Expression levels of sarcolemmal membrane repair proteins following prolonged exercise training in mice

Jenna Alloush¹, Steve R Roof¹, Eric X Beck¹, Mark T Ziolo¹ and Noah Weisleder^{1,2*}

¹The Dorothy M. Davis Heart and Lung Research Institute and Department of Physiology & Cell Biology,

The Ohio State University Wexner Medical Center, Columbus, OH 43210 USA

²TRIM-edicine, Inc., 675 US Highway 1, North Brunswick, NJ 08902 USA

Received 01 May 2013; revised 19 August 2013

Membrane repair is a conserved cellular process, where intracellular vesicles translocate to sites of plasma membrane injury to actively reseal membrane disruptions. Such membrane disruptions commonly occur in the course of normal physiology, particularly in skeletal muscles due to repeated contraction producing small tears in the sarcolemmal membrane. Here, we investigated whether prolonged exercise could produce adaptive changes in expression levels of proteins associated with the membrane repair process, including mitsugumin 53/tripartite motif-containing protein 72 (MG53/TRIM72), dysferlin and caveolin-3 (cav3). Mice were exercised using a treadmill running protocol and protein levels were measured by immunoblotting. The specificity of the antibodies used was established by immunoblot testing of various tissue lysates from both mice and rats. We found that MG53/TRIM72 immunostaining on isolated mouse skeletal muscle fibers showed protein localization at sites of membrane disruption created by the isolation of these muscle fibers. However, no significant changes in the expression levels of the tested membrane repair proteins were observed following prolonged treadmill running for eight weeks (30 to 80 min/day). These findings suggest that any compensation occurring in the membrane repair process in skeletal muscle following prolonged exercise does not affect the expression levels of these three key membrane repair proteins.

Keywords: Caveolin-3, Dysferlin, Exercise, Membrane repair, MG53, Mitsugumin 53, Repair patch, TRIM72, Tripartite motif, Vesicle trafficking

Mechanical disruptions of sarcolemmal membrane of striated muscle fibers, a common occurrence in the course of normal physiology are usually resealed through a membrane repair mechanism to allow for survival of affected muscle fibers¹⁻³. Membrane repair is a conserved cellular process, where intracellular vesicles translocate to disruptions in plasma membrane and fuse with each other and plasma membrane to create a repair patch that restores membrane integrity⁴. This mechanism is dependent on extracellular calcium (Ca²⁺) and has been compared to the neurotransmitter release process in neurons⁵. Membrane repair occurs in many different cell types. However, there appears to be an increased need for the membrane repair process in striated muscles due to their inherent contractile nature that produces consistent membrane disruptions in sarcolemmal membrane of these cells⁶.

Disruptions of sarcolemmal membrane and associated repair of these injuries occurs at a greater frequency during eccentric contractions of skeletal muscles¹. Eccentric contractions occur when the load on the muscle is massive enough to cause the muscle to lengthen, instead of shortening, while it is actively contracting. This results in increased strain on sarcolemmal membrane which increases appearance of transient disruptions in lipid bilayer of the cell. These disruptions result in the free exchange of components of intracellular cytosol with the extracellular space⁷. This can be observed by the appearance of many muscle resident soluble proteins in circulating blood.

Situations that result in increased eccentric contractions during exercise (e.g. weightlifting, downhill running) can increase the concentration of creatine kinase (CK) in the serum of the subject.

^{*}Author for correspondence:

Tel: +0 1 (614)292-5321

Fax +0 1 (614)247-7799

E-mail: noah.weisleder@osumc.edu

Abbreviations: cav3, caveolin-3; CK, creatine kinase; EDL, extensor digitorum longus; FDB, flexor digitorum brevis; gastroc, gastrocnemius; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MG53, mitsugumin 53;, TRIM, tripartite motif; TRIM72, tripartite motif-containing protein 72.

