Title: COMPOSITIONS AND METHODS TO PREVENT AND REPAIR ACUTE KIDNEY INJURY

Abstract: Disclosed herein are compositions and methods for repairing cell membranes. In particular, the description provides compositions and methods comprising MG53, a member of the TRIM-family of proteins, in the repair of kidney injury and its therapeutic use in the prevention and treatment of kidney injury. [Continued on next page]

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COMPOSITIONS AND METHODS TO PREVENT AND
REPAIR ACUTE KIDNEY INJURY

Cross-Reference to Related Applications
[001] The present application claims the benefit of U.S. Provisional Patent Application Serial No. 62/098,154 filed 30 December 2014, and titled: Compositions and Methods to Prevent and Repair Acute Kidney Injury, which is incorporated herein by reference.

Incorporation by Reference
[002] In compliance with 37 C.F.R. § 1.52(e)(5), the sequence information contained in electronic file name: Ma_2015utility_ST25.txt; size 57 KB; created on: December 29, 2015; using Patent-In 3.5, and Checker 4.4.0 is hereby incorporated herein by reference in its entirety.

Statement Regarding Federally Sponsored Research
[003] The U.S. Government has certain rights in this invention pursuant to the following grants: RO1-HL0691000 awarded to Dr. Jianjie Ma by the United States National Institutes of Health (NIH).

Background
[004] 1. Field of the discovery. This invention relates to polypeptide compositions and methods of use thereof for the modulation of cell membrane repair.

[005] 2. Background information. To maintain cellular homeostasis, eukaryotic cells must conserve the integrity of their plasma membrane through active recycling and repair in response to various sources of damage. For example, in response to external damage and internal degeneration, the cells of the body must repair the membrane surrounding the each individual cell in order to maintain their function and the health of the organism.

[006] Repair of damage to the plasma membrane is an active and dynamic process that requires several steps, including participation of molecular sensor(s) that can detect acute injury to the plasma membrane, nucleation of intracellular vesicles at the injury site and vesicle fusion to enable membrane patch formation. It has been demonstrated that entry of
extracellular calcium is involved in the fusion of intracellular vesicles to the plasma membrane, however, the molecular machinery involved in sensing the damaged membrane signal and the nucleation process for repair-patch formation have not been fully resolved.

[007] Defects in the ability of the cell to repair external membranes have been linked to a broad spectrum of diseases and pathological conditions, for example, neurodegenerative diseases (e.g., Parkinson's Disease, BSE, and Alzheimer's), heart attacks, heart failure, muscular dystrophy, bed sores, diabetic ulcers, oxidative damage, and tissue damage such as sinusitis that occurs as side effect from the administration of chemotherapeutic agents. Also, the muscle weakness and atrophy associated with various diseases, as well as the normal aging process, has been linked to altered membrane repair. In order for these cells to repair their membranes in response to acute damage they make use of small packets of membrane that are inside of the cell, referred to as vesicles. These vesicles are normally found within the cell, but upon damage to the cell membrane, these vesicles move to the damage site and form a patch to maintain the cell integrity. Without this essential function, the cell can die and the cumulative effect of this cellular injury can eventually result in dysfunction of the tissue or organ.

[008] Repair of injury to the plasma membrane is an important aspect of physiology and disruption of this process can result in pathophysiology in a number of human diseases including cardio-renal disorders. We previously identified a novel TRIM family protein, named MG53, as an essential component of the cell membrane repair machinery. Redox-dependent oligomerization of MG53 allows for nucleation of intracellular vesicles to the injury site for formation of a membrane repair patch. MG53 knockout mice (Mg53-/-) exhibit defective membrane repair in striated muscle that leads to progressive skeletal myopathy and increased vulnerability of cardiomyocytes to I/R induced injury. While MG53 is predominantly expressed in striated muscles, its expression in non-muscle cells and its physiological role in other organ protection are largely unknown. Our recent study identified an altered form of MG53 expressed in the lung tissue, and intravenous or inhalation delivery of recombinant human MG53 protein (rhMG53) could ameliorate acute lung injury in rodent models.

[009] During normal kidney function, active endocytosis and exocytosis occur in the brush border of the proximal tubular epithelium (PTE). The dynamic membrane trafficking and remodeling processes in PTE cells render them highly vulnerable to membrane injury, necessitating an intrinsic reparative mechanism to support normal renal function and to protect them from excessive damage when exposed to stresses such as ischemia-reperfusion
(I/R), nephrotoxin, chemotherapy, and sepsis. While the kidney has the ability to repair itself following mild injury, insufficient repair of PTE cells can trigger inflammatory response causing extensive damage and fibrotic remodeling, leading to progression to chronic renal failure.

[0010] Acute kidney injury (AKI) is commonly encountered in the hospital and outpatient settings, and is associated with a high rate of mortality. Currently, there is no effective means for preventing or treating AKI. As a result, patients who develop AKI in this setting require lengthy hospital stays, incurring high cost for treatment of AKI and in prevention of chronic renal failure. The knowledge gap in understanding the molecular mechanisms associated with repair of injury to PTE cells is a setback in the development of novel therapy for AKI.

[0011] Accordingly, there exists an ongoing need for the development of pharmaceutical modulators of the cell membrane repair process for the treatment of conditions related to acute and chronic cellular and tissue damage such as, but not limited to, AKI.

Summary

[0012] The present description relates to the surprising and unexpected discovery that MG53 constitutes a vital component of reno-protection. The description demonstrates that MG53 surprisingly and unexpectedly repairs injury to renal proximal tubular epithelium (PTE) cells. Thus, the description provides compositions and methods to repair the plasma membrane repair following injury to the PTE, which represents the underlying consequence of acute kidney injury (AKI), and represents a novel therapeutic approach for treatment and prevention of kidney injury. For example, the description provides MG53 nucleic acids, MG53 polypeptides, and therapeutic compositions comprising effective amounts of the same, which, alone or in combination with other components, can modulate or enhance the process of cell membrane resealing in PTE.

[0013] In certain aspects, the description relates to compositions useful as therapeutics for treating and prevention of diseases and disorders related to cellular and/or tissue damage. Therapeutic compositions of the invention comprise effective amounts of MG53 polypeptides, and/or nucleic acids encoding MG53 polypeptides, for example, the protein of SEQ ID NO. 1 and MG53 polypeptide mutants, homologs, fragments, truncations, pseudopeptides, peptide analogs, and peptidomimetics, as well as compounds that can modulate the activity of MG5.
In an exemplary embodiment, the description provides a pharmaceutical composition for the treatment of a kidney injury, disease or disorder comprising an effective amount of a recombinant MG53 polypeptide or fragment thereof possessing MG53-like activity sufficient to treat or ameliorate a symptom of the kidney injury, disease or disorder, and a pharmaceutically acceptable carrier. In certain embodiments, the kidney injury, disease or disorder is acute kidney injury (AKI). In still certain additional embodiments, the pharmaceutical composition is in a form suitable for intravenous injection. In certain embodiments, the composition can be in unitary dosage form or comprise approximately an entire daily, weekly, or monthly dose.

In certain additional aspects the description relates to methods for the treatment of kidney cell or tissue injury/damage. In certain exemplary embodiments, the method comprises, for example, the administration of a therapeutic composition as described herein for the prevention and/or treatment of kidney injury. In certain embodiments, the method comprises administering to a subject in need thereof, a composition comprising an effective amount of mitsugumin 53 (MG53), wherein the composition is effective in treating, preventing or ameliorating kidney injury. In certain embodiments, the kidney injury is acute kidney injury. In certain embodiments, the acute kidney injury includes at least one of surgery-related AKI, contrast media-induced AKI, drug or chemotherapy-induced AKI, toxin-induced AKI, dialysis, ischemia/reperfusion-induced AKI, sepsis-induced AKI, nephrotoxin exposure or a combination thereof.

In certain embodiments, the method includes a step whereby the subject is co-administered a chemotherapeutic agent (in the same or different formulation) and an effective amount of MG53. In certain embodiments, the chemotherapeutic agent is cisplatin.

In any of the aspects or embodiments described herein, the subject can be a human or a non-human primate.

The method of any one of the above claims, wherein the MG53 polypeptide or fragment thereof possessing MG53 activity is in an amount sufficient to enhance membrane repair in the proximal tubular epithelium (PTE) cells.

In any of the aspects or embodiments described herein, the MG53 polypeptide can be a recombinant MG53 polypeptide or fragment thereof possessing membrane repair activity.

In any of the aspects or embodiments as described herein, the MG53 polypeptide or fragment thereof possessing MG53 activity is recombinant human MG53 (rhMG53) polypeptide.
[0021] The preceding general areas of utility are given by way of example only and are not intended to be limiting on the scope of the present disclosure and appended claims. Additional objects and advantages of the present invention will be appreciated by one of ordinary skill in the art in light of the instant claims, description, and examples. For example, the various aspects and embodiments of the invention may be utilized in numerous combinations, all of which are expressly contemplated by the present description. These additional objects and advantages are expressly included within the scope of the present invention.

**Brief Description of the Drawings**

[0022] The accompanying drawings, which are incorporated into and form a part of the specification, illustrate several embodiments of the present invention and, together with the description, serve to explain the principles of the invention. The drawings are only for the purpose of illustrating an embodiment of the invention and are not to be construed as limiting the invention.

[0023] FIG. 1: MG53 is a muscle specific member of the TRIM protein family. An alignment of the protein sequence of MG53 from various organisms (See SEQ ID NOs.: 1, 3, 5, 9-16) reveals this protein to be a member of the TRIM family. Functional domains are boxed in grey while arrows indicate the domain continues onto another line of the sequence. Boxed Leucine residues indicate the location of a highly conserved Leucine zipper motif.

[0024] FIG. 2: Illustrates an exemplary domain comparison of some homologous proteins that contain one or more of the conserved tripartite motifs which are present in MG53. MG53 is unique in it's ability to translocate to an injury site at the cell membrane following multiple forms of insult and mediate repair of the damaged membrane - a function which is not exhibited by the other TRIM family proteins listed. While these TRIM proteins all contain similar domains and/or are expressed in striated muscle, none fully recapitulate the domain organization of MG53.

[0025] FIG. 3: MG53 contains unique TRIM and SPRY motifs and is predominantly expressed in muscle cells. A. Diagram of motif structure of MG53. From the results of cDNA cloning and homology searches, several motif sequences are detected in MG53 as shown. The sequences of rabbit and mouse MG53 cDNAs have been deposited in the databases under accession numbers AB231473 and AB231474, respectively. B. Western blot analysis shows the specific expression of MG53 in skeletal and cardiac muscles. Lysate (20 μg total protein per lane) from mouse tissues (lung, kidney, skeletal muscle, liver, heart, brain) were
analyzed using anti-mouse MG53 polyclonal antibody. C. Immunofluorescence staining of longitudinal transverse sections from mouse skeletal muscle cells. Scale bar is 125 μm.

[0026] FIG. 4. Progressive pathology is seen in mg53-/− skeletal muscle due to increased damage of cell membranes. A. Haematoxylin and Eosin (H/E) staining illustrates increased number of central nuclei (arrows) in aging mg53-/− muscle (10m) versus young (3m) wild type (wt) or mg53-/− mice. B. The diameter of muscle fibers in aged (8-10 month) mg53-/− mice (blue, n=541) decreased compared to aged (8-10 month) wild type controls (black, n=562) while there is no difference in young (3-5 months) wt (n=765) versus mg53-/− (n=673) muscle. Percentage of muscle fibers that display central nuclei in mg53-/− skeletal muscle increases with age when compared to wt. Data is mean ± s.e.m., *p < 0.05 by ANOVA. C. Trace recordings of contractile performance of intact soleus muscle obtained from mice subjected to 30 min down-hill exercise running was assessed using an in vitro voltage stimulation protocol, following described procedures. Black trace represents wt muscle, blue trace corresponds to mg53-/− muscle. D. Prior to fatigue stimulation (Pre, open bars), the maximal tetanic force, normalized in g/mg total protein, was significantly lower in aging mg53-/− muscle (blue) versus wt (black) (n=4). At 6 min after fatigue stimulation (After, closed bars), the wt muscle recovered significantly more than mg53-/− muscle. *p < 0.05 by ANOVA. E. Extensive Evans blue staining reveals serve damage in mg53-/− skeletal muscle subjected to down-hill running when compared to minimal staining in wt muscles. F. Chart of the quantity of Evans blue dye extracted by formamide from aging mg53-/− (blue) and wt (black) skeletal muscle following exercise. The data represents mean value of Evans blue (ng) per g of muscle ± s.e.m. n=8-12, *p < 0.005 by Student's t-test.

[0027] FIG. 5. Ablation of MG53 leads to defective muscle membrane repair function, (a) Immunostaining of MG53 in isolated wt FDB fibers to illustrate their colocalization at the injury site. These are representative images from >20 different muscle fibers which display damage during isolation, (b) Exclusion of membrane-impermeable FM-143 fluorescent dye in a FDB muscle fibers isolated from the wt mice following laser-induced damage of the sarcolemmal membrane, (c) Entry of FM-143 fluorescent dye into a FDB muscle fiber isolated from the mg53-/− mice following laser-induced damage. Times after laser injury were indicated, (d) Time-dependent accumulation of FM-143 inside the FDB muscle fiber induced by a laser damage of the sarcolemmal membrane. Data are means+s.e.m. for n=30 fibers obtained from wt mice and n=18 fibers from mg53-/− mice.

[0028] FIG. 6. MG53 knockout mice are susceptible to cardiac damage. Paraffin-embedded sections of myocardium from unexercised wild type mice show normal
morphology (left) and no Evans blue staining (right). In contrast, and mg53/- mice display a Evans blue infiltration into myocytes, indicating that there are significant defects in membrane integrity in the mg53/- heart.

FIG 7. Loss of MG53 increases susceptibility to cardiac ischemia reperfusion injury. Hearts from wild type (WT) and mg53/- mice were isolated and perfused on a Langendorff apparatus. Global ischemia was induced for 30 minutes by cessation of perfusate flow. The damage produced in the heart following restoration of perfusate flow (time 0) was measured by enzymatic assays for (a) creatine kinase (CK) or (b) lactate dehydrogenase (LDH). Hearts from mg53/- mice (dashed lines) show more damage than WT (solid lines). Data is presented as mean+S.D. for each listed time point.

FIG. 8. MG53 containing vesicles form a patch in the plasma membrane following physical insult, a) Damage of a C2C12 myoblast membrane using a micropipette leads to rapid accumulation of GFP-MG53 at the injury site (arrow). Images were representative of n=40 separate cells, b) Recovery of a mature C2C12 myotube in response to a severe damage, e.g. separation of the cell membrane, is associated with recruitment of GFP-MG53 toward the healing site (n=28). c) Comparison of survival rates of wild type and mg53/- primary skeletal myotubes. This data illustrates that MG53 is required for membrane resealing in striated muscle cells.

FIG. 9. Role of TRIM and SPRY domains in targeting of MG53 to the cell surface membrane of muscle cells. A. Scheme of the MG53 deletion fusion protein constructs with GFP fused to the N-terminus or C-terminus. With reference to SEQ ID NO. 1, "TRIM" represents a.a. 1-287 and "SPRY" represents a.a. 288-477 and includes both the PRY and SPRY motifs. B. Representative confocal images showing intracellular localization of each deletion construct in C2C12 cells. Scale bar is 5 μm. C. MG53 interacts with caveolin-3 through the TRIM motif. Cell lysate from CHO cells co-transfected with GFP-MG53 or GFP-TRIM and pcDNA-Cav-3 was subjected to IP with anti-caveolin-3 (mouse monoclonal antibody). (Lane 1, mixed cell lysate as positive control; Lane 2, normal mouse IgG as negative control; lane 3, lysate from cells overexpressing GFP-MG53; Lane 4, lysate from cells overexpressing GFP-TRIM).

FIG 10. Role of TRIM and SPRY domains in targeting of MG53 to the cell surface membrane in non-muscle CHO cells. Representative confocal images showing that GFP-MG53 exhibits intracellular vesicle, membrane targeting and budding, however MG53-GFP is mainly soluble in nature (upper panel); SPRY-GFP and GFP-SPRY are cytosolic (middle panel); TRIM-GFP and GFP-TRIM are mainly intracellular vesicle, and do not target
to plasma membrane (lower panel). "TRIM" represents a.a. 1-287 and "SPRY" represents a.a. 288-477 and includes both the PRY and SPRY motifs. Scale bar is 5 μm.

[0033] FIG 11. MG53 can interact with Kinesin family member 11 (Kif11). (a) Cell lysates were isolated from HEK293 cells stably expressing FLAG-tagged versions of either RFP (mRFP), RFP-MG53 (MG53) or C29L mutant RFP-MG53 (C29L). Extracts were co-immunoprecipitated with anti-FLAG antibody and then run on a SDS-PAGE gel. Coomassie staining revealed specific bands that would co-IP by this approach. One prominent band was for Kif11 (arrowhead). (b) Mass spectroscopy was used to identify particular bands from these gels. This representative mass spectroscopy tracing shows that MG53 can pull down Kif11 from cell lysates.

[0034] FIG 12. MG53 can interact with COP9 complex homolog subunit 6 (CSN6). HEK293 cells were transiently transfected with HA-tagged human MG53 and myc-tagged CSN6 and then used for co-immunoprecipitation (IP) using antibodies against the recombinant tags. The presence of the protein following pull down was confirmed using Western immunoblots (IB). MG53 can pull down CSN6 and that CSN6 can also pull down MG53. This provides evidence that these two proteins can interact within the cell. Lanes 1= HA-hMG53+hCSN6 +DMSO, Lanes 2= HA-hMG53+hCSN6 +MG132, Lanes 3= HA-mMG53+hCSN6 +DMSO, Lanes 4= HA-mMG53+hCSN6 +MG132.

[0035] FIG 13. MG53 can interact with myelin basic protein or periaxin. (a) Schematic diagrams of methods for biochemical isolation of vesicle fractions from either wild type (WT) or mg53(-/-) (KO) skeletal muscle, (b) Fractions isolated with methods presented in a were run on with 15% (left) of gradient (right) SDS-PAGE gels. Brilliant Blue (CBB) staining revealed specific bands that we differentially present in WT or KO muscle. Two prominent bands were identified as myelin basic protein or periaxin (arrows) by mass spectroscopy.

[0036] FIG 14. MG53 interacts with cellular membranes through an association with phosphatidylycerine to mediate vesicular trafficking. When GFP-MG53 is expressed in these mg53(-/-) myotubes, the protein will properly localize to the plasma membrane and intracellular vesicles (top). POVStrip lipid dot blot analysis reveals recombinant MG53 (1 μg/ml) specifically binds phosphatidylycerine (PS) and not other membrane lipids, including sphingosine-1-P, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine and various phosphoinositol metabolites (B). Using Annexin-V-GFP, we observed rapid labeling of Annexin-V-GFP at the C2C12 myoblast injury site. Annexin-V-GFP (a molecule with
well defined ability to bind PS) transfected into C2C12 myoblasts displays minimal translocation following cell wounding with a microelectrode (left), while co-expression of Annexin-V-GFP with RFP-MG53 (right) results in accelerated accumulation of Annexin-V-GFP (C). The accumulation of Annexin-V-GFP was accelerated by co-expression of RFP-MG53 (0.93±0.21 AF/F₀ control; 2.9±0.63 AF/F₀ +MG53). Entry of extracellular Ca²⁺ through the damaged plasma membrane allowed Annexin-V binding to PS, leading to its transition from a soluble pattern before cell injury to distinct localization to plasma membrane and intracellular vesicles (D). Removal of Ca²⁺ from the extracellular solution disrupted the labeling of PS by Annexin-V-GFP at the injury site, translocation of RFP-MG53 to the injury site was maintained (E).

**FIG 15.** Acute disruption of the plasma membrane leads to exposure of the cell interior to an external oxidized environment. (A and B) MG53 exists primarily as monomers in a reduced environment generated by the addition of dithiothreitol (DTT). (C) inclusion of 5 mM DTT in the extracellular solution produced drastic effects on the MG53-mediated membrane repair process in C2C12 cells. Thimerosal oxidizes sulfhydryl groups at cysteine residues. (E) multiple conserved cysteine residues were mutated into alanines - membrane targeting is maintained, but completely disrupts ability to facilitate the membrane repair process ; i.e., no accumulation of C242A was observed at the injury site.

**FIG 16.** (A) Under a reduced extracellular environment (+DTT), translocation of GFP-MG53 toward the injury site was largely disrupted. The addition of an oxidizing agent (Thimerosal) into the extracellular solution results in an increased translocation of GFP-MG53 to injury sites on the cell membrane. These experiments were conducted in C2C12 cells. (B) MG53 with a C242A mutation (GFP-C242A) cannot translocate to injury sites on the plasma membrane. Since a different conserved cysteine mutant, C313A, maintained oligomerization pattern under oxidized conditions and displayed similar translocation and membrane-repair function as the wild type GFP-MG53. Thus, the oxidation of Cys242 likely induces oligomerization of MG53, providing a nucleation site for repairosome formation at injury sites. These experiments were conducted with C2C12 cells. (C) Modulation of the extracellular redox state can affect the resealing of isolated muscle fiber membranes as the addition of DTT to the extracellular solution prevents membrane resealing, as measured by an increase in entry of FM-143 dye applied outside of the cell.

**FIG 17.** MG53-mediated repairosome formation and restoration of acute sarcolemma membrane damage. Entry of FM4-64, a red-shifted variant of FM1-43, was used
as an index of membrane repair capacity in mg53-/- myotubes transfected with GFP-MG53 and GFP-C242A. Following UV-bleaching of the green fluorescence, rapid translocation of GFP-MG53 took place at the injury site, whereas GFP-C242A remained static due to its defective oligomerization properties. Significantly less entry of FM4-64 was observed in cells transfected with GFP-MG53 compared with GFP-C242A, suggesting that the mutant was not able to restore membrane integrity following injury (B). Oligomerization of MG53 appears to be a step in repairosome formation, as the GFP-C242A mutant expressed in wt skeletal muscle displayed a dominant negative function over the native MG53 (C). Compared with GFP-MG53, overexpression of GFP-C242A in adult wt muscle fibers inhibited sarcolemmal membrane repair function (C).

FIG 18. Ubiquitination, in vitro, catalyzed by MG53. A recombinant maltose-binding protein (MBP) fusion protein for MG53 (MBP-MG53) was incubated with ATP, ubiquitin, E1 and E2 enzymes, and subjected to immunoblotting with the anti-MBP antibody. High molecular-mass ladders derived from ubiquitination were observed when MBP-MG53 was incubated with Ubc4 or UbcH5 as E2 (a). The intrinsic E3-ligase activity of MG53 was significantly reduced in C29L mutant (b). (c) Western blot demonstrated that the full-length GFP-MG53 and GFP-C29L proteins were present in the differentiated C2C12 myotubes, thus the C29L mutant is stable and it is unlikely that degradation of these fusion proteins contributes to the different subcellular distribution of GFP-C29L.

GFP-C29L displays predominantly a cytosolic pattern in C2C12 myotubes (d, left). Western blot demonstrated that the full-length GFP-MG53 and GFP-C29L proteins were present in the differentiated C2C12 myotubes (c), thus it is unlikely that degradation of these fusion proteins contributes to the different subcellular distribution of GFP-C29L and GFP-MG53 observed in (d). Similar phenomena were observed with transient expression of these fusion proteins into primary cultured skeletal myotubes derived from the mg53-/- neonates, where targeting of GFP-MG53 to sarcolemmal membrane and intracellular vesicles were attenuated for the GFP-C29L mutant (d, right). Following acute membrane damage, rapid accumulation of GFP-MG53 is observed in C2C12 myoblasts, whereas GFP-C29L appeared to be immobile and ineffective in repair of membrane injury (e, left). Similar defects with GFP-C29L were also observed in C2C12 myotubes (e, middle). Moreover, while GFP-MG53 could translocate to the plasma membrane following injury in primary cultured mg53-/- myotubes, GFP-C29L expressed in these cells remained generally unresponsive to acute cell injury (e, right).
FIG 19. Effect of removing zinc (Zn) from the extracellular solution before wounding C2C12 myoblasts expressing GFP-MG53. Chelating Zn with N,N,N,N-tetrais(2-pyridylmethyl)ethylenediamine (TPEN) could prevent the translocation of GFP-MG53 to the site of microelectrode penetration (A), indicating that Zn was necessary for MG53 function. Addition of a Zn ionophore, Zn-l-hydroxypyridine-2-thione (Zn-HPT), could induce the translocation of GFP-MG53 in C2C12 cells (B). Wild type FDB muscle fibers: + Zn-HPT reduces the amount of FM-1-43 dye that can enter the muscle fiber following injury induced by a UV laser (C).

FIG 20. Protective effect of zinc on membrane repair is lost in mg53-/ skeletal muscle, (a) individual flexor digitorum brevis (FDB) muscle fibers were isolated from wild type (WT) mice (3-6 months). A strong UV laser was applied to the FDB fiber that caused local damage to the muscle (arrow). Entry of FM1-43 fluorescent dye (2.5 µM) was used as an indicator for the measurement of membrane repair capacity. The images were taken 200 s following UV irradiation (control). Application of 2 µM zinc-ionophore (1-hydroxypyridine-2-thione) (+Zn-HPT) led to increased membrane repair capacity as reflected by the decreased amount of FM1-43 dye entry following UV-damage. Addition of 40 µM TPEN (Tetrakis-2-pyridylmethylenediamine), a specific buffer for zinc ions, led to compromised membrane repair capacity, as reflected by the significant increase in FM1-43 dye entry following UV-damage (+TPEN). (b) FDB muscle fibers isolated from the mg53-/ mice (3-6 months) exhibited defective membrane repair function, as shown by the elevated amount of FM1-43 dye entry following identical treatment of UV-damage (control). (c) with Ca-EDTA (100 µM), a reagent that buffers zinc without altering extracellular Ca concentration, also caused compromised membrane repair capacity in WT muscle (left). Treatment with Ca-EDTA did not produce any significant changes in membrane repair capacity in mg53-/ muscle. (d) Schematic diagram of zinc-binding motifs in MG53. The amino-terminus of MG53 contains two putative zinc-binding motifs: one located at the RING motif (a.a. 1-56, human cDNA), and the other located at the B-box motif (a.a. 86-117, human cDNA). The specific amino acids that participate in zinc-binding are indicated.

FIG 21. Extracellular zinc entry facilitates MG53-mediated vesicle translocation to acute membrane injury sites, a) GFP-MG53 fusion protein was expressed in C2C12 myoblast cells. GFP-MG53 displayed localization at the intracellular vesicles and the plasma membrane under resting condition (left). Acute injury of the cell generated by penetration of a microelectrode (arrow, right panel), b) Incubation of the C2C12 cell with 40
µM Ca-EDTA. c) Addition of 20 µM TPEN to the extracellular solution. d) C2C12 cells transiently transfected with GFP-MG53 were incubated with 20 µM Zn-HPT. Under control condition (0 min), prolonged incubation with Zn-HPT (15 min). e) Summary data with Ca-EDTA and TPEN on GFP-MG53 mediated membrane repair in C2C12 myoblast cells.

FIG 22. Zn-binding to RING and B-box motifs of MG53. a) site-specific mutations in the RING and B-box motifs of MG53 transiently expressed in C2C12 myoblast cells. 24 hours after transfection, the cells were harvested and the expression of the various GFP-MG53 mutants was assayed by Western blot with specific antibody against MG53; in the absence of DTT (left panel). Oligomeric patterns are marked "dimer." With the addition of 10 mM DTT, all mutant constructs displayed monomeric forms of ~75 kD (predicted molecular size of GFP-MG53).

FIG 23. MG53 can bind Zn through a RING motif, (a) MG53 contains a canonical TRIM domain that contains a Zn binding motif (Ring) and a Bbox motif, (b) Bacterial culture was lysed by sonication, centrifuged and bound to Amylose resin in column buffer containing 10 µM zinc for overnight at 4 degrees. The resin was then washed by zinc free column buffer followed by 50ml of zinc free column buffer with 0.3 mM maltose. Protein levels and stability were confirmed by SDS-PAGE gel as shown. Lane 1 (Marker), Lane 2 (mMG53), Lane 3 (mC29L-MG53 mutant), Lane 4 (mC29L/C105S double mutant DM clone1), Lane 5 (mC29L/C105S double mutant DM clone2), Lane 6 (10mg/ml BSA), Lane 7 (5mg/ml BSA), Lane 8 (2.5mg/ml BSA), Lane 9 (1mg/ml BSA). (c) The proteins on beads were first tested for the presence of free zinc in the solution (from 0.01 to 0.1 µM or ND depending on the preparation). The beads (aliquot) were stained with a zinc-specific probe TSQ and fluorescence was observed under the fluorescent microscope and relative fluorescence intensity taken. Then the proteins were denatured at 56 C for 5 min, vortexed, centrifuged, and the measurements were taken again from the solution. The assay uses TSQ (Mol Probe) and an atomic standard solution of zinc (Sigma) for calibration. Chart indicates the amount of Zn binding to recombinant wild type (WT) MG53, C29L mutant (C29L) and double mutant (DM). Both mutants are located in the Ring motif of the TRIM domain. Data presented as mean+S.D. *P<0.05, **P<0.001 compared to wt; n=4-5.

FIG 24. FDB muscle fibers isolated from the wild type mice were loaded with 2 µM TSQ, a specific fluorescent indicator for zinc in the intracellular solution (lower panels). A strong UV-laser was used to cause local damage to the FDB muscle fiber, as reflected by the accumulation of FM4-64 fluorescent day at the local injury site (top panels).
Notice that significant elevation of TSQ fluorescence (and therefore more zinc) was observed at the acute injury site.

[0048] FIG 25. Monoclonal antibody against hMG53 isolated from hybridoma (mAb 4A3F6F2) is highly effective at detecting human (and mouse) MG53 protein on a Western blot.

[0049] FIG 26. Recombinant expression of MG53. (a) Coomassie blue stained gel of recombinant human MG53 protein (arrow) fractions isolated from Sf9 insect cells with a Ni-NTA column. Input= cell extract, FT= flow through, M= marker, E= elution number. (b) Coomassie blue stained gel of recombinant human TAT-MG53 (arrow) isolated from Sf9 insect cells. (c) Coomassie blue stained gel of recombinant mouse TAT-MG53 (arrow) isolated from E.coli fermentation.

[0050] FIG 27. Illustrates that a signal-peptide at the amino-terminus of hMG53 allows export of the recombinant MG53 as a secretory protein. Western blot shows that abundant MG53 protein could be purified from conditioned media with CHO cells that are transiently transfected with the engineered hMG53 cDNA.

[0051] FIG 28. Co-immuno-precipitation (Co-IP) experiments in HEK293 cells transfected with a Flag-MG53 fusion protein construct and a series of HA-MG53 fusion protein mutants. (a) Co-IP was performed with an anti-Flag antibody on whole cell extracts followed by Western blot with an anti-HA antibody. (b) Co-IP experiments show that formation of MG53 dimers requires the presence of the coiled-coil domain.

[0052] FIG 29. Stable HEK293 (Human Embryonic Kidney) cell lines expressing RFP-MG53. (a) Cell lines that stably express an RFP (red fluorescent protein) control protein that shows a cytosolic expression pattern, (b) Injury of HEK293 cells expressing RFP only with a microelectrode results in no translocation of RFP to the injury site (arrow). Some bleaching of RFP fluorescence occurs from excessive entry of extracellular buffer (*). (c) HEK293 cells that are stably expressing RFP-MG53 show localization to intracellular vesicles, (d) Injury of HEK293 cells expressing RFP-MG53 results in massive translocation of MG53 to the injury site (arrow) in less than 90 seconds.

[0053] FIG 30. GFP-MG53 expressed in C2C12 cells, followed by perfusion with an alcohol extract from notoginseng. Application of notoginseng can rapidly induce MG53 translocation to the plasma membrane within 2 min after perfusion.

[0054] FIG 31. Therapeutic use of recombinant MG53 as a tissue repair reagent. RFP-MG53 (a MG53 fusion protein that contains a red fluorescent protein) was expressed in HEK293 cells, isolated, and applied to the external media surrounding C2C12 myoblasts in
culture. Cells were mechanically wounded with a microelectrode while the localization of the fusion protein was observed by confocal microscopy. RFP-MG53 can be observed to translocate to such sites of membrane damage (circles).

[0055] FIG 32. Genetic overexpression of MG53 prevents membrane damage. