Zinc Binding to MG53 Protein Facilitates Repair of Injury to Cell Membranes*

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Zinc is an essential trace element that participates in a wide range of biological functions, including wound healing. Although Zn²⁺ deficiency has been linked to compromised wound healing and tissue repair in human diseases, the molecular mechanisms underlying Zn²⁺-mediated tissue repair remain unknown. Our previous studies established that MG53, a TRIM (tripartite motif) family protein, is an essential component of the cell membrane repair machinery. Domain homology analysis revealed that MG53 contains two Zn²⁺-binding motifs. Here, we show that Zn²⁺ binding to MG53 is indispensable for assembly of the cell membrane repair machinery. Live cell imaging illustrated that Zn²⁺ entry from extracellular space is essential for translocation of MG53-containing vesicles to the acute membrane injury sites for formation of a repair patch. The effect of Zn²⁺ on membrane repair is abolished in mg53⁻/⁻ muscle fibers, suggesting that MG53 functions as a potential target for Zn²⁺ during membrane repair. Mutagenesis studies suggested that both RING and B-box motifs of MG53 constitute Zn²⁺-binding domains that contribute to MG53-mediated membrane repair. Overall, this study establishes a base for Zn²⁺ interaction with MG53 in protection against injury to the cell membrane.

Background: MG53, a zinc finger protein, is essential to cell membrane repair. It is not known whether zinc contributes to MG53-mediated membrane repair.

Results: Chelation of Zn²⁺ or mutation of Zn²⁺-binding motifs in MG53 affects membrane repair.

Conclusion: Zn²⁺ binding to MG53 is required for membrane repair.

Significance: This study establishes a base for Zn²⁺ interaction with MG53 in protection against injury to the cell membrane.

Zinc is an essential element for normal physiology (1), and nutritional Zn²⁺ deficiency and lack of bioavailability are associated with growth failure, dermatitis, impaired immunity, and delayed wound healing (2–4). One of the examples of impaired wound healing is acrodermatitis enteropathica, caused by reduced Zn²⁺ uptake in the small intestine and reduced serum Zn²⁺ levels (2, 3, 5). Surgical patients undergoing total hip replacement procedures have impaired wound healing that correlates with lower levels of serum Zn²⁺ (6, 7). Zn²⁺ deficiency also accompanies aging and a number of other pathological conditions, particularly those linked to oxidative stress (8).

Intracellular Zn²⁺ levels are tightly controlled by transporters and ion channels. Roughly 10% of the structures deposited in the Protein Data Bank have Zn²⁺ listed in their structure indexes (9, 10). Inside the cell, Zn²⁺ is bound to numerous structural and regulatory proteins, including transcription factors (11), regulators of hematopoietic stem cells (12) and immune cells (13), and proteins involved in intracellular signaling and neurotransmission (14). As such, metabolically active, labile Zn²⁺ is present inside cells at pico- to nanomolar concentrations, with extracellular concentrations in the sub-micromolar to micromolar range (15–17). Increased oxidative stress can release Zn²⁺ from its binding sites, executing the task of the so-called “redox Zn²⁺ switch” in performing its corresponding biological functions (18).

Dynamic membrane repair is a fundamental process in maintaining cellular integrity. Defective membrane repair is linked to compromised wound healing, muscular dystrophy, and cardiovascular diseases (19–23). We discovered that MG53, a TRIM (tripartite motif) family protein, is an essential component of the membrane repair machinery (24). MG53 acts as a sensor of oxidation to nucleate recruitment of intracellular vesicles to the injury site for membrane patch formation. MG53 ablation results in defective membrane repair, with progressive pathological consequences to skeletal and cardiac muscles (24–27).

Domain homology analysis showed that MG53 contains two Zn²⁺-binding domains in the RING finger and B-box motifs (24), but whether MG53 binds with Zn²⁺ to regulate membrane repair is unknown. Here, we present evidence that Zn²⁺
binding to MG53 is indispensable for repair of cell membrane
injury. Chelation of extracellular Zn$^{2+}$ impacts the movement
of intracellular vesicles to the acute membrane injury sites in a
MG53-dependent manner. Defective membrane repair was
observed in the absence of extracellular Zn$^{2+}$ or in response to
disruption of Zn$^{2+}$-binding motifs in MG53. Our data suggest
that MG53 serves as an acceptor for Zn$^{2+}$ during cell mem-
brane repair and provide mechanistic insight in the biology of
Zn$^{2+}$ in wound healing and regenerative medicine.