Various muscle-related diseases can exacerbate injuries to sarcolemmal membrane that lead to a loss of membrane integrity, myocyte death and degeneration of the muscle tissue. Duchenne muscular dystrophy is a genetic disorder produced by mutations in the dystrophin gene that increase the fragility of sarcolemmal membrane, making it more likely to undergo disruption⁸⁻¹⁰. Other muscular dystrophies are known to develop following mutation of specific genes, such as dysferlin¹¹⁻¹³ and caveolin-3 (cav3)¹⁴⁻¹⁷, involved in the sarcolemmal membrane repair process. In the heart, the loss of cardiomyocytes following myocardial infarction is linked to the breakdown of sarcolemmal membrane during necrotic cell death¹⁸⁻²⁰.

Dysferlin is a member of the ferlin gene family that contains multiple C2 domains that are expressed in several tissue types^{21,22}. While the molecular mechanism of dysferlin function remains to be fully clarified, it has been hypothesized to act as a fusogen to facilitate vesicle fusion with the plasma membrane to allow for myotube formation and assembly of a membrane repair patch^{23,24}. Recent studies have also found additional functions for dysferlin in the immune system that may contribute to development of dystrophy in the mouse knockout model^{25,26}. Cav3 is a muscle-specific member of the caveolin family of proteins that participates in the formation of caveolae and other structures in the sarcolemmal membrane²⁷⁻²⁹. Studies have linked the expression of Cav3 with membrane repair, as mutations in Cav3 are known to produce muscular dystrophy and can also compromise the membrane repair capacity of skeletal muscle fibers^{22,30,31}

TRIM72, a member of tripartite motif (TRIM) gene family has been linked to these proteins and the sarcolemmal membrane repair process. It was initially isolated as mitsugumin 53(MG53)³² and recent studies show that MG53/TRIM72 is an essential component of the membrane repair process in mammalian striated muscles^{30,33-38}. Genetic ablation of TRIM72 gene from mice results in compromised membrane repair capacity in skeletal³³ and cardiac muscle³⁶. TRIM72 knockout (trim72-/-) mice develop age-dependent myopathy in their skeletal muscles and cannot effectively resist the results of ischemia/reperfusion injury to the heart^{36,39}. When expressed in muscle or non-muscle cells, the MG53/TRIM72 protein contributes to vesicle trafficking to sites of membrane disruptions to allow for membrane repair patch

formation⁴⁰. MG53/TRIM72 can functionally interact with dysferlin and is required for dysferlin to be able to translocate to membrane injury sites in cultured skeletal muscle cells³³. MG53/TRIM72 is also known to functionally interact with Cav3 to mediate membrane repair in skeletal muscle³⁰. Considering the importance of MG53/TRIM72, dysferlin and Cav3 in the resealing of membrane disruptions in skeletal muscle and the need for repair of sarcolemmal membrane following exercise, it is possible that there are adaptive changes to the expression of these key membrane repair proteins following prolonged exercise training.

In this study, we have aimed to determine, if there are changes in the expression of these genes by establishing the specificity of the antibodies used for quantification of protein expression and then determining the expression levels in isolated muscle extracts from mice exercised on a rodent treadmill. Previous results show that custom-produced antibodies can be effective in detection of MG53/TRIM72 protein with immunoblotting and immunostaining approaches^{31,33}. These studies show that MG53 expression is restricted primarily to the striated muscles, with roughly equivalent levels of expression seen in cardiac and representative skeletal muscles. While these antibodies show good specificity to MG53/TRIM72 they are all either monoclonal antibodies raised against an unknown epitope or polyclonal antibodies raised against a protein containing large portions of the complete MG53/TRIM72 protein sequence. We have also determined the specificity of these novel antibodies and investigated, if they detect MG53 at sites of membrane disruption.

