by manipulating the fiber in and out of the field of view with a micrometer attached to a micromanipulator. Mean fiber diameter was used to estimate fiber cross-sectional area, assuming fibers were cylindrical. Fiber length was also determined by transilluminating the fiber with a He-Ne laser (Melles Griot, Irvine, CA) to permit measurement of the sarcomere length at slack length and after 0.5-mm stretch. Sarcomere length was determined by projecting the diffraction pattern onto a photodiode array (PDA; model S2048, Reticon) positioned above the fiber. The position of the PDA was calibrated by using 2.50-, 3.33-, and 5.0- μ m diffraction gratings before the experiment, and first-order diffraction peaks were used to calculate sarcomere length according to standard grating equations. Fiber length was calculated, assuming constant sarcomere number, with the formula fiber length = $0.5 \text{ mm}/(\text{SL}_{\text{stretch}}/\text{SL}_{\text{slack}} - 1)$, where SL is sarcomere length. The fiber length used in the final analysis was the average of both the visually determined and the calculated fiber length, which were within 0.5 mm of each other. The fiber was carefully checked for mechanical integrity such that any discoloration, abrasion, swelling, or disrupted diffraction pattern disqualified the fiber from further analysis. All experiments were performed at room temperature.

Dynamic experiments were performed using a Labview program (National Instruments, Austin, TX) to control the motor, record force from the force transducer, and record the diffraction pattern from the PDA. A preconditioning bout of stretches was performed by imposing five stretches at 20% strain and 20%/s strain rate. After 5-min rest, two stretch protocols consisting of five cycles of stretch and release at 35% strain were imposed on the muscle: low velocity (10%/s strain rate) and high velocity (100%/s strain rate). Sarcomere length and force were recorded in real time for both strain rates and analyzed with MATLAB (MathWorks, Natick, MA) for tangent modulus (final slope in the last 5% of each stretch cycle), hysteresis loop area, starting sarcomere length, peak sarcomere length, peak stress, and peak strain. After the dynamic stretch protocols, the muscle fibers were stretched to failure in 2-min intervals and 250- μ m increments with the micrometer attached to the motor. Sarcomere length and tension were recorded at the end of each 2-min interval.

A separate set of EDL fibers were subjected to a progressive protocol of five cycles of stretch at 20%, 30%, 40%, 50%, and 60% strain and 5%/s strain rate in order to look for strain softening behavior and for tangent modulus differences at a slower strain rate. After this progression, stress relaxation was recorded by using the motor to stretch the fiber 20%, 30%, or 40% and hold at that length for 2 min. MATLAB was then used to calculate peak stress and steady-state stress and to fit an exponential equation to the stress relaxation decay in order to compare stress relaxation time constants among the genotypes. After mechanical testing, the fibers were placed in 10 μ l of sample buffer and stored for fiber type analysis as described above.

Exercise protocol. The exercise apparatus and regimen are described in detail elsewhere (4). In brief, mice were positioned in a specially designed jig that allowed measurement of ankle dorsiflexion torque and control of ankle position (to control muscle length) via an ergometer (custom-modified model 360B, Aurora Scientific) while under general anesthesia (2% isoflurane). The TA and EDL muscles were stimulated by sterile subcutaneous 28-gauge needle electrodes (Grass Instruments, Braintree, MA) placed in the vicinity of the right peroneal nerve, ~ 0.5 mm under the skin, just lateral to the midline and distal to the knee joint. Stimulation voltage was usually 10 V, and optimum stimulation frequency (resulting in a flat and fused isometric torque record) was usually determined to be 200 Hz.

Before all exercise paradigms, maximal isometric torque was measured as the mean of two isometric contractions. The eccentric bout consisted of 50 ECs, 1/min, with the footplate forcing 78° of plantarflexion at ~ 1 fiber length/s, starting 150 ms after the muscle was activated. During the EC bout, isometric twitches were recorded every five contractions and needle electrodes were repositioned if necessary to achieve a maximal twitch. The contralateral leg received only an isometric testing protocol, consisting of just enough isometric contractions (~ 5) to determine and record maximum isometric torque, without ECs.

Mice were subjected to one of two protocols. The preliminary protocol followed four animals over time as they recovered from the injury protocol. Thus these animals were returned to their cage after the initial exercise bout and then subjected to the isometric testing protocol described above on both the eccentrically exercised and contralateral legs 6, 24, 72, 120, 168, and 216 h (9 days) after the initial bout. After the last testing time point, the animals were euthanized by cervical dislocation while under anesthesia. The second protocol collected muscles for analysis from the animals 6, 12, or 24 h after the initial exercise bout, with no intermediate testing. At the defined time point, TA muscles were removed and frozen in liquid nitrogen-cooled isopentane for further analysis, and the animals were euthanized by cervical dislocation while under anesthesia. The following seven genotypes were subjected to both protocols: wild type, CARPKO, Ankrd2KO, DARP KO, CARP/Ankrd2 double KO, CARP/DARP double KO, and MARF triple KO (MKO; Table 1).