Experimental Procedures

Plasmid Construction—Cloning and construction of MG53
expression plasmids were performed as described previously
(24, 28). The various MG53 mutants (C29L, H31A, C53A/
C55A/C56A, C86A, C105S, and C29L/C105S) were
constructed by replacing the appropriate residues in GFP-MG53
using the method described previously (29). The coding
sequence of WT MG53 was cloned into the pMAL-p2 vector
(New England Biolabs) for expression of the recombinant
maltose-binding protein (MBP)$^4$-MG53 fusion protein in Esch-
erichia coli (30). The coding sequences of mutants C29L and
C29L/C105S were cloned into the pMAL-p2 vector in the same
manner to yield plasmids pMAL-C29L and pMAL-C29L/
C105S for production of the MBP-MG53(C29L) and MBP-
MG53(C29L/C105S) fusion proteins using E. coli fermentation.
All plasmids were confirmed by sequencing.

Flexor Digitorum Brevis (FDB) Muscle Fiber Isolation and
Membrane Repair Assay—All animal care and usage followed
National Institutes of Health guidelines and was approved by the
institutional animal care and use committees of Rutgers
University and The Ohio State University. Mice null for MG53
(mg53$^{-/}$) or age-matched WT control animals were produced
as described previously (24). FDB muscle fiber isolation was
performed with a previously published protocol (24, 30, 31).
Membrane repair capacity in the FDB muscle was determined
using an established technique (32, 33). Briefly, prior to cell
wounding, 2.5 μM FM 1-43 (Life Technologies, Inc.) was added
to Tyrode’s solution containing 140 mM NaCl, 5 mM KCl, 2.5
mM CaCl$_2$, 2 mM MgCl$_2$, and 10 mM HEPES (pH 7.2), and mem-
branes in FDB fibers were damaged using an UV laser on the
stage of a Zeiss LSM 510 confocal microscope (Enterprise)
to irradiate a 5 × 5 pixel area at a maximum power for 5 s (80
milliwatts, 351/364 nm). Confocal images were acquired (x-y
images captured at 6.6-s intervals) to monitor FM 1-43 dye
entry at the irradiation site. The mean fluorescence intensity
at the irradiation site was calculated based on the following equa-
tion: $\Delta F/F_o = (F_t - F_o)/F_o$, where $F_o$ is the fluorescence
intensity at the initial time point, and $F_t$ is the fluorescence intensity
at the various time points afterward. In some experiments, the
fibers were preincubated with a cell-impermeable cation cation-
ator (Ca-EDTA, Sigma), a Zn$^{2+}$ ionophore (1-hydroxyppyrimidine-
2-thione zinc salt (Zn-HPT), Sigma), or a high affinity Zn$^{2+}$
cation (tetakis(2-pyridylmethyl)enediamine (TPEN), Sigma)

4 The abbreviations used are: MBP, maltose-binding protein; FDB, flexor digito-
rum brevis; Zn-HPT, 1-hydroxyppyrimidine-2-thione zinc salt; TPEN, tetakis(2-
pyridylmethyl)enediamine; LDH, lactate dehydrogenase; rhMG53, recombi-
nant human MG53; TSQ, 6-methoxy-(8-p-toluenesulfonylamo)quinolone.

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Zn\(^{2+}\) Binding Assessment—Zn\(^{2+}\) contents in the recombinant MBP-MG53 proteins were measured by 6-methoxy-(8- p-toluensulphonamido)quinolone (TSQ) fluorescence assay according to our previous protocol (37). In brief, the Zn\(^{2+}\)-specific fluorescent probe TSQ (Life Technologies, Inc.) was added to 2–10 μM MBP-MG53 fusion proteins bound on the amylose resin beads in phosphate-buffered saline to a final concentration of 10 μM. After removal of the unbound TSQ probe, the thiol-bound Zn\(^{2+}\) contents of the MBP-MG53 fusion proteins on the beads were analyzed with the thiol-reactive reagent p-hydroxymercurophenylsulfonate (Sigma) for 10 min at room temperature to liberate the intramolecular Zn\(^{2+}\) content. Stained beads were excited at 334 nm, and fluorescence emission was recorded at 465 nm with an Axiovert 200M motorized fluorescence microscope (Zeiss) equipped with a mercury lamp. A standard Zn\(^{2+}\) solution (Sigma) was used to calibrate the assay system. The quantification of relative Zn\(^{2+}\) content was performed with MetaMorph imaging analysis software (Molecular Devices).

Western Blotting—C2C12 cells expressing the different GFP-MG53 proteins were harvested and lysed with ice-cold modified radioimmune precipitation assay buffer (150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and 20 mM Tris-HCl (pH 7.5)) supplemented with protease inhibitor mixture. The total protein lysates (10 μg each) were separated on 4–12% SDS-polyacrylamide gradient gels (Invitrogen). Proteins were transferred onto PVDF membrane (Millipore) and probed with a custom-made rabbit anti-MG53 polyclonal antibody as described previously (24), and detection was conducted with an ECL Plus kit (Thermo Scientific). To assay the effect of chelating Zn\(^{2+}\) concentration on the redox-dependent oligomerization of MG53, water-dissolved rhMG53 protein (in lyophilization buffer containing 3% mannitol, 1% sucrose, 0.005% Tween 80, and 5 mM phosphate buffer) (30) was treated with varying concentrations of Ca-EDTA or TPEN as indicated. The protein samples were then incubated with sample loading buffer (62.5 mM Tris-Cl (pH 6.8), 2% SDS, 10% glycerol, and 0.002% bromphenol blue with Ca-EDTA or TPEN and with or without 10 mM DTT as indicated), followed by SDS-PAGE separation.