Materials and Methods

Treadmill exercise

All animal studies were conducted with the approval of the Ohio State University Institutional Animal Care and Use Committee and conformed to local and national regulations on the use of animals for scientific studies. C57Bl/6 mice underwent a high intensity aerobic interval treadmill training protocol for 8 weeks as previously described⁴¹. Briefly, mice (5 months of age at sacrifice) underwent treadmill (Columbus Instruments, Columbus, OH) training 5 days a week for 8 weeks starting at 30 min/day and increased to 80 min/day. Mice were challenged at a high intensity fast pace for 4 min, followed by

1 min of low intensity recovery pace and repeated until the designated time was up. Soleus muscles were removed within 2 days of the last training day.

Immunoblotting

Samples from skeletal muscles, heart and non-muscle tissues were dissected from 3 months old male C57Bl/6 mice (Jackson Labs) or 100-200 g male Lewis inbred rats (Charles River) after they were sacrificed by CO₂ inhalation. Immunoblotting was performed as previously described⁴² with the following modifications. Tissue samples were flash frozen and then homogenized in ice-cold RIPA buffer containing 150 mMNaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 (Sigma) and centrifuged at 14,000 rpm for 10 min at 4°C. For sample preparation, protein lysates were mixed with 2X SDS sample buffer containing 125 mMTris-HCl, pH 6.8, 4% SDS, 20% (vol./vol.) glycerol and 0.004% bromophenol blue. Samples were placed on a heating block for 5 min at 95°C. 50 µg total protein was separated on Novex 10% Tris-Glycine gels and transferred to PVDF membrane using transfer buffer containing 25 mM Tris, 192 mM glycine and 20% methanol overnight at 4°C at 25V. Membranes were stained with Ponceau Red (Sigma) to confirm transfer of protein to the membrane. For immunoblotting, membranes were blocked in phosphate buffered saline containing 0.1% Tween-20 and 5% non-fat dry milk for 1 h at room temperature. Primary and secondary antibodies were diluted in blocking solution as follows: anti-dysferlin (1:500, Novocastra Laboratories) overnight at 4°C, anti-TRIM72 (1:1000, Pro-Sci) overnight at 4°C, anti-Cav3 (1:5000.BD) overnight at 4°C. anti-GAPDH (1:1000, Cell Signaling D16H11), anti-actin (1:2500, Santa Cruz Biotechnology sc-1616) for 1 h at room temperature, HRP-conjugated secondary antibodies (1:10000, Santa Cruz Biotechnology) for 1 h at room temperature. Proteins were detected using ECL prime detection reagent (GE Healthcare).

Densitometry measurements were made from digital files collected on a Chemidoc MP system (BioRad) with various exposure times to avoid saturation of the signal from each band. The density of each band was analyzed using ImageJ software (NIH) and the relative signal strength of each band was calculated by dividing the density of each sample by the density of the band for each respective loading control. Both actin and GAPDH levels were used as separate loading controls to avoid any potential variation in the level of the loading controls between animals and conditions.

Immunostaining

Intact flexor digitorum brevis (FDB) muscle fibers were isolated by enzymatic digestion using a previously described method⁴³. Briefly, FDB muscles were dissected from C57Bl/6 male mice (3 months of age) and placed in modified Tyrode buffer (140 mM NaCl, 5 mM KCl, 10 mM HEPES (free acid), 5.5 mM D-glucose, 2 mM MgCl₂, pH 7.2) supplemented with 2 mg/mL collagenase type I (#4196 from Worthington). Muscles were incubated at 37°C with orbital shaking at 160 rpm for 60-90 min until the muscles developed a frayed appearance. Muscles were then washed in modified Tyrode buffer and isolated by gentle trituration through a series of pipette tips with a decreasing diameter. Thereafter, muscle fibers were plated out on 35-mm Delta T dishes (Bioptechs, Inc) and immunostained as previously described⁴⁴.

Isolated muscle fibers were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) overnight at 4°C and permeabilized with 0.05% Triton X100 (Sigma). Fibers were blocked with 5% goat serum in phosphate buffered saline for 1 h at room temperature before primary anti-MG53 antibody (1:100 dilution) was applied for 2 h at room temperature. After three washings of 5 min each, AlexaFluor 488 conjugated secondary antibody (Invitrogen) was applied for 2 h at room temperature. After three rounds of washing, fibers were examined and images were collected on a Zeiss Axiovert 100 epifluorescent microscope.