RNA analysis. RNA was extracted from the TA muscles of exercised and contralateral legs with a combination of the standard TRIzol (Invitrogen, Carlsbad, CA) and RNeasy (Qiagen, Valencia, CA) protocols, described elsewhere (4). Five hundred nanograms of RNA from each sample was reverse transcribed with the manufacturer's protocol (Superscript II, Invitrogen). Quantitative real-time PCR was done with the Cepheid SmartCycler (Sunnyvale, CA), using primers specific to the genes of interest, and quantified by monitoring the fluorescence levels of the intercalating dye SYBR Green (Sigma) compared with a PCR product standard, as described elsewhere (4). Primers are listed in Table 2. All samples were run at least in duplicate, along with a standard. Amplification conditions were as follows: an initial hold at 95°C for 5 min was followed by 40 cycles of denaturing at 95°C for 15 s followed by annealing/extension at 66°C for 40 s (CARP and MyoD), 70°C for 40 s (Ankrd2), 68°C for

Table 2. Primers used for quantitative real-time polymerase chain reaction

Gene	Accession No.	Sense Primer (5'-3')	Antisense Primer (5'-3')	Product Length, bp
MLP*	D88791	ATCAGAGAAGTGCCACGATG	GTAAGCCCTCCAAACCCAAT	213
CARP*	AF041847	GAAGTTAATGGAGGCTGGAGC	TTCTCTGCTCTGGCATTGAG	244
Ankrd2*	AJ011118	CGTGAGACTCAACCGCTACAA	TGCAGGCAGCTCATAGTAGG	269
DARP*	AF492400	AAGGCACAGGATAAGGAAGG	ATCCACAGGTGCCAGTCGTCA	228
Myogenin	D90156	TCCCAACCCAGGAGATCAT	TGCAGGCAGCTCATAGTAGG	269
MyoD	DM84918	ACCCAGGAACCTGGGATATGGA	AAGTCGTCTGCTGTCTCAA	102
Desmin	L22550	CAGGACCTGCTCAATGTGAA	TGTGTAGCCTCGCTGACAAC	227

MLP, muscle LIM protein. *Primers are the same as used in Ref. 4.

30 s (*Desmin*), 66°C for 30 s (*Myogenin*), or 69°C for 40 s [muscle LIM protein (*MLP*)], followed by a melt curve. Success of each reaction was deduced based on observing a single reaction product on an agarose gel and a single peak on the DNA melting temperature curve determined at the end of the reaction.

Titin molecular mass determination. EDL and soleus muscles from unexercised MKO and wild-type controls were removed and frozen in liquid nitrogen. One percent agarose gel electrophoresis was used as previously described (44) to evaluate the molecular mass of titin in the samples, which was calibrated with a standard containing rat cardiac muscle and human soleus, two titin isoforms for which the molecular mass has been unequivocally determined. The gels were run in duplicate.

Statistical analysis. Comparisons among genotypes were made with analysis of variance (ANOVA; Statview, SAS Institute), and a repeated-measures ANOVA was used when the same parameter was measured over time. Fisher's PLSD was used when ANOVA showed significant differences. Comparisons between the distribution of fiber types tested among genotypes for the single-fiber mechanical analysis were done with a χ^2 analysis. $P < 0.05$ was considered to be significant, and all values are presented as means \pm SE.

RESULTS

Histology, histochemistry, and fiber type analysis. No obvious histological abnormalities, such as centralized nuclei, necrosis, increased fiber size variability, abnormal fiber shape, or altered fiber density, were seen in the MKO model compared with wild-type mice with H&E staining (data not shown). No significant differences were seen in the size of either type 1 or type 2 fibers between genotypes as determined by myosin ATPase staining (Fig. 1A). In addition, there were no statistically significant differences in the fiber type composition of the two genotypes as determined by SDS-PAGE measurements of the different isoforms of myosin heavy chain in the soleus, EDL, and TA (Fig. 1, B–D), although there was a trend in all three muscles toward MKO muscles being slower than wild-type muscles. These results were mirrored in the fiber type composition and fiber size of the single and double KO animals.

Single-fiber mechanics. Analysis of MKO single-fiber mechanics showed some subtle effects of the lack of these genes on the passive characteristics of muscle, suggesting that these genes play a role in the structure and mechanics of normal muscle. No differences between genotypes were found in the elastic modulus as determined by the slope of the stress-strain curve when the fibers were stretched in 2-min intervals (Fig. 2A). MKO muscle fibers, from both the EDL and the soleus, had a smaller tangent modulus at 10%/s strain rate (Fig. 2B) than wild-type muscle fibers (Fig. 2C) that closely approached significance with repeated-measures ANOVA ($P = 0.056$) and was significant by *t*-test ($P < 0.05$) for all individual stretch cycles except for cycle 4. When tangent modulus was compared among genotypes by fiber type, the same trend was seen: MKO fibers were less stiff than wild-type fibers. No differences were seen at 100%/s strain rate. Interestingly, there was a significant difference between the goodness of fit (r^2) value for the tangent modulus at 5 and 10%/s strain rates, suggesting that wild-type fibers have a more linear tangent modulus than MKO fibers ($P < 0.001$). There was a small but significant positive correlation by linear regression between tangent modulus and the r^2 value ($r^2 = 0.191$, $P < 0.0001$), suggesting that the increased goodness of fit of the

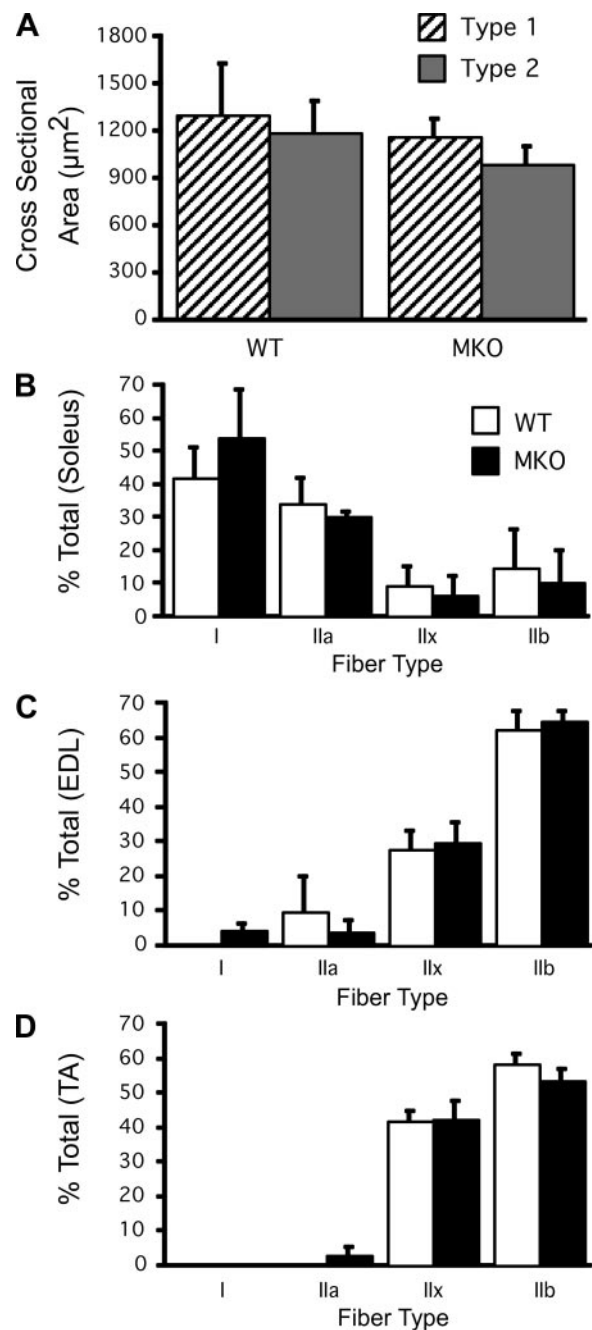
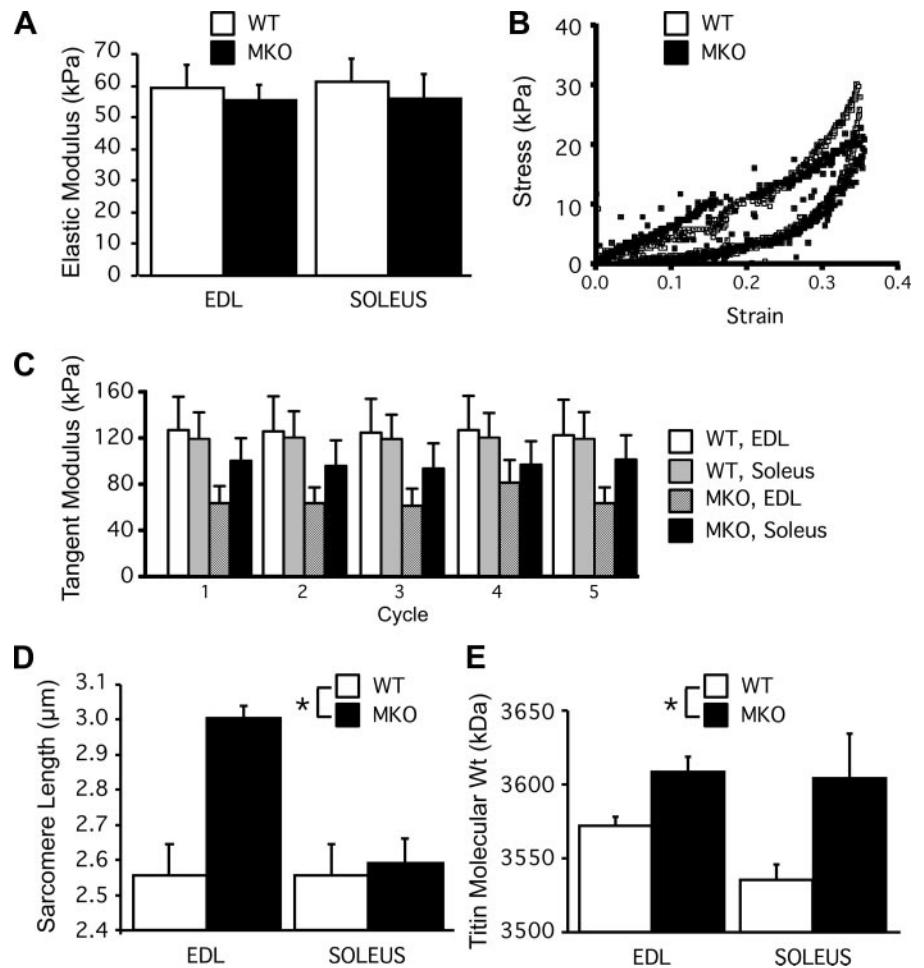


Fig. 1. Fiber size and fiber type distribution in triple muscle ankyrin repeat protein (*MARF*) family knockout (MKO) and wild-type (WT) muscle fibers. A: cross-sectional area of type 1 and type 2 fibers in the soleus muscle of WT and MKO mice. No significant differences were found. ($n = 4$ or 5 per group). B–D: fiber type distribution in the soleus (B), extensor digitorum longus (EDL; C), and tibialis anterior (TA; D) muscles of WT and MKO mice. No significant differences between genotypes were found ($n = 3$ –5 per group).

tangent modulus accounted for $<20\%$ of the variability of the tangent modulus itself, and probably was not a significant factor in the genotype effect.

The EDL fibers from MKO mice showed a significantly longer resting sarcomere length than EDL fibers from wild-type mice of the same genetic background or soleus fibers from either genotype ($P < 0.002$, Fig. 2D). EDL muscles from mice heterozygous for all three *MARF* genes and with the same

Fig. 2. Passive mechanical behavior of WT and MKO mice. *A*: elastic modulus as determined by the slope of the stress-strain curve when single WT and MKO soleus or EDL fibers were stretched in 250- μm increments to failure. No significant differences were found among genotypes or muscle types as determined by 2-way ANOVA ($n = 12\text{--}17/\text{group}$). *B*: sample stress-strain curves from a single cycle (*cycle 1*) of a 5-cycle series of stretches of a single soleus fiber from a WT and a MKO mouse. Strain was $\sim 35\%$ at 10%/s strain rate. *C*: tangent modulus determined by the end slope of the stress-strain curve (such as that depicted in *B*) of 5 cycles of stretch and release of 35% strain and 10%/s strain rate. Repeated-measures ANOVA revealed that fibers from MKO mice tend to have smaller tangent moduli than WT fibers ($P = 0.0560$; $n = 12\text{--}16$ fibers/group). *D*: resting sarcomere length of EDL and soleus fibers from WT and MKO mice. Two-way ANOVA showed a significant effect of genotype and muscle type and a significant interaction between genotype and muscle type ($*P < 0.01$ in all cases), with MKO EDL fibers having a longer resting sarcomere length than any other group ($n = 12\text{--}14/\text{group}$). *E*: titin length of WT and MKO EDL and soleus fibers. Two-way ANOVA showed a significant effect of genotype ($*P < 0.01$) but not muscle type or interaction between genotype and muscle type ($P = 0.26$ and 0.35 , respectively; $n = 4/\text{group}$).



genetic background as the MKO mice also had a trend toward shorter resting sarcomere length compared with MKO mice (2.71 ± 0.03 and $2.79 \pm 0.05 \mu\text{m}$, respectively; $P = 0.19$) and a significantly larger tangent modulus compared with MKO mice (201 ± 17 and 129 ± 20 kPa, respectively; $P < 0.05$). This indicates that the differences between genotypes are due to the complete loss of the MARF proteins and not the slight differences in background strain. There were no significant differences between genotypes in the distribution of fiber types tested ($P = 0.2723$), although fibers expressing multiple myosin heavy chain isoforms (for example, both types I and IIa) were only found in wild-type muscle.

MKO muscles from both the soleus and the EDL had significantly longer titin lengths compared with wild-type controls ($P < 0.01$, Fig. 2E). No differences between genotypes were seen in any of the stress relaxation parameters, including peak stress, steady-state stress, or the time constants of decay during the 2-min hold after stretch (data not shown). In addition, there was no evidence of strain-softening behavior, because the tangent modulus did not change significantly between the first and second stretch cycle for any genotype.

Injury and recovery after ECs. No significant differences in the recovery of wild-type and MKO mice from EC-induced muscle injury when the same mice were followed over time were found by two-way ANOVA over time and between the two genotypes (Fig. 3A), indicating that the MARF family is

not required for a normal recovery after injury. MKO mice showed a significantly greater degree of injury, however, as shown by the percentage drop from the initial torque produced by the muscle, immediately after the EC bout than wild-type mice (Fig. 3C). This greater injury was not a result of greater torque produced by and therefore borne by the muscle, because the initial torques were not significantly different between groups (Fig. 3B). No significant differences were seen with any of the other KO genotypes (Fig. 3, B and C). Torque loss and recovery after ECs was not significantly different when animals were subjected to the second protocol, with separate animals euthanized at each time point, as when they were subjected to the preliminary exercise protocol (Fig. 4A). It is unlikely that the increased injury seen in the MKO mice is due to increased fatigue susceptibility, because there was no difference between the response of MKO and wild-type mice to 50 isometric contractions (Fig. 4B). In addition, torque production in the contralateral legs of both genotypes was not significantly affected by the exercise protocol, suggesting that any differences between genotypes were not due to differential response to the stress of anesthesia or systemic exercise tolerance (Fig. 4C).

mRNA quantification. Both MKO and wild-type mice showed a robust increase in *MyoD* and *MLP* expression after the EC bout, and the expression levels of *MLP* and *MyoD* were increased significantly more in MKO than wild-type mice

