Localization and function of Xino in mouse skeletal muscle

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Feng HZ, Wang Q, Reiter RS, Lin JL, Lin JJ, Jin JP. Localization and function of Xino in mouse skeletal muscle. Am J Physiol Cell Physiol 2013; doi:10.1152/ajpcell.00005.2013.—The Xin repeat-containing proteins were originally found in the intercalated discs of cardiac muscle with implicated roles in cardiac development and function. A pair of paralogous genes, Xinα (Xirp1) and Xinβ (Xirp2), is present in mammals. Ablation of the mouse Xinα (mXinα) did not affect heart development but caused late-onset adulthood cardiac hypertrophy and cardiomyopathy with conductive defects. Both mXinα and mXinβ are also found in the myotendinous junction (MTJ) of skeletal muscle. Here we investigated the structural and functional significance of mXinα in skeletal muscle. In addition to MTJ and the contact sites between muscle and perimysium, mXinα but not mXinβ was found in the blood vessel walls, whereas both proteins were absent in neuro-muscular junctions and nerve fascicles. Coimmunoprecipitation suggested association of mXinα with talin, vinculin, and filamin, but not β-catenin, in adult skeletal muscle, consistent with our previous report of colocalization of mXinα with vinculin. Loss of mXinα in mXinα-null mice had subtle effects on the MTJ structure and the levels of several MTJ components. Diaphragm muscle of mXinα-null mice showed hypertrophy. Compared with wild-type controls, mouse extensor digitorum longus (EDL) muscle lacking mXinα exhibited no overt change in contractile and relaxation velocities or maximum force development but better tolerance to fatigue. Loaded fatigued contractions generated stretch injury in wild-type EDL muscle as indicated by a fragmentation of troponin T. This effect was blunted in mXinα-null EDL muscle. The results suggest that mXinα play a role in MTJ conductance of contractile and stretching forces.

Xino; myotendinous junction; muscle fatigue; fast troponin T fragmentation

THE FAMILY OF XIN REPEAT-CONTAINING PROTEINS is a relatively new class of actin-binding protein primarily found in striated muscle cells (10, 19, 33, 44, 57, 59, 62). The majority of Xin proteins in muscle cells is present in adherence junctions: the intercalated discs of cardiac muscle and the myotendinous junction (MTJ) of skeletal muscle (20, 44, 49, 57, 59). In mammals, a pair of paralogous genes encode two protein isoforms, Xinα [also called cardiomypathy-associated 1 (Cmya1)] or Xin actin-binding repeat-containing 1 (Xirp1)) and Xinβ (also called Cmya3, myomaxin, or Xirp2; Ref. 19). Through alternative splicing, each gene is capable of producing several protein variants (20, 43, 57, 61).

In the heart, mXinα directly interacts with β-catenin at the intercalated discs, providing a link between N-cadherin/catenin complex and the underlying actin cytoskeleton. Mouse hearts deficient in mXinα exhibited progressive structural defects in the intercalated discs and late-onset cardiac hypertrophy and cardiomyopathy with conductive abnormalities (6, 20, 30, 43). Therefore, mXinα may play an important role in the N-cadherin-mediated adhesion and signaling between cardiomyocytes (10, 20, 43). Mouse hearts without mXinβ had mislocalization of mXinα and N-cadherin and severe growth retardation, diastolic dysfunction, and postnatal lethality (61).

In skeletal muscle, which has no intercalated discs, the majority of mXinα is localized to the MTJs. MTJ is the interface between skeletal muscle cells and tendon and as such bears the contractile and stretching forces. The molecular mechanisms for the MTJ components to regulate and/or transmit these forces remain largely unknown. The mouse Xinα (mXinα) is a modular protein located at MTJ, capable of binding to actin filaments and interacting with many actin-binding proteins, including filamin, Mena/VASP, α-actinin, tropomyosin, gelsolin, and vinculin (10, 19, 24, 44, 57).

During development, skeletal muscles differentially express three types of cadherin (M-, N-, and R-cadherin), and each type of cadherin-mediated adhesion is specifically required for the cellular events such as commitment, recognition, alignment, fusion, and terminal differentiation in myogenesis (8, 17, 40, 46, 64). However, none of them was detected at the MTJs of adult skeletal muscle (27), suggesting minimal contribution to MTJ adhesion by the cadherin/catenin-mediated system. Therefore, the role of mXinα in the function of MTJ requires further investigation.