Results

MG53/TRIM72 expression levels in skeletal and cardiac muscles in mice and rats

Although several polyclonal antibodies against specific peptides in the MG53/TRIM72 sequence are commercially available, these antibodies have not been examined for their efficacy or specificity. The characterization of such antibodies may allow us to assess MG53 levels in appropriate experimental systems. Here, we tested if a commercial TRIM72 antibody could recognize MG53/TRIM72 specifically within skeletal and cardiac muscles. Various tissue lysates from the mouse and rat were prepared and used for immunoblot analysis (Fig. 1). Rat tissues were included here to determine, if there were species-specific aspects of the antibody function.



Fig. 1—MG53 expression levels vary between different murine anatomical muscles [(A) Whole tissue lysates were isolated from the indicated mouse tissues and then used for immunoblotting. MG53/TRIM72 bands of appropriate molecular weight (*arrow*) were observed in lysates from various anatomical muscles. Numbers provided (*left*) indicate the position of molecular weight markers; and (B) Whole tissue lysates of rat samples were used for immunoblotting for MG53/TRIM72 expression. A MG53/TRIM72 band of appropriate molecular weight (*arrow*) appears in rat skeletal (Gastroc) and cardiac muscle. Numbers provided (*left*) indicate molecular weight markers. Immunoblots for actin levels in tissue lysates were also provided as a control for loading]

We found that a TRIM72 commercial antibody produced appropriate sized bands (53 kDa) specifically with the striated muscle extracts. In addition, smaller molecular weight bands were recognized by the antibody were also found across a variety of tissues. Given that there do not appear to be spliced forms of MG53/TRIM72³³, the antibody might recognize other members of the well conserved TRIM gene family. This was likely for one of these bands that appeared at approximately 45 kDa in all tissues tested, since MG53/TRIM72 derived proteins should appear primarily in striated muscles³³. The other lower molecular weight band at approximately 20 kDa appeared only in some of the skeletal muscles tested, however, the smaller size of the fragment indicated that this band resulted from modification of MG53/TRIM72 or from cross

reaction with another small TRIM family protein. Examination of different anatomical muscles in the mouse showed that MG53/TRIM72 levels could vary from one muscle type to the other, with the highest level seen in the extensor digitorumlongus (EDL), while the lowest in the FDB.

Localization of MG53/TRIM72to sites of membrane disruption in muscle fibers

Since the protein expression levels in whole tissue extracts mirrored the expected expression pattern for MG53/TRIM72, we further tested the localization of MG53/TRIM72 in single skeletal muscle fibers. Previous study has shown MG53/TRIM72 localization in the cystosol and broadly throughout the sarcolemmal membrane of myocytes in uninjured skeletal tissue sections³³. Tissue sections myocardium following ischemia/reperfusion of injury show enrichment at sites on the presumably injured sarcolemmal membrane³⁶. However, it has not been previously shown that native MG53/TRIM72 localizes to injury sites in isolated skeletal muscle fibers. Individual intact muscle fibers were enzymatically isolated from the FDB muscle of wild-type mice and then stained for localization of MG53/TRIM72. Many of these muscle fibers were damaged during the isolation procedure and while these muscle fibers were not useful for functional studies, they did provide the opportunity to observe, if native MG53/TRIM72 protein accumulated at sites of physical membrane injury similar to those that occur following exercise.

We found that MG53 protein accumulated at the sarcolemma in fibers, where there were clear sites of membrane damages, such as where a fiber would bend due to damage that occurred during isolation (Fig. 2). In contrast, muscle fibers that showed a healthy, rod-like appearance showed foci of MG53 protein only at the ends of the fiber. Since these fibers were isolated by gentle trituration, the resulting fibers tended to be broken fragments of the longer muscle fibers present in the anatomical muscle. When these fibers are broken during isolation, the ends of the fibers must reseal in order to prevent the death of the cell³³, thus it is likely that there would be accumulation of MG53/TRIM72 at the ends of isolated muscle fibers. The observed localization pattern of native MG53/TRIM72 in isolated muscle fibers was consistent with the expected pattern that would follow mechanical damage to the sarcolemmal membrane.