Statistical Analysis—All data are expressed as mean ± S.E. unless indicated otherwise. Statistical analyses were performed using Student’s t test (unpaired and two-tailed). Analysis of variance was used for comparisons between more than two groups. A value of p < 0.05 was considered statistically significant.

Results

Chelation of Zn\(^{2+}\) Impacts MG53-mediated Membrane Repair in Skeletal Muscle—To investigate the role of Zn\(^{2+}\) in muscle membrane repair, the FM 1-43 fluorescent dye entry assay following UV laser damage was performed on isolated FDB muscle fibers from WT and mg53\(^{-/-}\) mice as described previously (24, 34). Changes in FM 1-43 fluorescent dye entry (ΔF/F\(_{0}\)) at the damage sites were assessed by confocal microscope imaging. As shown in Fig. 1A, detectable fluorescent dye entry into WT muscle fiber was observed under control conditions. The addition of 20 μM Zn-HPT increased membrane repair capacity as reflected by the diminished FM 1-43 dye entry following injury compared with the control. Removal of Zn\(^{2+}\) ions by chelation with 40 μM TPEN, a high affinity Zn\(^{2+}\) chelator, led to a significant increase in FM 1-43 dye entry following UV damage, indicating compromised membrane repair capacity in the absence of Zn\(^{2+}\).

Consistent with our previous study (24), the mg53\(^{-/-}\) FDB muscle fibers exhibited defective membrane repair function, as shown by the elevated amount of FM 1-43 dye entry following identical treatment (Fig. 1B, left panel). Unlike the WT muscle fibers, the membrane repair capacity observed in mg53\(^{-/-}\) muscle fibers did not show Zn\(^{2+}\) dependence: neither Zn-HPT (Fig. 1B, middle panel) nor TPEN (right panel) affected FM 1-43 dye entry following UV damage. Furthermore, the membrane-impermeable Zn\(^{2+}\) chelator Ca-EDTA, which buffers external Zn\(^{2+}\), was also found to cause compromised membrane repair capacity in WT muscle, whereas it had no significant effect on membrane repair capacity in mg53\(^{-/-}\) muscle (Fig. 1C). These data suggest that MG53 is a critical player in membrane repair, and Zn\(^{2+}\) ions are part of the regulatory component of the repair machinery.

Extracellular Zn\(^{2+}\) Regulates MG53-mediated Vesicle Translocation to Membrane Injury Sites—Our previous study (24) demonstrated that MG53 facilitates intracellular vesicle trafficking to the membrane disruption site for formation of a repair patch. To examine the role of Zn\(^{2+}\) in the process of MG53-mediated vesicle translocation, we expressed GFP-MG53 fusion protein in C2C12 myoblasts and used live cell imaging to assess microelectrode-generated mechanical membrane damage. As shown in Fig. 2A, under resting conditions, GFP-MG53 localized to intracellular vesicles in proximity to the plasma membrane. Acute injury to the cell membrane caused rapid translocation of MG53-containing vesicles to the injury site. Removal of extracellular Zn\(^{2+}\) by preincubation with 40 μM Ca-EDTA impaired GFP-MG53 translocation to membrane injury sites (Fig. 2B). Moreover, the addition of 20 μM TPEN significantly reduced translocation of GFP-MG53-containing vesicles to the mechanical injury site (Fig. 2C). Data from multiple experiments assessing the role of Ca-EDTA and TPEN in GFP-MG53-mediated membrane repair in C2C12 myoblast cells are summarized in Fig. 2E. Clearly, chelation of extracellular Zn\(^{2+}\) produced significant defects in membrane repair patch formation.

GFP-MG53-mediated membrane repair patch formation was also examined in the presence of the Zn\(^{2+}\) ionophore Zn-HPT. Interestingly, after a 15-min incubation with Zn-HPT, GFP-MG53 was redistributed toward the cell surface membrane and concentrated in the intracellular membrane compartments (Fig. 2D), suggesting that elevation of intracellular Zn\(^{2+}\) may facilitate the translocation of GFP-MG53 to intracellular vesicles and to the plasma membrane.

Molecular Analysis of Zn\(^{2+}\) Binding to MG53—MG53 is a member of the TRIM family of proteins comprising two to three Zn\(^{2+}\)-binding domains (38–40). It has been reported that Zn\(^{2+}\)-binding motifs coordinate Zn\(^{2+}\) ions with a cluster of cysteine and histidine residues. The Cys\(_{3}\)-His\(_{2}\)-like structure is by far the best characterized class of zinc fingers and is commonly found in mammalian transcription factors. These domains adopt a simple ββα-fold and have the amino acid
sequence motif $X_2$-Cys-$X_{2,4}$-Cys-$X_{12}$-His-$X_{3,4,5}$-His (40), which is present in MG53. As shown in the schematic diagram in Fig. 3A, domain homology analysis revealed that MG53 contains one Zn$^{2+}$-binding domain in the RING finger motif (amino acids 1–56) and another in the B-box motif (amino acids 86–117).

To understand the molecular mechanisms underlying the role of Zn$^{2+}$ binding to MG53 in repair of cell membrane injury, we generated several site-specific mutations of the possible Zn$^{2+}$-binding residues in the RING and B-box motifs of GFP-MG53, including GFP-MG53(C29L), GFP-MG53(H31A), and GFP-MG53(C53A/C55A/C56A) in the RING motif; GFP-MG53(C86A) and GFP-MG53(C105S) in the B-box motif; and GFP-MG53(C29L/C105S) in both motifs. These constructs were transiently transfected into C2C12 myoblasts, and expression of the mutant MG53 proteins was determined by Western blotting. As shown in Fig. 3B, all mutant constructs could be expressed in C2C12 cells, with a predicted molecular mass of ~75 kDa for GFP-MG53. Although all mutant proteins showed a monomeric form in a reduced environment (with 10 mM DTT) (Fig. 3B, left panel), removal of DTT (right panel) resulted in the appearance of oligomeric forms of GFP-MG53, which were observed for all mutants generated, except for mutant C242A, which was used as a control here because it has been shown to exist only in monomeric form under both reduced and oxidized conditions (24, 33). This finding indicates that all mutants with changes in the Zn$^{2+}$-binding motifs maintained their intermolecular oligomerization properties, which would be crucial for MG53-mediated membrane repair function.

To examine the Zn$^{2+}$-binding properties of MG53 in vitro, we purified the recombinant MBP-MG53 fusion proteins following E. coli fermentation. MBP has been proven to enhance the solubility of proteins expressed in E. coli (41). The addition of MBP to the N terminus of MG53 enabled proper folding and purification of the MBP-MG53 fusion proteins. Approximately 100 mg of fusion protein/liter of bacterial culture could be
To characterize the Zn\(^{2+}\)/H11001-binding capacity, we used the Zn\(^{2+}\)/H11001-specific fluorescent probe TSQ (42) to quantify the amount of Zn\(^{2+}\)/H11001 that was bound to the MBP-MG53 proteins. The linearity of TSQ fluorescence as a function of Zn\(^{2+}\)/H11001 content was established in our previous publication (37). The thiol-bound Zn\(^{2+}\)/H11001 in MBP-MG53, MBP-MG53(C29L), or MBP-MG53(C29L/C105S) attached to the amylose resin beads was liberated with p-hydroxymercuriphenylsulfonate treatment. After washing away the free TSQ fluorescent dye, the MBP-MG53-bound beads were analyzed by fluorescence microscopy. The images show strong fluorescent signals corresponding to Zn\(^{2+}\)/H11001 amounts liberated from the beads in striking contrast to the beads under the control conditions (Fig. 4A). Compared with MBP-MG53, TSQ fluorescence was less in the MBP-MG53(C29L) mutant and further decreased in the MBP-MG53(C29L/C105S) double mutant. Quantitative data are presented in Fig. 4C. The Zn\(^{2+}\)/H11001 content was diminished by approximately half in MBP-MG53(C29L), and Zn\(^{2+}\)/H11001 binding was further reduced in the MBP-MG53(C29L/C105S) double mutant. Taken together, these data indicate that disruption of zinc finger domains in the RING and B-box motifs of MG53 compromises its Zn\(^{2+}\)/H11001-binding capacity.

Due to technical difficulty with expression of the MG53(C105S) mutant in E. coli, its Zn\(^{2+}\)/H11001-binding capacity could not be determined. In addition, we found that an MG53 mutant with defective E3 ligase activity (C14A), according to our previous work (43), also could not be expressed in sufficient quantity using E. coli fermentation, likely due to protein-misfolding problems.
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The GFP-MG53(C105S) mutant with a mutation in the B-box motif also failed to move to the acute injury site following microelectrode penetration in an extracellular nominal zinc-free solution (Fig. 5B, middle panel). Similarly, the addition of Zn-HPT led to partial rescue of the membrane repair capacity of the GFP-MG53(C105S) mutant, as evidenced by its movement toward the injury site (Fig. 5B, right panel).

Further study showed that the GFP-MG53(C29L/C105S) double mutant failed to move to the plasma membrane upon acute membrane damage in nominal zinc-free solution (Fig. 5C, middle panel) and following the addition of extracellular Zn$^{2+}$ (right panel). Data from multiple experiments are summarized in Fig. 5D. These results demonstrated that the membrane repair function of the MG53(C29L/C105S) mutant was completely abolished and was insensitive to Zn$^{2+}$ (Fig. 5D, right panel).

We next performed a quantitative LDH release assay to assess the impact of Zn$^{2+}$-binding motif mutations on MG53-mediated cell membrane repair (35, 36). Populations of C2C12 cells ($5 \times 10^5$) transfected with GFP-MG53, GFP-MG53(C29L), GFP-MG53(C105S), or GFP-MG53(C29L/C105S) were subjected to glass microbead-induced cell membrane damage. Membrane damage-induced release of LDH into the culture medium was measured using a LDH detection kit. As shown in Fig. 5E, C2C12 cells expressing GFP-MG53(C29L) or GFP-MG53(C105S) did not show a significant change in LDH release compared with cells expressing GFP-MG53. However, C2C12 cells expressing GFP-MG53(C29L/C105S) displayed a significant increase in LDH release, indicating more cell injury. These data are consistent with our live cell imaging of MG53-mediated vesicle translocation to acute membrane injury sites, where mutations in Zn$^{2+}$-binding to both RING and B-box motifs are required for complete disruption of the membrane repair function of MG53.

Chelation of Zn$^{2+}$ by Either Ca-EDTA or TPEN Does Not Affect Redox-dependent Oligomerization of MG53—In our previous studies (24, 33), we determined that the redox-dependent oligomerization of MG53 plays an important role in the nucleation process of cell membrane repair. To address whether Zn$^{2+}$ binding affects the oligomerization process of MG53, we conducted the following experiments. We recently developed a protocol for scale-up production of the rhMG53 protein from CHO cells. Purified rhMG53 showed redox-dependent oligomerization, as removal of DTT from the sample buffer led to the appearance of dimers and oligomers of rhMG53 (Fig. 6). To test whether chelation of Zn$^{2+}$ affects the oligomerization of MG53, we supplemented the sample buffer with increasing concentrations of Ca-EDTA and TPEN. As shown in Fig. 6, these treatments did not affect the oligomerization of rhMG53.

On the basis of these results, we conclude that both Zn$^{2+}$ binding, MG53 oligomerization, and repair function for the various mutant constructs are summarized in Table 1. WT MG53 possesses the ability to form oligomers, bind Zn$^{2+}$ ions, and function as a membrane repair molecule. MG53(C242A) lost the ability to form oligomers and is defective in membrane repair.

Disruption of Zn$^{2+}$ Binding to MG53 Correlates with Defects in MG53-mediated Repair of Membrane Damage—To examine the functional role of Zn$^{2+}$ binding in MG53-mediated membrane repair, we mechanically damaged membranes of C2C12 cells transfected with the various GFP-MG53 mutants by microelectrode penetration. As shown in Fig. 5A, the GFP-MG53(C29L) mutant displayed compromised movement toward the acute injury site in a nominal zinc-free extracellular solution (middle panel). The addition of Zn-HPT to the extracellular solution partially restored the movement of GFP-MG53(C29L) toward the acute injury site (Fig. 5A, right panel).
function (24, 33). The MG53(C29L) and MG53(C105S) single mutants can form dimers, but they have diminished ability to bind Zn$^{2+}$ and thus cannot repair the injured membrane in the absence of extracellular Zn$^{2+}$. However, the repair function can be partially recovered in the presence of extracellular Zn$^{2+}$. The MG53(C29L/C105S) double mutant can form dimers;
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Interestingly, the initial movement of MG53 in response to membrane injury is Ca\(^{2+}\)-independent (24). Like Ca\(^{2+}\), Zn\(^{2+}\) is involved in a wide array of physiological functions (46), and the deregulation of intracellular Zn\(^{2+}\) has an important role in pathophysiology, including wound healing. Evidence has been obtained that Zn\(^{2+}\) delivered locally provides therapeutic advantages in treatment of both acute and chronic wounds (4, 47–49). However, the definitive role of Zn\(^{2+}\) in the process of plasma membrane repair has not yet been determined. In this study, we established a base for Zn\(^{2+}\) interaction with MG53 in protection against injury to the cell membrane.

Our studies showed that different motifs serve distinct roles in MG53-mediated membrane repair. The C242A mutation in MG53 causes oligomerization loss and has a dominant-negative effect on MG53-mediated membrane repair, indicating that protein oligomerization is important for vesicular nucleation by MG53 at the membrane disruption sites (24, 33). In addition to redox-dependent oligomerization, Zn\(^{2+}\) binding constitutes an important factor for cell membrane repair. We have demonstrated that MG53 is a potential target for Zn\(^{2+}\) during the membrane repair process. We have shown that removing extracellular Zn\(^{2+}\) or disrupting the Zn\(^{2+}\)-binding motifs in MG53 alters MG53-mediated vesicular translocation and membrane repair function in muscle cells. We have also shown that the effect of Zn\(^{2+}\) on cell membrane repair was lost in mg53\(^{-/-}\) muscle fibers, suggesting that MG53 probably serves as a receptor for Zn\(^{2+}\) during cell membrane repair. Because chelation of free Zn\(^{2+}\) did not appear to affect the redox-dependent oligomerization of MG53, we conclude that both Zn\(^{2+}\) binding to MG53 and redox-dependent oligomerization of MG53 contribute to the nucleation process of cell membrane repair.

Zn\(^{2+}\) deficiency has been linked to many human diseases (50), including cardiovascular diseases (51) and Alzheimer disease (52). Defective membrane repair has been associated with muscular dystrophy and cardiomyopathy (32, 53). Our study highlights a novel potential therapeutic approach in targeting the functional interaction between Zn\(^{2+}\) and MG53 for treatment of human diseases associated with compromised membrane repair capacity.

As a TRIM family protein, MG53 possesses an intrinsic E3 ligase function, enabling it to execute the ubiquitination of specific target proteins (38–40). Recent studies showed that MG53-mediated IRS-1 ubiquitination negatively regulates insulin signaling in skeletal muscle (43, 54). A single mutation in the Zn\(^{2+}\)-binding motif of MG53, C14A, causes complete disruption of E3 ligase function. The data presented in this study show that Zn\(^{2+}\)-binding single mutations do not completely abolish the membrane repair function of MG53, unlike the double mutation MG53(C29L/C105S), which results in loss

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**TABLE 1**

Summary data of MG53 zinc-binding mutants and their corresponding membrane repair capacity

| MG53 protein | Oligomerization | Zinc binding | Membrane repair
|--------------|----------------|--------------|----------------
|              |                | Without Zn\(^{2+}\) | With Zn\(^{2+}\) |
| WT           | +              | +             | +             |
| C29L         | +              | +             | +             |
| H31A         | +              | NA            | 0             |
| C29L/C105S   | +              | -             | -             |
| C105S        | +              | NA            | -             |
| C242A        | -              | NA            | 0             |
| C53A/C55A/C56A | +           | NA            | +             |
| C86A         | +              | NA            | 0             |

+ +, normal function; +, ~50% reduction of function; −, defective function; NA, not assayed.

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**FIGURE 5.** Zn\(^{2+}\)-binding to RING and B-box motifs in MG53 is critical for membrane repair. The effect of Zn\(^{2+}\)-binding on MG53-mediated membrane repair was analyzed with different Zn\(^{2+}\)-binding motif mutants: GFP-MG53(C29L) (A), GFP-MG53(C105S) (B), and GFP-MG53(C29L/C105S) (C). Confocal images of GFP-MG53 translocation before (0 s; left panels) and after (50 s; middle panels) microelectrode-induced acute membrane damage with extracellular nominal zinc-free solution are shown. In separate experiments, the addition of 20 μM Zn-HP (right panels) rescued the membrane translocation properties of single mutants, but failed in double mutants. D, summary of mutant GFP-MG53 protein translocation upon acute membrane damage: GFP-MG53(C29L) (left panel), GFP-MG53(C105S) (middle panel), and GFP-MG53(C29L/C105S) (right panel). **, p < 0.001. E, LDH release after glass microbead-induced injury to C2C12 cells transfected with WT GFP-MG53 or the zinc-binding mutants. Data represent the normalized LDH activity relative to cells transfected with WT GFP-MG53 (n = four independent experiments). *, p < 0.05 versus WT.

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**FIGURE 6.** Chelating Zn\(^{2+}\) does not alter the redox-dependent oligomerization of MG53. Purified rhMG53 (1 μg) was run on a SDS-polyacrylamide gel. Colloidal blue staining revealed that DTT (10 mM) treatment disrupted oligomer formation of rhMG53. The redox-dependent oligomerization of rhMG53 was not affected by increasing concentrations of TPEN (0 – 400 μM) or Ca-EDTA (0 – 400 μM).

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**FIGURE 5.** Zn\(^{2+}\)-binding to RING and B-box motifs in MG53 is critical for membrane repair. The effect of Zn\(^{2+}\)-binding on MG53-mediated membrane repair was analyzed with different Zn\(^{2+}\)-binding motif mutants: GFP-MG53(C29L) (A), GFP-MG53(C105S) (B), and GFP-MG53(C29L/C105S) (C). Confocal images of GFP-MG53 translocation before (0 s; left panels) and after (50 s; middle panels) microelectrode-induced acute membrane damage with extracellular nominal zinc-free solution are shown. In separate experiments, the addition of 20 μM Zn-HP (right panels) rescued the membrane translocation properties of single mutants, but failed in double mutants. D, summary of mutant GFP-MG53 protein translocation upon acute membrane damage: GFP-MG53(C29L) (left panel), GFP-MG53(C105S) (middle panel), and GFP-MG53(C29L/C105S) (right panel). **, p < 0.001. E, LDH release after glass microbead-induced injury to C2C12 cells transfected with WT GFP-MG53 or the zinc-binding mutants. Data represent the normalized LDH activity relative to cells transfected with WT GFP-MG53 (n = four independent experiments). *, p < 0.05 versus WT.
of repair function. Thus, therapeutic approaches for increased expression of MG53 in tissues that bypasses its interaction with IRS-1 may be a novel way to treat human diseases linked to compromised membrane repair capacity.

References

1. Chasapis, C. T., Loutsidou, A. C., Spiliopoulou, C. A., and Stefanidou, M. E. (2012) Zinc and human health: an update. Arch. Toxicol. 86, 521–534

2. Moynahan, E. J. (1974) Letter: Acrodermatitis enteropathica: a lethal inherited human zinc-deficiency disorder. Lancet 2, 399 – 400

3. Braun, O. H., Heilmann, K., Pauli, W., Rossner, J. A., and Bergmann, K. E. (1976) Acrodermatitis enteropathica: recent findings concerning clinical features, pathogenesis, diagnosis and therapy. Eur. J. Pediatr. 121, 247 – 261

4. Landsdown, A. B., Mirastschijski, U., Stubbs, N., Scanlon, E., and Agren, M. S. (2007) Zinc in wound healing: theoretical, experimental, and clinical aspects. Wound Repair Regen. 15, 2 – 16

5. Gupta, M., Mahajan, V. K., Mehta, K. S., and Chauhan, P. S. (2014) Zinc and human health: an update. Arch. Toxicol. 88, 521 – 534

6. Zorrilla, P., Gómez, L. A., Salido, J. A., Silva, A., and López-Alonso, A. (2006) Low serum zinc level as a predictive factor of delayed wound healing in total hip replacement. Wound Repair Regen. 14, 119 – 122

7. Mozzillo, N., Ayala, F., Formato, A., Forestieri, P., and Mazzeo, F. (1984) First full blown syndrome of acute zinc deficiency in course of long term total parenteral nutrition: a clinical case. Ital. J. Surg. Sci. 14, 229 – 231

8. Herbein, G., Varin, A., and Fulop, T. (2006) NF-κB, AP-1, zinc-deficiency and aging. Biogerontology 7, 409 – 419

9. Wang, C., Vernon, R., Lange, O., Tyka, M., and Baker, D. (2010) Prediction of structures of zinc-binding proteins through explicit modeling of metal coordination geometry. Protein Sci. 19, 494 – 506

10. Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., and Bourne, P. E. (2000) The Protein Data Bank. Nucleic Acids Res. 28, 235 – 242

11. Berg, J. M., and Shi, Y. (1996) The galvanization of biology: a growing total parenteral nutrition: a clinical case. J. Biol. Chem. 271, 1081 – 1085

12. van der Meer, L. T., Jansen, J. H., and van der Reijden, B. A. (2010) Gfi1 and Gfi1b: key regulators of hematopoiesis. Leukemia 24, 1834 – 1843

13. Haase, H., and Rink, L. (2014) Multiple impacts of zinc on immune function. Metallomics 6, 1175 – 1180

14. Marger, L., Schubert, C. R., and Bertrand, D. (2014) Zinc: an underappreciated modulatory factor of brain function. Biochem. Pharmacol. 91, 426 – 435

15. Vallee, B. L., and Falchuk, K. H. (1993) The biochemical basis of zinc physiology. Physiol. Rev. 73, 79 – 118

16. Frederickson, C. J., Giblin, L. J., Krezel, A., McAdoo, D. J., Mueller, R. N., Zeng, Y., Balaji, R. V., Fierke, C. A., and Sarvey, Y., Balaji, R. V., Masalha, R., Thompson, R. B., Fierke, C. A., Yan, R., Sterling, M., Zhao, X., Hwang, M., Takeshima, M., Cai, C., Cheng, H., Takeshima, H., Xiao, R. P., and Ma, J. (2012) Redominant MG53 protein modulates therapeutic cell membrane repair in treatment of muscular dystrophy. Sci. Transl. Med. 4, 139ra185

17. McNeil, P. L., Vogel, S. S., Miyake, K., and Terasaki, M. (2000) Patching plasma membrane disruptions with cytoplasmic membrane. J. Cell Sci. 113, 1891 – 1902

18. Cai, C., Masumuya, 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., and Ma, J. (2009) MG53 nucleates assembly of cell membrane repair machinery. Nat. Cell Biol. 11, 56 – 64

19. Cao, C. M., Zhang, Y., Weisleder, N., Ferrante, C., Wang, X., Lv, F., Zeng, Y., Song, R., Hwang, M., Lin, J., Guo, J., Peng, W., Li, G., Nishi, M., Takeshima, H., Ma, J., and Xiao, R. P. (2010) MG53 constitutes a primary determinant of cardiac ischemic preconditioning. Circulation 121, 2565 – 2574

20. Towler, M. C., Kaufman, S. J., and Brodsky, F. M. (2004) Membrane traffic and patch repair. Traffic 5, 129 – 139

21. Glover, L., and Brown, R. H., Jr. (2007) Dyserlin in membrane trafficking and patch repair. Traffic 8, 785 – 794

22. Shen, S. S., Tucker, W. C., Chapman, E. R., and Steinhardt, R. A. (2005) Molecular regulation of membrane rescaling in 3T3 fibroblasts. J. Biol. Chem. 280, 1652 – 1660

23. McNeil, P. L., Vogel, S. S., Miyake, K., and Terasaki, M. (2000) Patching
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and Lippard, S. J. (2004) Method for identifying neuronal cells suffering zinc toxicity by use of a novel fluorescent sensor. J. Neurosci. Methods 139, 79–89

43. Yi, J. S., Park, J. S., Ham, Y. M., Nguyen, N., Lee, N. R., Hong, J., Kim, B. W., Lee, H., Lee, C. S., Jeong, B. C., Song, H. K., Cho, H., Kim, Y. K., Lee, I. S., Park, K. S., Shin, H., Choi, I., Lee, S. H., Park, W. J., Park, S. Y., Choi, C. S., Lin, P., Karunasiri, M., Tan, T., Duann, P., Zhu, H., Ma, J., and Ko, Y. G. (2013) MG53-induced IRS-1 ubiquitination negatively regulates skeletal myogenesis and insulin signalling. Nat. Commun. 4, 2354

44. Steinhardt, R. A., Bi, G., and Alderton, J. M. (1994) Cell membrane resealing by a vesicular mechanism similar to neurotransmitter release. Science 263, 390–393

45. Terasaki, M., Miyake, K., and McNeil, P. L. (1997) Large plasma membrane disruptions are rapidly resealed by Ca2+-dependent vesicle-vesicle fusion events. J. Cell Biol. 139, 63–74

46. Sensi, S. L., Paolelli, P., Bush, A. I., and Sekler, I. (2009) Zinc in the physiology and pathology of the CNS. Nat. Rev. Neurosci. 10, 780–791

47. Agren, M. S., Ostenfeld, U., Kallehave, F., Gong, Y., Raffn, K., Crawford, M. E., Kiss, K., Friis-Møller, A., Gluud, C., and Jorgensen, L. N. (2006) A randomized, double-blind, placebo-controlled multicenter trial evaluating topical zinc oxide for acute open wounds following pilonidal disease excision. Wound Repair Regen. 14, 526–535

48. Lansdown, A. B. (1993) Influence of zinc oxide in the closure of open skin wounds. Int. J. Cosmet. Sci. 15, 83–85

49. Grommes, J., Binnebösel, M., Klink, C. D., von Trotha, K. T., Rosch, R., Oettinger, A. P., Lindlar, L., and Krones, C. J. (2011) Balancing zinc deficiency leads to an improved healing of colon anastomosis in rats. Int. J. Colorectal Dis. 26, 295–301

50. Prasad, A. S. (2013) Discovery of human zinc deficiency: its impact on human health and disease. Adv. Nutr. 4, 176–190

51. Frustaci, A., Sabbioni, E., Fortaner, S., Farina, M., del Torchio, R., Tafani, M., Morgante, E., Ciriolo, M. R., Russo, M. A., and Chimenti, C. (2012) Selenium- and zinc-deficient cardiomyopathy in human intestinal malabsorption: preliminary results of selenium/zinc infusion. Eur. J. Heart Fail. 14, 202–210

52. Brewer, G. J., and Kaur, S. (2013) Zinc deficiency and zinc therapy efficacy with reduction of serum free copper in Alzheimer’s disease. Int. J. Alzheimers Dis. 2013, 586365

53. Han, R., Bansal, D., Miyake, K., Muniz, V. P., Weiss, R. M., McNeil, P. L., and Campbell, K. P. (2007) Dysferlin-mediated membrane repair protects the heart from stress-induced left ventricular injury. J. Clin. Invest. 117, 1805–1813

54. Song, R., Peng, W., Zhang, Y., Lv, F., Wu, H. K., Guo, J., Cao, Y., Pi, Y., Zhang, X., Jin, L., Zhang, M., Jiang, P., Liu, F., Meng, S., Zhang, X., Jiang, P., Cao, C. M., and Xiao, R. P. (2013) Central role of E3 ubiquitin ligase MG53 in insulin resistance and metabolic disorders. Nature 494, 375–379