Several components of the dystrophin-glycoprotein complex-mediated and integrin-mediated adhesion systems have been shown to concentrate at the MTJs (1, 7, 9, 11, 29, 48, 55). The integrin specifically enriched in the MTJ is αβ1 (4, 22, 45). The β1-subunit of integrin has been further shown to bind talin (23, 53) and filamin (18, 37, 56). We (10) and others (57) previously showed that mXinα was capable of interacting with filamin and vinculin but not talin. It has been demonstrated that filamin can bind γδ- sarcoglycans of dystrophin-glycoprotein complex (21, 54), whereas vinculin is a known talin-binding protein (5). Therefore, mXinα may potentially play a role at the MTJ in linking these two adhesion systems for the transmission of forces.

We previously reported that in pressure overload-induced hypertrophic hearts both mXinα and mXinβ are significantly upregulated and integrated in the intercalated discs (62), suggesting a load-related function. In skeletal muscle, eccentric contractions involving stretched/loaded shortenings cause injuries in myofibril and cytoskeleton components (15, 31, 32, 42). The morphological and molecular changes detected in the stretch-induced muscle injury include Z-band dissolution, sarcolemmal damage, misorganized desmin intermediate filaments, misaligned myofibrils, and upregulation of talin and vinculin. In contrast, electron microscopy detected only very moderate morphological alteration at MTJ (15). Despite the...
minimal alteration at MTJ, studies of human and rabbit muscle strain injury have demonstrated that the region of muscle near MTJ is most vulnerable to eccentric contractions (41, 52).

In the present study, we investigated the localization and function of mXin in skeletal muscle. Immunofluorescence microscopy using Xin isoform-specific antibodies determined the detailed localization of mXin. Coimmunoprecipitation revealed the association of mXin with known MTJ-associated proteins. Contractility and fatigability were investigated in muscles from wild-type and mXin-null mice. Compared with wild-type control, mXin-null muscle was more tolerant to fatigue and more resistant to stretch.

RESULTS

The detailed localization of mXin in skeletal muscle by immunofluorescence microscopy

### MATERIALS AND METHODS

**Animals.** All animal procedures were performed using protocols approved by the Wayne State University and University of Iowa Institutional Animal Care and Use Committees. The mXin-null mouse line was generated as described previously (20) and has been backcrossed to and maintained in C57BL/6J strain. Age-matched adult wild-type and mXin-deficient mice were used in all experiments for comparisons.

**Histology and immunofluorescence assay.** Immediately after euthanasia, gastrocnemius (Ga), tibialis anterior (TA), extensor digitorum longus (EDL), soleus (Sol), tongue (Ton), and diaphragm (Di) muscles were rapidly dissected and frozen in liquid nitrogen-cooled isopentane as described previously (26). Seven-micrometer cryosections were examined with hematoxylin and eosin, Masson’s trichrome, and immunofluorescence staining as previously described (20).

Single- and double-label indirect immunofluorescence microscopy was carried out as previously described (20, 49). Several rabbit polyclonal antibodies against Xin isoforms were used: U1013 recognizing both mXin and Xinβ (49), U1697 recognizing only mXin, U1741 recognizing only mXinβ, and U1040 recognizing only mXinβ (20, 61). The specificities of the anti-Xin antibodies have been characterized in details in the cited publications. The endogenous mouse IgG in mouse muscle samples studied is not recognized by the goat anti-rabbit IgG second antibody used in our study.

Other antibodies used were mouse monoclonal antibody (mAb) CGβ recognizing smooth muscle and nonmuscle tropomyosins (16, 34) and anti-neurofilament 200 mAb N52 (Sigma, St Louis, MO). Alexa Fluor 488-conjugated α-bungarotoxin (B-13422) was purchased from Molecular Probes (Eugene, OR).

**Coimmunoprecipitation and Western blot analysis.** Gastrocnemius muscle of adult mice was homogenized in an immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, and 0.1% SDS plus protease inhibitor mixture; Roche Applied Science, Indianapolis, IN). After preincubation with protein G-Sepharose beads, total muscle fiber cross-sectional area was measured with a caliper, based on defined anatomical landmarks. Fiber length (Lf) of mouse EDL muscle was estimated by multiplying Lf by the Lf/Ls ratio of 0.45 (2). The mean LF values of EDL were 5.58 ± 0.03 and 5.61 ± 0.02 mm (means ± SE) for muscles of adult wild-type and mXin-KO groups, respectively.

**Fatigue contraction protocol.** After baseline contractions were measured, the EDL muscle was treated with a fatigue protocol consisting of 150 repeats of 200 ms/s stimulation at 300 Hz. After the fatigue protocol, the muscle was allowed to recover under 200-ms 300 Hz stimulation/min for 20 min when a plateau of recovery in tetanic force development occurred.

**Stretch-contraction protocol.** A stretch-contraction protocol mimicking eccentric contractions was carried out after the baseline functional measurement or fatigue protocol. During each of the 200 ms/s 300-Hz stimulations, stretch was applied from the 100th ms to 200th picking eccentric contractions was carried out after the baseline functional measurement or fatigue protocol. During each of the 200 ms/s 300-Hz stimulations, stretch was applied from the 100th ms to 200th ms in a ramp to increase the muscle fiber length (Lf) by 20%. This contraction-stretch cycle was repeated for 150 times. The length changes corresponded to 4–20% of the EDL muscle fiber length and are within the physiological range for most skeletal muscles (12). The same protocol as that used for recovery after fatigue contractions was applied to allow the muscle to recover from the stretch contraction protocol and to evaluate the tolerance of muscles to such loaded contractions.

After in situ contractile measurements, the mice were euthanized under anesthesia and the EDL muscles were rapidly dissected, trimmed to remove tendons, weighed after blotting dry, and frozen at −80°C for protein analysis. Total muscle fiber cross-sectional area was calculated by dividing the muscle wet mass by the product of the Lf and the specific density of skeletal muscle tissue, 1.06 mg/mm3. The EDL muscles subjected to contractile measurements, fatigue and/or stretch-contractions were examined compared with the control muscle from the resting leg.

**Examination of the integrity of myofilament proteins.** Immediately after being taken out from deep freezer, the frozen muscle tissue was homogenized in 40 vol (wt/vol) of SDS-gel sample buffer containing 2% SDS, 10% glycerol, 50 mM Tris-base, and 2% 2-mercaptoethanol, pH 8.8, using a high speed mechanical homogenizer (PRO Scientific, Oxford, CT). The homogenized muscle samples were immediately heated at 80°C for 5 min, clarified by high speed centrifugation, and stored at −80°C for SDS-PAGE and

In situ measurement of muscle contraction. Littermates of adult wild-type and mXin−/− KO mice of body weight ~22 g were used to study muscle function employing an in situ force measurement protocol (3). The mouse was anesthetized with intraperitoneal injection of pentobarbital (100 mg/kg body wt). A small incision was made at the ankle to expose the distal tendon of EDL muscle. Carefully to avoid stretch injury, the tendon was dissected from surrounding connective tissues and cut near the bone attachment. The animal was then placed on a platform maintained at 37°C with circulating warm water, and the knee was immobilized by mounting between a pair of screws instrumented on the platform (Aurora Scientific, Aurora, Ontario, Canada). The end segment of tendon was folded back and tied with 3–0 silk suture to securely attach to the lever arm of a servomotor (model 300B; Aurora Scientific) that controls the length of the muscle and measures the force development. The small incision site and the exposed muscle and tendon tissue were kept under moisture with dripping Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.25 mM MgSO4, 1.2 mM KH2PO4, 2.25 mM CaCl2, 11 mM glucose, and 21 mM NaHC03) equilibrated in 5% CO2-95% O2, pH 7.4 at 37°C.

The endogenous mouse IgG in mouse muscle samples was cleared by centrifugation at 12,000 g at 4°C, the homogenate (150 µl) was folded back and tied with 3–0 silk suture to securely attach to the lever arm of a servomotor (model 300B; Aurora Scientific) that controls the length of the muscle and measures the force development. The small incision site and the exposed muscle and tendon tissue were kept under moisture with dripping Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.25 mM MgSO4, 1.2 mM KH2PO4, 2.25 mM CaCl2, 11 mM glucose, and 21 mM NaHC03) equilibrated in 5% CO2-95% O2, pH 7.4 at 37°C.

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Western blot analysis. The total muscle protein extracts were resolved on 14% Laemmli gel with an acrylamide:bisacrylamide ratio of 180:1. The protein bands were visualized by staining the gel with Coomassie Brilliant Blue R250.