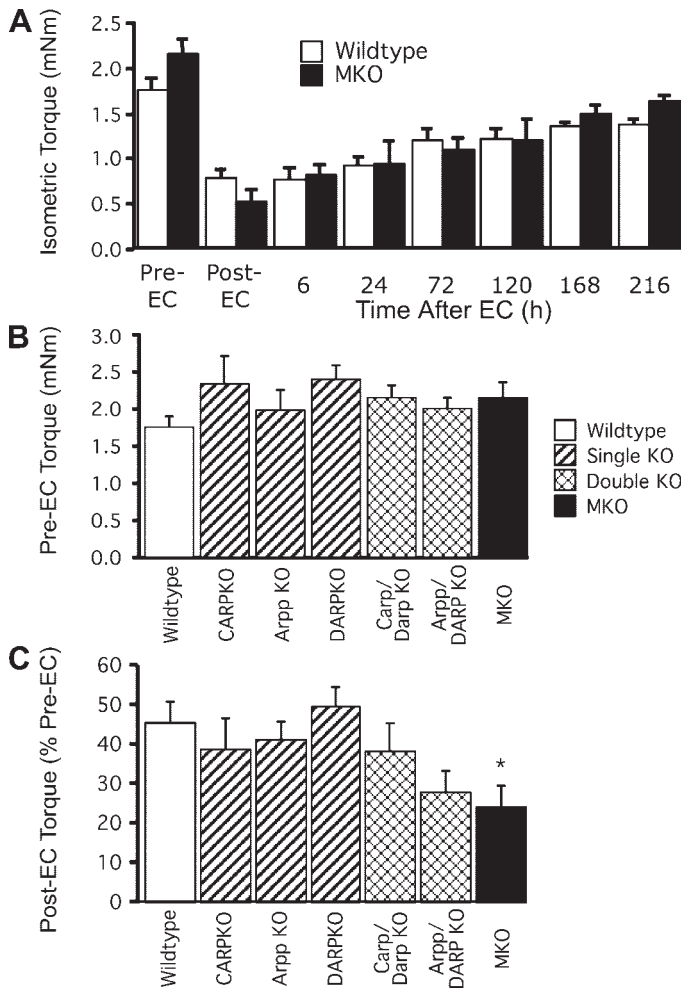


Fig. 3. Physiological response of WT and MKO mice to a bout of 50 eccentric contractions (ECs). *A*: isometric torque measured before (pre-EC), immediately after (post-EC), and then 6, 24, 72, 120, 168, and 216 h (9 days) after the eccentric bout in 4 WT and 4 MKO mice. Torque was measured in the same animals over time. Torque decreased significantly immediately after the EC bout ($P < 0.0001$) and remained significantly decreased even 9 days later ($P < 0.05$). No overall difference in the response of the different genotypes to the EC bout was found by 2-way ANOVA. Single and double knockouts (KOs) were also tested, with similar results (data not shown). *B*: isometric torque before commencing the exercise bout. No significant differences among genotypes were seen. *C*: torque immediately after the EC bout, presented as % of the pre-EC torque. MKO mice showed significantly decreased torque (i.e., greater injury) immediately after the EC bout compared with WT controls, and this decrease was not seen in either the single KOs or double KOs. $n = 4$ for all figures; * $P < 0.05$ compared with WT. *CARP*, cardiac ankyrin repeat protein; *DARP*, diabetes-associated ankyrin repeat protein; *Arpp*, ankyrin repeat protein with PEST and proline-rich region.

(Fig. 5, *A* and *B*, respectively). This increase was not seen in any of the three single or two double KO genotypes. For *MLP*, expression levels in contralateral legs for both genotypes remained low, not significantly different from expression levels in true control muscles that were not exposed to any exercise or significantly different between genotypes. For *MyoD*, expression levels were higher in MKO muscles in both the exercised and contralateral leg compared with wild-type muscles at 6 and 12 h after EC, and in only the EC leg at 24 h. Interestingly enough, however, the initial expression levels of *MyoD*, before the animal was subjected to any exercise, were

actually lower in MKO compared with wild-type muscles ($P < 0.05$). No increased expression was seen in any genotype at any time point after EC for either desmin or myogenin (data not shown).

DISCUSSION

In this study, we present an investigation into the skeletal muscle phenotype of mice lacking all three members of the *MARP* family, as single, double, and, ultimately, triple KO mouse models. These three genes are upregulated in skeletal muscle in many different physiological settings, ranging from muscular dystrophy to mechanical manipulation (2, 10, 21, 42), but this is the first report examining the effects of this gene family on normal skeletal muscle physiology. Although the phenotypic changes seen when all three *MARP* family members are knocked out are

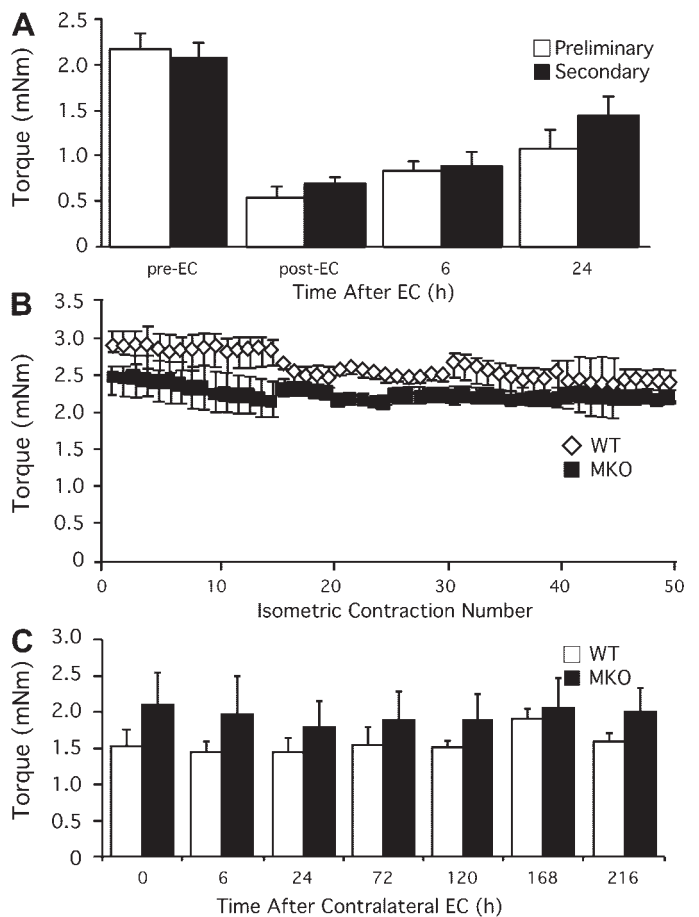


Fig. 4. Controls for the response of WT and MKO mice to ECs. *A*: isometric torque before (pre-EC), immediately after (post-EC), and then 6 and 24 h after the eccentric bout compared between the preliminary protocol (same animals over time) and the secondary protocol (separate animals euthanized for each time point such that each animal was only tested twice, at initial injury and then at a defined time period afterward; see METHODS for details). Two-way ANOVA showed a significant effect of time ($P < 0.0001$) but no effect of exercise protocol. *B*: isometric torque loss during a bout of 50 isometric contractions. Two-way ANOVA revealed a significant drop over time for both genotypes ($P < 0.05$) but no significant difference between genotypes. *C*: isometric torque of legs contralateral to those exercised by EC, up to 9 days (216 h) after 1 EC bout. Two-way ANOVA showed a significant effect of genotype ($P < 0.05$) but no effect of time. There were no significant differences between genotypes at any individual time point. $n = 4$ for all panels.

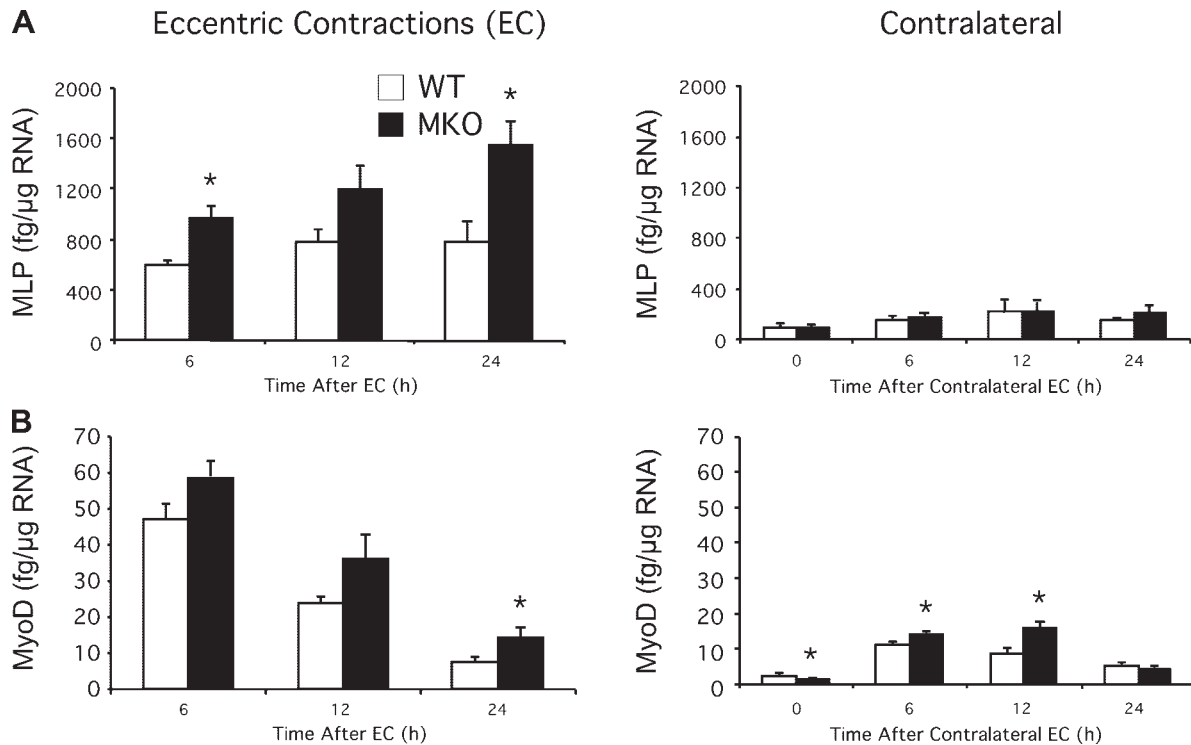


Fig. 5. *MLP* and *MyoD* expression in WT and MKO TA. All RNA analysis was also performed on exercised and contralateral TAs from single *CARP*, *Ankrd2*, and *DARP* KOs, and double *CARP/DARP* and *Ankrd2/DARP* KOs, with expression levels similar to those seen in WT mice. **A:** *MLP* expression levels after EC (left) or in control contralateral legs (right). A 3-way ANOVA was performed, with significant effects of leg (exercised vs. contralateral; $P < 0.0001$), time point ($P < 0.05$), and genotype ($P < 0.0001$). *MLP* expression in the contralateral unexercised leg was not significantly different between genotypes, and expression levels did not increase above levels at time 0 in the contralateral legs. **B:** *MyoD* expression levels after EC (left) or in control contralateral legs (right). A 3-way ANOVA was performed, with significant effects of leg (exercised vs. contralateral; $P < 0.0001$), time point ($P < 0.0001$), and genotype ($P < 0.0005$). *MyoD* expression increased more in the EC leg of MKO mice than WT mice, although this difference was only significant at 24 h ($P < 0.05$). WT mice showed a slightly higher basal expression level of *MyoD* than MKO mice ($P < 0.05$, time 0), but *MyoD* expression increased more in MKO mice than WT mice, even in the contralateral leg ($P < 0.0001$). *MyoD* expression returned to baseline levels in WT animals by 24 h after the exercise but remained elevated in MKO mice at this time point ($P < 0.02$). $n = 4$ for all panels. * $P < 0.05$ between WT and MKO at that specific time point.

mild, they nonetheless provide intriguing insight into the function of the *MARPs* in skeletal muscle.

The passive mechanical data suggest that *MARPs* serve a structural role, such that in their absence skeletal muscle fibers are less stiff. The effect is subtle, and we did not detect it with the stretch to failure method (although there is a trend toward MKO being less stiff than wild type, Fig. 2A), which allows the fibers to stress-relax for 2 min before being stretched again. The viscoelastic phenomena that occur during these 2 min are complex and poorly understood, and it is possible that the absence of the *MARPs* has a small effect compared with the other mechanical events. When stretched at faster speeds, however, the absence of the *MARPs* was quite noticeable (Fig. 2, B and C), indicating that the *MARPs* play a role in regulating single muscle fiber stiffness. One possibility for how this regulation occurs is suggested by the data showing a longer resting sarcomere length in MKO mice compared with controls (Fig. 2D), which would directly result in the observed decrease in muscle fiber stiffness. The longer sarcomere length could be due to the expression of a longer isoform of titin in the triple *MARP* KO (Fig. 2E). Titin is the main molecule responsible for passive tension in striated muscle, and may be involved in setting resting sarcomere length. Different lengths of titin have been shown to be expressed in different tissues (34, 43), suggesting that there is precise, complex, and poorly understood regulation of titin

isoform expression in skeletal muscle. These data suggest an intriguing connection between titin length expression and the *MARP* proteins.

In addition to altering the expression of titin isoforms, the *MARPs* may also have a mechanical contribution to passive tension, potentially mediated through their direct binding to the I band region of titin (26). In this scenario, the *MARPs* act to “clamp down” upon titin, perhaps stiffening it directly or tethering it more tightly to actin. When the *MARPs* are removed, therefore, titin would be somewhat more flexible, resulting in a decreased fiber tangent modulus. How the *MARPs* influence the length of titin and therefore the passive mechanical behavior of skeletal muscle fibers requires further testing.

In addition to the effect on in vitro passive mechanics, the larger than normal drop in torque after ECs (Fig. 3C) suggests that the *MARPs* may play a role in the stability of skeletal muscle under high active stress. The cause of decreased torque immediately after ECs is under much debate (35, 45), with explanations ranging from mechanical to neurological to energetic, and we are not certain of the cause of the decreased torque in our model. One obvious possibility for the large decrease in torque after exercise in the MKO mice compared with controls was increased fatigue. This is unlikely to be the case, however, because when the mice are exercised isometri-

cally, which should be a larger metabolic load on the muscle than ECs, the drop in torque is if anything larger in wild-type than MKO mice (Fig. 4B). We are unaware of any role that the *MARPs* might play in excitation-contraction coupling. We believe, therefore, that it is most likely that the larger drop in torque after ECs in MKO mice is due to mechanical differences between the genotypes. The decreased passive tension, as described above, could be due to the absence of the *MARPs* causing titin instability and increased titin length. This instability could lead to increased sarcomere length inhomogeneity, which would result in a greater amount of damage to the fibers under high stress during EC, perhaps through increased numbers of "popped" sarcomeres (28). This would result in decreased torque immediately after exercise, as seen experimentally. Increased expression of this gene family after ECs would be consistent with their providing structural support for the muscle fiber: if absence of these genes leads to increased structural injury (i.e., sarcomere length instability), perhaps increased expression leads to protection from EC-induced injury. Further studies are required to test this hypothesis.

