Localization and function of Xinn in mouse skeletal muscle

Han-Zhong Feng,1 Qinchuan Wang,2 Rebecca S. Reiter,2 Jenny L.-C. Lin,1,2 Jim J.-C. Lin,2 and J.-P. Jin1

1Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan; and 2Department of Biology, University of Iowa, Iowa City, Iowa

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Feng HZ, Wang Q, Reiter RS, Lin JL, Lin JJ, Jin JP. Localization and function of Xinn in mouse skeletal muscle. Am J Physiol Cell Physiol 304: C1002–C1012, 2013. First published March 13, 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 (mXinn) did not affect heart development but caused late-onset adulthood cardiac hypertrophy and cardiomyopathy with conductive defects. Both mXinn and mXinβ are also found in the myotendinous junction (MTJ) of skeletal muscle. Here we investigated the structural and functional significance of mXinn in skeletal muscle. In addition to MTJ and the contact sites between muscle and perimysium, mXinn 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 mXinn with talin, vinculin, and filamin, but not β-catenin, in adult skeletal muscle, consistent with our previous report of colocalization of mXinn with vinculin. Loss of mXinn in mXinn-null mice had subtle effects on the MTJ structure and the levels of several MTJ components. Diaphragm muscle of mXinn-null mice showed hypertrophy. Compared with wild-type controls, mouse extensor digitorum longus (EDL) muscle lacking mXinn exhibited no overt change in contractile and relaxation velocities or maximum force development but better tolerance to fatigue. Loaded fatigue contractions generated stretch injury in wild-type EDL muscle as indicated by a fragmentation of troponin T. This effect was blunted in mXinn-null EDL muscle. The results suggest that mXinn play a role in MTJ conductance of contractile and stretching forces.

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


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-CATENIN, TROPOMYOSIN, GELSOLIN, AND VINCULIN (10, 19, 24, 44, 57).

DURING DEVELOPMENT, SKELETAL MUSCLES DIFFERENTIALLY EXPRESS THREE TYPES OF CADHERIN (M-, N-, 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 α1β1 (4, 22, 45). THE α1-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, SARCOLEMMA 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 isofrom-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 injury. The results suggest a role of mXin in skeletal muscle force transductions.

**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 mXinα and mXinβ (49), U1697 recognizing only mXinα, 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) CGβ6 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, pH7.5, 1% Nonidet P-40, and 0.1% SDS plus protease inhibitor mixture; Roche Applied Science, Indianapolis, IN). After preincubation with protein G-Sepharose beads (GE Healthcare, Piscataway, NJ) for 30 min at 4°C, the homogenate (150 μg of total protein/immunoprecipitation) was cleared by centrifugation at 12,000 × g for 15 min. The precleared homogenate was incubated with anti-tropomyosin mAb LC24 (16, 35, 47, 63), anti-filamin mAb FLMN01 (Abcam, Cambridge, MA), anti-vinculin mAb hVIN-1 (Sigma), anti-talin mAb 8D4 (Sigma), anti-β-catenin mAb 15B8 (Sigma), or normal mouse serum control at 4°C overnight. Incubated again with protein G-Sepharose beads at 4°C for 1 h, the beads were collected by centrifugation and washed three times with immunoprecipitation buffer and one time with PBS. The bound proteins were eluted with SDS-PAGE sample buffer, resolved on 7.5% SDS-PAGE (49), and transferred to nitrocellulose membrane for Western blot analysis using anti-mXin U1013 as described previously (20).

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 NaHCO3) equilibrated in 5% CO2–95% O2, pH 7.4 at 37°C.

Contraction of EDL muscle was induced with electrical stimulation of the peroneal or sciatic nerve through a pair of wire electrodes. The stimulation voltage and, subsequently, muscle length were adjusted to optimum for the development of maximum isometric twitch force. The muscle was stimulated to contract at increasing frequencies until the force development reached plateau (Po), typically at 300 Hz. The optimal in situ muscle length (Lo) was measured with a caliper, based on defined anatomical landmarks. Fiber length (Lf) of mouse EDL muscle was estimated by multiplying Lo by the Ld/Lo, 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/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 stretch-induced muscle contraction cycle was repeated for 150 times. The length changes corresponded to a 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 Lo 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
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 troponin T (TnT; Ref. 36) and mAb Tnl-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), anti-filamin, 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
mXinα IN MYOTENDINOUS JUNCTION

Fig. 3. mXinα 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-mXin antibody. mXinα 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 mXinα from total protein extract of adult mouse heart (10).

overt difference in histology of these muscles was observed. At a similar plane, the roundness values (as a measure of fiber shape complexity) for wild-type and mXinα-null diaphragm fibers were the same (Table 1). Using dynamic image analysis system (DIAS) software (50, 51) to analyze muscle size, we found that the cross-sectional area of myofibers was significantly larger for the mXinα-null diaphragm muscle (Fig. 4, A and B, and Table 1). However, hypertrophy was not observed in any other muscles examined in mXinα-null mice.

Western blots showed that wild-type mouse diaphragm muscle does not contain particularly high level of Xinα. Actually, the relative expression levels of mXinα and mXinβ in diaphragm were 2.6−4.4 folds lower than the highest expression detected in soleus muscle (data not shown). Therefore, the expression level could not account for the hypertrophic phenotype in mXinα-null diaphragm but not soleus muscle. An alternative hypothesis is that diaphragm is a muscle that continuously works rhythmically to sustain respiration, which could be a reason for its sensitivity to the loss of Xinα. The hypertrophic phenotype of Xinα-null diaphragm muscle may be analogous to the progressive myocardial hypertrophy seen in Xinα-deficient hearts (20).