Duplicate gels were electrically blotted on nitrocellulose membranes. TBS containing 1% BSA was used to block the nitrocellulose membranes at room temperature for 30 min. The membranes were then incubated with mAb T12 recognizing fast skeletal muscle tropinin T (TnT; Ref. 36) and mAb TnI-1 recognizing all three muscle type TnI isoforms (25), both diluted in TBS containing 0.1% BSA, at 4°C overnight. After high stringency washes with TBS plus 0.5% Triton X-100 and 0.05% SDS, the membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG second antibody (Santa Cruz Biotechnology), washed again, and developed in 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium substrate solution.

Data analysis. Two-dimensional densitometry was carried out to quantify SDS-PAGE gel and Western blots on images scanned at 600 dots/in. Statistical significance for all quantitative data was determined using Student’s t-test or two-way ANOVA test.

RESULTS

Similar but distinguishable localizations for mXinα and mXinβ in mouse skeletal muscles. Immunofluorescence microscopy using anti-mXin polyclonal antibodies showed major staining in the MTJ of gastrocnemius (Ga; Fig. 1, B and D), TA (data not shown), EDL (data not shown), and Ton (Fig. 1M). An additional contact site between the muscle fibers and perimysium of EDL was stained with anti-mXinα U1697 and with anti-mXinβ U1040 (arrowheads in Fig. 1, F and H, respectively). Therefore, mXinα and mXinβ were similarly localized to the MTJs and the contact sites between muscle and perimysium. It should be noted that the presence of these two proteins was uneven at these contact sites, particularly at the...
perimysium sites. Not all of the cells in contact with the perimysium were labeled (Fig. 1, F and H). This variation suggests that cells surrounding the perimysium make focal contacts at different planes of the section throughout the perimysium.

In addition to MTJ and the contact sites, U1013 (recognizing mXinα and mXinβ) and U1697 (specific to mXinα) both stained blood vessels within the perimysium of wild-type EDL, and Ga muscles (Fig. 1, F and K, arrows). These blood vessels were confirmed by double-staining of mAb CGβ6 against smooth muscle and nonmuscle tropomyosins (Fig. 1J). In contrast, U1040 (mXinβ-specific) antibody did not stain the blood vessel within the perimysium (Fig. 1H, arrow). Moreover, blood vessels in mXinα-null muscles were not stained by any of these anti-mXin antibodies (data not shown). The results suggest that mXinα but not mXinβ is present in the blood vessel walls.

Absence of mXin proteins in neuromuscular junctions and nerve fascicles. In adult skeletal muscle, neuromuscular junctions are specialized contact sites for motor neuron to control muscle activity, and nerve fascicles are formed by a bundle of nerves running through the perimysium. Double-label immunofluorescence microscopy on wild-type gastrocnemius muscle cryosections using anti-mXin antibody U1013 together with either fluorescence-tagged α-bungarotoxin for neuromuscular junction or anti-neurofilament 200 for nerve fascicle showed that neither of these structures (Fig. 2, B, C, E, and F) contained a detectable amount of mXin protein.

Coimmunoprecipitation of mXin with known MTJ components. Several known adhesion components, such as filamin, talin, vinculin, β1-integrin, and dystrophin also concentrate in MTJs of skeletal muscle (49, 55, 57). Our previous immunofluorescence microscopy on muscle sections showed the colocalization of mXinα with vinculin (49). Yeast two-hybrid assay using a heart cDNA library identified filamin, vinculin, and tropomyosin as mXinα-interacting partners (10). As shown in Fig. 3A, mXinα was coimmunoprecipitated from gastrocnemius muscle extracts using anti-tropomyosin (LC24), antifilamin, anti-vinculin, or anti-talin mAb but not anti-β-catenin mAb or normal mouse serum control. mAb LC24 recognizes tropomyosin isoform 4 that is known to locate together with γ-actin external to the sarcomere but adjacent to the Z-line and in the subsarcolemmal region including MTJ in skeletal muscle cells (28, 58). The antibodies used in our experiments specifically precipitated their respective antigens from skeletal muscle extract except for anti-β-catenin (Fig. 3B and data not shown for LC24), while the same anti-β-catenin mAb was able to coimmunoprecipitate mXinα from heart extract (Fig. 3C) (10). These results suggested that mXinα formed complexes with talin, vinculin, filamin, and nonmuscle tropomyosin at the contact sites with potential roles in adhesion and/or cell signaling. The fact that skeletal muscle extract contained very little β-catenin suggests that the N-cadherin/β-catenin-mediated adhesion may play only minor roles at those contact sites in adult skeletal muscle.