These data suggest that, in addition to their structural role, *MARPs* have a physiological influence on the gene expression program of skeletal muscle cells. The evidence for this is based on a number of observations. First, fiber type distribution in three different MKO muscles, the soleus, TA, and EDL, is shifted toward being more slow (Fig. 1, B–D). Although this shift was not statistically significant, the trend toward slower fiber type is consistent across all three muscles and is unusual, as fiber type distribution is usually quite invariant in mice (8). Fiber type is determined by a complicated set of genes, many of which are still being determined (5, 33, 36), and a shift in fiber type is suggestive of a fundamental change in the "thinking" of the muscle cell, either during development or in response to normal use. In addition, we have provided direct evidence that MKO mice have enhanced expression of two genes known to increase after ECs, keeping a similar time course of regulation known to occur after ECs (4, 32), *MyoD* and *MLP*. Both genes play an important positive role in either the regeneration of muscle (*MyoD*; Refs. 25, 47), or the response of skeletal muscle to exercise (*MLP*; Refs. 38, 46). Increased expression in the absence of the *MARPs* indicates that the *MARPs* have an inhibitory influence on these genes. Further evidence for this comes from the physiological response of the muscle to ECs: although the MKO mice showed greater injury (perhaps due to structural weakness resulting from the absence of *MARPs*), if anything they recovered faster and more completely from the injury compared with their wild-type counterparts (Fig. 3A; perhaps due to an enhanced gene expression response resulting from the absence of *MARPs*). Whether or not this increased recovery despite increased initial injury is due to increased *MyoD* and/or *MLP* expression remains unknown. The long-term effect of the absence of *MARPs* on muscle function after ECs is also unknown, because this study only examines changes that occur up to 9 days after injury.

The experiments presented here do not offer direct evidence as to how this influence on gene expression programs could occur. While it is possible that *MARPs* act directly as transcription inhibitors in the nucleus (19, 23, 48), this is not certain. Other possibilities include simply greater structural instability leading to greater transcription stimulation, or al-

tered mechanical behavior of individual muscle fibers causing other stress sensors to transduce an altered gene expression message. In addition, the *MARPs* have at least two known binding partners in the sarcomere, titin and myopalladin (26), and there is some suggestion that even more interactions may occur (23). The extent of interconnectedness of various proteins within the sarcomere is immense (12), and it is therefore possible that the absence of the *MARPs* causes a general disorganization within the sarcomere, leading to altered gene expression among other effects.

It is interesting to speculate as to why normal muscle fiber would increase expression of this gene family in response to exercise, if it in fact inhibits the response to the exercise (4). There are examples in muscle physiology of phenomena having a short-term benefit but being detrimental in the long term, such as is possible with nonsteroidal anti-inflammatory drugs (27). In the absence of *MARPs*, while there seems to be a short-term increase in the expression of some genes involved with the regeneration of muscle and normal or possibly even enhanced functional torque recovery soon after injury, it is unknown whether MKO mice could show, for example, protection from injury due to ECs after training with ECs, or if they could show other normal training effects such as increased oxidative capacity or hypertrophy with exercise, all of which may require a more complicated and carefully regulated gene expression response. It is conceivable, for example, that the *MARPs* may help stimulate the immune response to injury. Without a robust immune response, the tissue would appear to recover faster than occurs with a complete response, but would not necessarily have a complete recovery at the cellular level and therefore might show a long-term deficit in function. It is also possible that the *MARPs* promote another beneficial response of the muscle to ECs that has not yet been determined, or that the increased expression of *MARPs* after EC is primarily due to the structural effects of this protein described above and the effect on gene expression is only secondary or is an epiphenomenon associated with the knockout model. More studies are needed to determine whether any of these possibilities are correct, because the studies presented here were limited to a single EC bout with torque measured only up to 9 days later.

The results presented here also offer some intriguing insights into how closely related the *MARP* family really is. While 50% homology is relatively high, the tissue distribution of the three *MARPs* is different (*CARP* highest in cardiac muscle, *Ankrd2* and *DARP* in skeletal), and there is not any upregulation or compensation of the remaining *MARP* when one or more are removed (data not shown), at least at the mRNA level. The question of the functional homology of the three genes, therefore, remains unclear. All experiments except for the passive mechanics were performed on single KO of *CARP*, *Ankrd2*, and *DARP* and double KO of *CARP* and *DARP* or *Ankrd2* and *DARP*, and yet the only significant changes were seen with the triple KO lacking all three proteins. There are two possible explanations for this phenomenon. The first is that the three proteins act independently from one another, and the phenotype of individual and double KO is too mild to be detected in our experiments. When all three are missing, however, a significant effect is seen. The second possibility is that the *MARPs* are, in fact, able to compensate for each other physiologically, such that it becomes necessary to remove all

three in order to see an effect. Given that these three genes do have similar binding partners and intracellular distributions (26), the latter seems likely. It should be noted, however, that double KO's of *CARP* and *Ankrd2* were not examined, and it therefore remains possible that only these two *MARF* family members compensate for each other. Given that only these genes, and not *DARF*, are upregulated after ECs (4), this remains a distinct possibility.

In summary, the *MARF* family plays an important role in the normal structure and function of skeletal muscle. Our data suggest that these genes are structural, providing stability within the sarcomere and protecting the muscle against EC-induced muscle injury, and perhaps also gene regulatory, modulating the expression of genes such as titin, *MLP*, and *MyoD* known to be important for the response of muscle to exercise and even the basal functioning of the muscle cell. Whether these effects are direct (i.e., direct transcriptional regulation) or indirect (i.e., resulting from structural instability leading to increased injury and therefore increased stimulation for gene expression or alterations in the behavior of *MARF* family binding partners) remains to be determined.

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