Fig. 2—MG53 protein appears at sites of membrane disruption in isolated skeletal muscle fibers [(**A**) FDB muscle fibers were isolated from C57Bl/6 mice and immunostained for the localization of MG53/TRIM72 protein (*right*). When observing the sealed end of the isolated muscle fibers (*left*) concentration of MG53 protein wasobserved (*arrow*). Scale bar represents 10 μ m; and (**B**) During FDB fiber isolation, there were membrane disruptions created in some fibers that could be observed in bright field microscopy (*left*). Such disruptions showed concentrations of MG53/TRIM72 immunostaining (*right*) at sites of membrane damage (*arrowheads*) as well as the resealed end of the fibers (*arrows*). Scale bar represents 10 μ m]

Intensive exercise does not alter expression of cell membrane repair proteins

Since native MG53/TRIM72 protein localizes to mechanical membrane disruptions, the membrane repair process might have a role following the contractions in skeletal muscle associated with exercise. The repeated membrane disruptions associated with prolonged exercise could cause increased expression of various proteins involved in the membrane repair process. To test if prolonged exercise could alter expression of MG53, dysferlin and Cav3, we used a rodent treadmill to exercise mice 5 days a week for 8 weeks starting at 30 min/day and increased to 80 min/day. This protocol allowed for intense prolonged stimulation that could have the potential to modify expression of these genes. Multiple mice were exercised to determine, if there would be variation in the response to this exercise protocol between individual animals. These mice were then sacrificed and the soleus was isolated and processed for immunoblotting.

We used the soleus muscles for this analysis, as we observed moderate levels of expression of MG53 in these muscles in our previous immunoblots and that intermediate level of expression would allow for



Fig. 3—Expression levels of cell membrane repair associated proteins do not change following prolonged exercise [(A) Whole tissue lysates of soleus muscles from exercised (E) and sedentary (S) mice were used for immunoblot analysis of the protein levels of MG53/TRIM72, dysferlin and cav3. Both actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels in these tissue lysates were used for a loading control; (B, C) Densitometry measurements were used to determine the relative concentration of the proteins of interest in the tissue lysates. A bar graph represents the data for each group (n = 4 mice per group) relative to expression levels of GAPDH (B) or actin (C). Analysis using Student's t-test did not show any statistically significant differences between the exercised and sedentary groups for the proteins tested]

monitoring increased or decreased levels of the protein. Additionally, the nature of the soleus suggested that the mice might extensively use these muscles in our treadmill exercise approach. In these muscles, we found that there were no significant changes in the levels of MG53/TRIM72, dysferlin and Cav3 in mouse soleus muscles, following 8 weeks of treadmill exercise (Fig. 3). Quantification of the levels

of protein expression by densitometry confirmed that levels of these three proteins did not change following a course of prolonged exercise.

Discussion

While membrane repair contributes to normal muscle physiology by resealing transient disruption in the sarcolemmal membrane during muscle contraction, the role of membrane repair in adaptation to prolonged exercise has not vet been fully determined. In this study, we examined if the expression levels of several proteins known to be involved in the membrane repair process are altered in mouse skeletal muscle following prolonged endurance exercise training. The results show that the levels of three proteins known to be involved in membrane repair in striated muscle do not show altered expression following prolonged exercise. It is possible that increased membrane repair protein expression is not required to adapt to prolonged exercise. The immunostaining results presented here suggest it may not be necessary for levels of membrane repair proteins to increase with exercise, as there is ample MG53/TRIM72 in non-exercised muscle fibers to repair even the large membrane disruptions produced during the isolation of the muscle fibers. Another potential explanation is that increased membrane repair may not be necessary during prolonged exercise. Indeed, increased membrane repair capacity could potentially have detrimental effects during exercise. One of the factors contributing to beneficial hypertrophy of skeletal muscle after exercise is the formation of micro-tears in the sarcolemma. If the membrane repair processes are accelerated in skeletal muscle following exercise then this beneficial response might be abrogated. While it appears that there is no increase in the expression of proteins associated with membrane repair following prolonged exercise, there could be changes in expression of other proteins that could contribute to membrane repair. Additionally, other functional changes in the membrane repair process could affect membrane repair capacity without changes in protein expression level. Future studies are needed to determine, if there are functional alterations in the membrane repair capacity of skeletal muscle following exercise.

We have reported previously that the concentration of MG53 in mouse serum increases following acute bouts of exhaustive exercise, as membrane disruptions created during exercise releases MG53/TRIM72 into the bloodstream⁴⁰. Given our findings here that MG53 levels in muscle do not increase with prolonged exercise, this suggests that the increase in serum MG53 levels is likely due to more protein released from the myocyte, rather than from changes in the expression level in the muscle. However, we cannot rule out this possibility as there could be an acute increase in the levels of MG53, following the termination of exercise that is not detected in the long-term running protocol used in this study.

study used a commercially available Our anti-TRIM72 antibody raised against a specific peptide antigen. We found that the antibody recognizes the MG53/TRIM72 protein, as the appropriately sized band appears in striated muscles. However, there is a visible off-target band that appears in multiple tissues types at a molecular weight slightly below that of MG53. Given the size of the TRIM family of proteins and the similarities in domain structure and primary amino acid sequence of many of the family members^{45,46}, it is quite possible that this antibody might recognize other family members, in addition to MG53/TRIM72. While we found that the anti-TRIM72 antibody primarily recognizes MG53/TRIM72 in the skeletal and cardiac tissues, it cannot be ruled out that cells appearing in other tissues in low abundance could express MG53. In the whole tissue extracts used here, the appearance of MG53/TRIM72 in a minority of cells in the tissue may not reveal expression by immunoblot analysis. More comprehensive immunohistochemical analysis is required to clearly establish the specificity of expression of the TRIM72 gene.

Our results also show the relative expression of MG53/TRIM72 in different anatomical muscles of the mouse. We found higher levels of MG53/TRIM72 in the EDL and gastrocnemius than in the soleus and FDB muscles. Since there are lower levels in the soleus muscle that is enriched for slow twitch muscle fibers, one interpretation would be that MG53/TRIM72 is expressed in lower levels in slow twitch myocytes. However, the low levels of MG53/TRIM72 expression in the fast twitch enriched FDB muscle argues against this interpretation. Another interpretation would be that the increased MG53/TRIM72levels in certain muscle fibers corresponds with the need for membrane repair in a particular muscle type. However, further studies on isolated muscle fibers are needed to establish, if these differences in MG53 expression level correspond with the membrane repair capacity of muscle fibers from different anatomical muscles.

In conclusion, this study has demonstrated that native MG53 protein localizes to membrane disruptions in isolated skeletal muscle fibers, however, the membrane disruptions associated with prolonged exercise do not produce significant changes in the expression of proteins associated with membrane repair. As the role of MG53/TRIM72 in membrane repair as part of normal physiology becomes clearer, the application of these proteins may aid in addressing aspects of pathophysiology of musculoskeletal and cardiovascular diseases.