Structure and molecular changes in mXinα-null muscles. To study the role of mXinα in skeletal muscle, we histologically and molecularly characterized muscle tissues from wild-type and mXinα knockout mice. Figure 4 shows the comparisons of hematoxylin and eosin-stained muscle sections from Ton, Di, and Sol of wild-type (mXinα+/+) and mXinα−/− mice. No
mXino IN MYOTENDINOUS JUNCTION

Fig. 3. mXino is associated with tropomyosin, filamin, vinculin and talin in skeletal muscle extract. A: coimmunoprecipitates (IP) from total proteins extracted from gastrocnemius muscle using various antibodies were analyzed by immunoblot (IB) using anti-mXino antibody. mXino was present in the immunoprecipitates of anti-tropomyosin, anti-filamin, anti-vinculin, and anti-talin but not anti-β-catenin antibodies or control normal mouse serum. B: various IP products were examined with Western blot using respective antibodies. Results showed that the gastrocnemius muscle extract contained very little β-catenin. C: same anti-β-catenin antibody was able to coimmunoprecipitate mXino from total protein extract of adult mouse heart (10).

Fig. 4. mXino-null diaphragm muscle fibers showed hypertrophy. Hematoxylin and eosin-stained cryosections were prepared from Ton (A and B), diaphragm (D; C and D), and soleus (Sol; E and F) muscles of 7-mo-old wild-type (mXina+/+) and mXina−/− mice. No centrally localized nuclei or obvious alterations in gross morphology was detected in mXina-null muscles. However, the cross-sectional area of mXina−/− diaphragm muscle fibers appeared larger than the wild-type counterparts, indicative of hypertrophy. Bar in A for A and B = 250 μm; bar in D for C and D = 20 μm; bar in F for E and F = 50 μm.

crisco. There was no obvious alteration in the location of talin in MTJ of mXino-deficient muscles (data not shown). In addition, no overt changes in the other MTJ protein components were found in the mXino-null muscle (data not shown). The results that talin, vinculin, and integrin were still coassociated in mXino-null muscle indicate that mXino was not required for the association or the stabilization of the protein complex.

The lack of changes in expression levels of mXinβ, dystrophin, talin, metavinculin, vinculin, filamin, α-tubulin, and β-tubulin was further confirmed by Western blot analyses of Ga, EDL, and Di muscle samples from 5.5 and 8.5-mo-old wild-type and mXino−/− and mXino+/− mice (data not shown).

Effects of mXino-null on muscle function. Isometric and stretched/eccentric type contractile protocols (Fig. 5) were used to compare the function and fatigability of mXino-null and wild-type muscles. mXino-null and wild-type EDL mus-
cles showed no significant difference in contractility (Table 2), indicating that deletion of mXin did not affect the baseline contractility.

In isometric fatigability test, a higher resistance to fatigue with better recovery was found for mXin-null EDL muscle compared with that of wild-type controls (Fig. 6). In the stretch-loaded fatigue contraction test (Fig. 5B), EDL muscle of mXin-null mice showed the same fatigability, as well as recovery, as that of wild-type control muscle (data not shown).

mXin-null EDL muscle exhibited minimized fast TnT fragmentation. Western blot analysis detected a fragment of fast skeletal muscle TnT (fsTnT) in wild-type mouse EDL muscles, which increased after loaded fatigue contractions (Fig. 7). In contrast, no significant production of the fast TnT fragment after loaded nonfatigue contractions (Fig. 7A) or unloaded fatigue contractions (Fig. 7B). Stretch-loaded nonfatigue contractions did not increase the level of fast TnT fragmentation either (Fig. 7C).

SDS-PAGE showed no apparent change of protein profile in mXin-null EDL muscle compared with the wild-type control (Fig. 8A). The fast TnT fragmentation in wild-type EDL muscles reflecting an injury after loaded fatigue contractions was not detected in EDL muscle of the resting leg. No fragmentation was found for TnI, another subunit of the troponin complex (Fig. 8A).