The SDS gels and Western blots further showed no difference in the overall protein profile or the expression of representative thick and thin filament proteins in the diaphragm muscle of mXinα-null mice compared with wild-type controls (data not shown).

The effect of mXinα loss on the structure and protein components of MTJ was examined in 5.5 to 13.5-mo-old mXinα-null mice using double-label immunofluorescence mi-

Fig. 4. mXinα-null diaphragm muscle fibers showed hypertrophy. Hematoxylin and cosin-stained cryosections were prepared from Ton (A and B), diaphragm (Di; C and D), and soleus (Sol; E and F) muscles of 7-mo-old wild-type (mXinα+/+) and mXinα−/− mice. No centrally localized nuclei or obvious alterations in gross morphology was detected in mXinα-null muscles. However, the cross-sectional area of mXinα−/− 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.
cles showed no significant difference in contractility (Table 2), indicating that deletion of mXina did not affect the baseline contractility. In isometric fatigability test, a higher resistance to fatigue with better recovery was found for mXina-null EDL muscle compared with that of wild-type controls (Fig. 6). In the stretch-loaded fatigability test (Fig. 5B), EDL muscle of mXina-null mice showed the same fatigability, as well as recovery, as that of wild-type control muscle (data not shown).

mXina-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 mXina-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 mXina-null mice

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<td>n</td>
<td>CSA, μm²</td>
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<tr>
<td>mXina⁺/⁺</td>
<td>60</td>
<td>1,506.14 ± 71.16</td>
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<tr>
<td>mXina⁻/⁻</td>
<td>72</td>
<td>1,433.79 ± 39.26</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 (DIAS) software (50, 51). Fiber cross-sectional area (CSA) was computed from the perimeters, whereas the roundness was computed using the formula 100 × 4π(area/perimeter²), 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).

Fig. 5. Protocol of contractile studies. A: experimental protocol used on EDL muscle for in situ functional measurement is outlined with representative muscle length and force traces. During initial twitch contractions, the muscle was stimulated with single pulse of 0.1-ms duration at constant voltage of 25 V. Muscle length was increased to reach the maximum activation of twitch force. Tetanic contractions were then induced with 200 ms of such pulse stimulations at 300 Hz every minute for 20 min. A fatigue protocol was followed consisting of 150 repeats of 200 ms/s tetanic contractions for 20 min. Eccentric contractions were induced at last with the same fatigue stimulations together with 20% stretching of fiber length (L₀) during the last 100 ms. Eccentric contractions were induced at last with the same fatigue stimulations together with 20% stretching of fiber length (L₀) during the last 100 ms. The above recovery protocol was applied 1 min after the eccentric contractions. B: eccentric contraction cycle is illustrated to show the ramp increase of muscle length during each cycle of tetanic contractions. Isometric peak force was measured during the first 100 ms under the optimal muscle length whereas peak force was shown at the end of stretch. Muscle length was returned to the optimal length by a ramp decrease in 100 ms.
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 rhythmic 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...
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 mXina-null muscles (data not shown), suggesting an analogous mechanism for the MTJ formation. The presence of mXinβ in mXina-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 mXina-null EDL muscles, there was no significant difference in baseline contractile parameters (Table 2). However, during isometric tetanic contraction, mXina-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 β1-subunit of integrin (18, 23, 37, 53, 56). αβ1-Integrin is a transmembrane structural protein concentrated at the MTJ, linking to actin cytoskeleton through talin/vinculin. Alterations in compliance have been observed in α7-integrin (Igα7) knockout mouse muscle (38), consistent with the possibility of the αβ1-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 mXina-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 mXinα mouse EDL muscle is a highly selective response, rather different from the universal housekeeping function of the ubiquitin-proteasome activity (39).

μ-Calpain associated with the myofilaments contributes to the production of cardiac TnT truncation in the heart (65). Since the level of free Ca2+ naturally fluctuates during contraction and relaxation in striated muscle cells, the peak concentration of cytosolic Ca2+ would be sufficient to activate μ-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 μ-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 myofilbrils 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 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 was examined using Student’s t-test. Values are presented as means ± SE; n = 3 mice each in wild-type and mXin-null groups. **P < 0.01 vs. wild-type; #P < 0.05 vs. resting control.

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

AUTHOR CONTRIBUTIONS
Author contributions: H. Z. F., Q. W., R. S. R., and J. L. L. performed experiments; H. Z. F. analyzed data; H. Z. F., J. L. L., and J. P. J. interpreted results of experiments; H. Z. F., J. J. L., and J. P. J. prepared figures; H. Z. F., J. L. L., and J. P. J. drafted manuscript; H. Z. F., J. L. L., and J. P. J. edited and revised manuscript; H. Z. F., Q. W., R. S. R., J. L. L., J. J. L., and J. P. J. approved final version of manuscript; J. L. L. and J. P. J. conception and design of research.

Fig. 8. EDL muscle of mXin-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 mXin-null (mXin<sup>−/−</sup>) 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 mXin-null EDL muscle than that in wild-type muscle. No degradation of TnI 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 mXin-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 mXin-null groups. **P < 0.01 vs. wild-type; #P < 0.05 vs. resting control.
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