Acknowledgements

Noah Weisleder is a Co-Founder and Chief Scientific Officer at TRIM-edicine, Inc, which is developing MG53 as a therapeutic strategy. Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, part of the National Institutes of Health, under Award Number R01-AR063084. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

- 1 McNeil P L & Khakee R (1992) Am J Pathol 140, 1097-1109
- 2 Miyake K & McNeil P L (1995) J Cell Biol 131, 1737-1745
- 3 McCarter G C & Steinhardt R A (2000) *J Membr Biol* 176, 169-174
- 4 McNeil P L & Steinhardt R A (2003) Annu Rev Cell Dev Biol 19, 697-731
- 5 Bi G Q, Alderton J M & Steinhardt R A (1995) *J Cell Biol* 131, 1747-1758
- 6 McNeil P L & Kirchhausen T (2005) Nat Rev Mol Cell Biol 6, 499-505
- 7 Banfi G, Colombini A, Lombardi G & Lubkowska A (2012) Adv Clin Chem 56, 1-54
- 8 Pasternak C, Wong S & Elson E L (1995) J Cell Biol 128, 355-361
- 9 Hamer P W, McGeachie J M, Davies M J & Grounds M D (2002) J Anat 200, 69-79
- 10 Lapidos K A, Kakkar R & McNally E M (2004) Circ Res 94, 1023-1031
- 11 Bansal D, Miyake K, Vogel S S, Groh S, Chen C C, Williamson R, McNeil P L & Campbell K P (2003) Nature 423, 168-172
- 12 Cagliani R, Fortunato F, Giorda R, Rodolico C, Bonaglia M C, Sironi M, D'Angelo M G, Prelle A, Locatelli F, Toscano A, Bresolin N & Comi G P (2003) *Neuromuscul Disord* 13, 788-795
- 13 Bansal D & Campbell K P (2004) Trends Cell Biol 14, 206-213
- 14 McNally E M, de Sa Moreira E, Duggan D J, Bonnemann C G, Lisanti M P, Lidov H G, Vainzof M, Passos-Bueno M R, Hoffman E P & Zatz M & Kunkel L M (1998) *Hum Mol Genet* 7, 871-877

- 15 Minetti C, Sotgia F, Bruno C, Scartezzini P, Broda P, Bado M, Masetti E, Mazzocco M, Egeo A, Donati M A, Volonte D, Galbiati F, Cordone G, Bricarelli F D, Lisanti M P & Zara F (1998) Nat Genet 18, 365-368
- 16 Matsuda C, Hayashi Y K, Ogawa M, Aoki M, Murayama K, Nishino I, Nonaka I, Arahata K & Brown R H Jr. (2001) *Hum Mol Genet* 10, 1761-1766
- 17 Jaiswal J K, Marlow G, Summerill G, Mahjneh I, Mueller S, Hill M, Miyake K, Haase H, Anderson L V, Richard I, Kiuru-Enari S, McNeil P L, Simon S M & Bashir R (2007) *Traffic* 8, 77-88
- 18 Tavazzi B, Di Pierro D, Bartolini M, Marino M, Distefano S, Galvano M, Villani C, Giardina B & Lazzarino G (1998) *Free Radic Res* 28, 25-37
- 19 Ferrari R, Guardigli G, Mele D, Percoco G F, Ceconi C & Curello S (2004) *Curr Pharm Des* 10, 1699-1711
- 20 Han R, Bansal D, Miyake K, Muniz V P, Weiss R M, McNeil P L & Campbell K P (2007) J Clin Invest 117, 1805-1813
- 21 Davis D B, Doherty K R, Delmonte A J & McNally E M (2002) *J Biol Chem* 277, 22883-22888
- 22 Capanni C, Sabatelli P, Mattioli E, Ognibene A, Columbaro M, Lattanzi G, Merlini L, Minetti C, Maraldi N M & Squarzoni S (2003) *Exp Mol Med* 35, 538-544
- 23 Posey A D, Jr., Demonbreun A & McNally E M (2011) Curr Top Dev Biol 96, 203-230
- 24 Lek A, Evesson F J, Sutton R B, North K N & Cooper S T (2012) Traffic 13, 185-194
- 25 Han R (2011) Skelet Muscle 1, 10
- 26 Han R, Frett E M, Levy J R, Rader E P, Lueck J D, Bansal D, Moore S A, Ng R, Beltran-Valero de Bernabe D, Faulkner J A & Campbell K P (2010) *J Clin Invest* 120, 4366-4374
- 27 Song K S, Scherer P E, Tang Z, Okamoto T, Li S, Chafel M, Chu C, Kohtz D S & Lisanti M P (1996) J Biol Chem 271, 15160-15165
- 28 Parton R G, Way M, Zorzi N & Stang E (1997) J Cell Biol 136, 137-154
- 29 Repetto S, Bado M, Broda P, Lucania G, Masetti E, Sotgia F, Carbone I, Pavan A, Bonilla E, Cordone G, Lisanti M P & Minetti C (1999) *Biochem Biophys Res Commun* 261, 547-550
- 30 Cai C, Weisleder N, Ko J K, Komazaki S, Sunada Y, Nishi M, Takeshima H & Ma J (2009) J Biol Chem 284, 15894-15902
- 31 Waddell L B, Lemckert F A, Zheng X F, Tran J, Evesson F J, Hawkes J M, Lek A, Street N E, Lin P, Clarke N F, Landstrom A P, Ackerman M J, Weisleder N, Ma J, North K N & Cooper S T (2011) J Neuropathol Exp Neurol 70, 302-313
- 32 Weisleder N, Takeshima H & Ma J (2008) *Cell Calcium* 43, 1-8
- 33 Cai C, Masumiya H, Weisleder N, Matsuda N, Nishi M, Hwang M, Ko J K, Lin P, Thornton A, Zhao X, Pan Z, Komazaki S, Brotto M, Takeshima H & Ma J (2009) *Nat Cell Biol* 11, 56-64
- 34 Cai C, Masumiya H, Weisleder N, Pan Z, Nishi M, Komazaki S, Takeshima H & Ma J (2009) J Biol Chem 284, 3314-3322
- 35 Weisleder N, Takeshima H & Ma J (2009) Commun Integr Biol 2, 225-226
- 36 Wang X, Xie W, Zhang Y, Lin P, Han L, Han P, Wang Y, Chen Z, Ji G, Zheng M, Weisleder N, Xiao R P, Takeshima H, Ma J & Cheng H (2010) *Circ Res* 107, 76-83