Table 1. Comparisons of cross-sectional area and roundness of soleus and diaphragm muscle fibers of wild-type and mXin-null mice

<table>
<thead>
<tr>
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<th>CSA, µm²</th>
<th>Roundness, %</th>
<th></th>
<th>n</th>
<th>CSA, µm²</th>
<th>Roundness, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>mXin+/+</td>
<td>60</td>
<td>1,506.14 ± 71.16</td>
<td>73.78 ± 1.23</td>
<td>mXin+/+</td>
<td>38</td>
<td>809.1 ± 59.75</td>
<td>70.28 ± 1.72</td>
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<tr>
<td>mXin−/−</td>
<td>72</td>
<td>1,433.79 ± 39.26</td>
<td>75.43 ± 1.27</td>
<td>mXin−/−</td>
<td>32</td>
<td>1,113.61 ± 82.6</td>
<td>70.29 ± 1.81</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
<td>NS</td>
<td>0.003</td>
<td></td>
<td>NS</td>
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Data are expressed as means ± SE. Cross sections of muscle fibers were stained with hematoxylin and eosin and examined under Leitz Laborlux 12 microscope equipped with Leica DFC320 digital camera. Images were taken and the fiber perimeters were traced manually and analyzed using the dynamic image analysis system (DIAAS) software (50, 51). Fiber cross-sectional area (CSA) was computed from the perimeters, whereas the roundness was computed using the formula $100 \times 4\pi \left(\frac{\text{area}}{\text{perimeter}^2}\right)$, with a perfect circle having 100% roundness and a straight line having 0% of roundness value. NS, not significant ($P > 0.5$ in Student’s $t$-test).
In comparison with the wild-type control, mXinα-null mouse EDL muscle had a significantly lower level of fast TnT fragmentation (Fig. 8A). The results were quantified in Fig. 8B, and the data suggest that the lack of mXinα in MTJ altered force transduction and minimized the stretch injury. In contrast to the fast fiber muscle EDL, the fast TnT fragmentation was minimum in wild-type mouse soleus muscle (slow/oxidative fiber) after the loaded fatigue protocol (data not shown), possibly due to the lower number of fast muscle fibers present in slow type muscle or the different tissue environment.

DISCUSSION

The vast majority of both mXinα and mXinβ was localized to the MTJ and the contact sites between muscle and perimysium. mXinα but not mXinβ was found in the blood vessel walls in the muscle tissues. Similar blood vessel localization of mXinα was observed in mouse heart (data not shown). It was previously reported by Otten et al. (43) that a transiently increased perivascular fibrosis occurred in mXinα knockout mouse hearts. Our mXinα knockout mouse hearts and skeletal muscles did not show any detectable fibrosis phenotype. This discrepancy may be due to the different knockout alleles used. Nonetheless, the function of mXinα in the blood vessel walls merits further investigation.

In mXinα-null mice at 5.5 to 13.5 mo of age, there were subtle histological and molecular changes in most muscles examined (data not shown). Hypertrophy was only detected in mXinα-null diaphragm fibers. Neither the relative expression level of mXinα in diaphragm muscle (data not shown) nor the subtle changes in myofibrillar proteins in mXinα-null muscle (data not shown) could account for the hypertrophy of diaphragm muscle. Previously, we reported that mXinα-null hearts exhibited defects in intercalated discs between 1 and 3 mo of age. During aging, they developed progressive cardiac hypertrophy and cardiomyopathy (20). Therefore, the continuous periodic contractions of diaphragm muscle analogous to that of the cardiac muscle may correlate to the development of hypertrophy in these muscles of mXinα-null mice.

MTJ is an essential structure for the function of skeletal muscle. It transduces forces during muscle contraction and stretch. The presence of the vast majority of both isoforms of mXin in MTJ may suggest a role in the structural integrity as that seen for mXin proteins in the formation of intercalated discs and heart chambers. The evolutionarily more conserved mXinβ is essential for initiating the formation and maturation of intercalated discs (19, 60–62). mXinβ-null mice die postnatally with cardiac chamber defects (61). During the second

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**Table 2. Baseline contractile parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>mXinα Null</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>21.44 ± 0.32</td>
<td>21.57 ± 1.28</td>
</tr>
<tr>
<td>Muscle mass, mg</td>
<td>9.20 ± 0.31</td>
<td>8.73 ± 0.47</td>
</tr>
<tr>
<td>Length, mm</td>
<td>12.40 ± 0.06</td>
<td>12.49 ± 0.05</td>
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<tr>
<td>CSA, mass/length</td>
<td>0.74 ± 0.03</td>
<td>0.70 ± 0.04</td>
</tr>
<tr>
<td>Twitch force, mN</td>
<td>22.23 ± 1.27</td>
<td>19.97 ± 7.46</td>
</tr>
<tr>
<td>Twitch force/CSA</td>
<td>30.07 ± 2.21</td>
<td>29.49 ± 12.27</td>
</tr>
<tr>
<td>Twitch +dF/dt, mN/s</td>
<td>4,281.13 ± 365.71</td>
<td>3,892.86 ± 984.91</td>
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<tr>
<td>Twitch –dF/dt, mN/s</td>
<td>−2,756.17 ± 161.36</td>
<td>−2,204.42 ± 565.63</td>
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<tr>
<td>Twitch TPT, ms</td>
<td>10.13 ± 1.38</td>
<td>10.00 ± 0.69</td>
</tr>
<tr>
<td>Twitch TP50, ms</td>
<td>4.30 ± 0.26</td>
<td>4.20 ± 0.38</td>
</tr>
<tr>
<td>Twitch TR75, ms</td>
<td>13.33 ± 0.57</td>
<td>14.50 ± 1.77</td>
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<tr>
<td>Tetanic force, mN</td>
<td>238.55 ± 6.34</td>
<td>225.12 ± 22.06</td>
</tr>
<tr>
<td>Tetanic/CSA, mN/mm²</td>
<td>322.86 ± 20.08</td>
<td>326.80 ± 47.17</td>
</tr>
<tr>
<td>Tetanic +dF/dt, mN/s</td>
<td>8,965.34 ± 747.94</td>
<td>9,904.12 ± 955.34</td>
</tr>
<tr>
<td>Tetanic –dF/dt, mN/s</td>
<td>−16,426.45 ± 955.81</td>
<td>−15,824.13 ± 1,190.50</td>
</tr>
<tr>
<td>Stretch Force, mN</td>
<td>412.79 ± 15.90</td>
<td>396.61 ± 9.90</td>
</tr>
<tr>
<td>Stretch/CSA, mN/mm²</td>
<td>557.23 ± 23.94</td>
<td>569.73 ± 28.07</td>
</tr>
<tr>
<td>Stretch +dF/dt, mN/s</td>
<td>5,156.26 ± 152.62</td>
<td>5,250.16 ± 489.19</td>
</tr>
<tr>
<td>Stretch –dF/dt, mN/s</td>
<td>−16,349.41 ± 725.86</td>
<td>−14,847.85 ± 450.22</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE; n = 3 mice each in mXinα-null and wild-type groups. Contractile function of extensor digitorum longus muscle was examined in situ under central anesthesia. No difference was found in the twitch contraction of mXinα-null muscle as compared with wild-type control. Tetanic contractions with stretch or without stretch also did not show significant difference between mXinα-null and wild-type muscles. TPT, contractile time to peak twitch tension; TP50, contractile time to 50% of peak twitch tension; TR75, relaxation time to 25% peak tension. Statistical analysis was done using Student’s t-test.

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**Fig. 6. Fatigability and recovery of mXinα-null and wild-type mouse EDL muscles.** During isometric tetanic contractions in situ, force data were sampled every 15 s to plot the curve of fatigability. Shown as the percentage of the maximum force before fatigue, tetanic force decreased rapidly during the 150-s protocol (A) with an 85–90% recovery within 20 min (B). EDL muscle of mXinα-null (mXinα−/−) mice showed less fatigability and better recovery than that of wild-type muscle (*P < 0.05; **P < 0.01 as tested using two-way ANOVA). Values are presented as means ± SE; n = 3 mice each in wild-type and mXinα−/− groups.
week postnatal, upregulated mXinβ colocalized with N-cadherin puncta to form bigger aggregates along the developing intercalated discs between cardiomyocytes (60). In this regard, we also observed similar mXinβ aggregates along the MTJ of mXinα-null muscles (data not shown), suggesting an analogous mechanism for the MTJ formation. The presence of mXinβ in mXinα-null muscle may account for its ability to form MTJs and to maintain functionality.

The potential role of mXinα in the mechanical property of MTJ may also be of functional significance. When twitch and tetanic contractions were compared between wild-type and mXinα/H9251-null EDL muscles, there was no significant difference in baseline contractile parameters (Table 2). However, during isometric tetanic contraction, mXinα-null EDL muscles fatigued slower and recovered from fatigue faster (Fig. 6). The results suggest that the deletion of mXinα at the MTJ may have caused a functional change in transducing contractile forces.

Our coimmunoprecipitation results suggest that mXinα can form a complex with filamin, vinculin, metavinculin, and talin. Both filamin and talin are known to bind the \( \beta_\text{v} \) subunit of integrin (18, 23, 37, 53, 56). \( \alpha_\text{v}\beta_1 \)-Integrin is a transmembrane structural protein concentrated at the MTJ, linking to actin cytoskeleton through talin/vinculin. Alterations in compliance have been observed in \( \alpha_\text{v}\beta_1 \)-integrin (Itga7) knockout mouse muscle (38), consistent with the possibility of the \( \alpha_\text{v}\beta_1 \)-integrin being a load-bearing protein. In our previous yeast two-hybrid study, we showed that mXinα interacted with vinculin and filamin (10). Therefore, it is likely that mXinα associates with filamin, vinculin, and talin at MTJ and plays structural roles and mXinα-null MTJ may have an altered mechanical compliance.

We previously demonstrated that in cardiac muscle pressure overload induced a restrictive proteolytic truncation of cardiac TnT (13). Since pressure overload increases the resistance to the ejection force of the ventricle and thus applies resistance to the shortening of activated cardiac muscle, this stress condition may be considered analogous to that in the loaded fatigue contraction of skeletal muscle. Therefore, the fragmentation of fast skeletal muscle TnT in EDL muscle during loaded fatigue contractions may be a physiological adaptation to this stress condition rather than a simple sign of muscle injury.

The ubiquitin-proteasome system is not anticipated to have a major change here since the outcome of reduced fast TnT truncation in Xin−/− mouse EDL muscle is a highly selective response, rather different from the universal housekeeping function of the ubiquitin-proteasome activity (39).

\( \mu \)-Calpain associated with the myofilaments contributes to the production of cardiac TnT truncation in the heart (65). Since the level of free Ca\(^{2+} \) naturally fluctuates during contraction and relaxation in striated muscle cells, the peak concentration of cytosolic Ca\(^{2+} \) would be sufficient to activate \( \mu \)-calpain. Therefore, we proposed that induction of the restrictive fragmentation of cardiac TnT in pressure overload of cardiac muscle is most likely regulated by the substrate sensitivity to the protease rather than by increasing \( \mu \)-calpain activity. This mechanism may guide future investigations on the production of fast TnT fragmentation in skeletal muscle cells during loaded fatigue contraction, in which high loads on myofibrils during activated contraction may alter the molecular conformation of TnT and increase the sensitivity to proteases.

It is known that the truncated cardiac TnT remains in the myofilaments (65) to have a functional effect on decreasing the
contractile velocity, which is energetically compensatory (14). A decrease in contractility due to such truncation of fast skeletal muscle TnT could also be protective in the cases of loaded fatigue contractions in skeletal muscle, whereas future structural and functional characterizations of the fast TnT fragmentation will verify this hypothesis.

In summary, our study documented detailed information for the location of mXin/H9251 in MTJs and its association with other cell junction proteins. The implication of the role of Xin in the structure and compliance of the MTJ is supported by the finding that the mXin-null mouse EDL muscles had higher tolerance to fatigue and a protective phenotype during loaded fatigue contraction as shown by the significantly reduced fragmentation of fast TnT. The location and function of mXin in skeletal muscle MTJ suggest that it is an interesting target to understand muscle function and injury. The physiological and pathophysiological significance of mXin in skeletal muscle function and adaptation merits further investigation.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Fig. 8. EDL muscle of mXinα-null mice had minimized fast TnT fragmentation during loaded fatigue contractions. A: SDS-PAGE and Western blot of EDL muscles from the testing and control legs of mXinα-null (mXinα−/−) and wild-type (WT) mice showed that the fragmentation of fast TnT detected by mAb T12 in muscles undergone loaded fatigue contractions compared with that in the control muscle. The level of fast TnT fragmentation was significantly lower in mXinα-null EDL muscle than that in wild-type muscle. No degradation of Tnl was detected in the Western blot using mAb TnI-1. B: densitometry analysis quantified the difference and statistical significance of the reduced level of fast TnT fragmentation in mXinα-null EDL muscle during loaded fatigue contraction was examined using Student’s t-test. Values are presented as means ± SE; n = 3 mice each in wild-type and mXinα-null groups. **P < 0.01 vs. wild-type; #P < 0.05 vs. resting control.
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