- 37 Hwang M, Ko J K, Weisleder N, Takeshima H & Ma J (2011) *Am J Physiol Cell Physiol* 301, C106-114
- 38 He B, Tang R H, Weisleder N, Xiao B, Yuan Z, Cai C, Zhu H, Lin P, Qiao C, Li J, Mayer C, Ma J & Xiao X (2012) *Mol Ther* 20, 727-735
- 39 Cao C M, Zhang Y, Weisleder N, Ferrante C, Wang X, Lv F, Song R, Hwang M, Jin L, Guo J, Peng W, Li G, Nishi M, Takeshima H, Ma J & Xiao R P (2010) *Circulation* 121, 2565-2574
- 40 Weisleder N, Takizawa N, Lin P, Wang X, Cao C, Zhang Y, Tan T, Ferrante C, Zhu H, Chen P J, Yan R, Sterling M, Zhao X, Hwang M, Takeshima M, Cai C, Cheng H, Takeshima H, Xiao R P & Ma J (2012) *Sci Transl Med* 4, 139ra185
- 41 Roof S R, Tang L, Ostler J E, Periasamy M, Gyorke S, Billman G E & Ziolo M T (2013) Basic Res Cardiol 108, 332-341
- 42 Weisleder N, Ferrante C, Hirata Y, Collet C, Chu Y, Cheng H, Takeshima H & Ma J (2007) *Cell Calcium* 42, 548-555
- 43 Weisleder N, Zhou J & Ma J (2012) Methods Mol Biol 798, 395-410
- 44 Tjondrokoesoemo A, Li N, Lin P H, Pan Z, Ferrante C J, Shirokova N, Brotto M, Weisleder N & Ma J (2013) *J Biol Chem* 288,2103-2109
- 45 Meroni G & Diez-Roux G (2005) Bioessays 27, 1147-1157
- 46 Napolitano L M & Meroni G (2012) IUBMB Life 64, 64-71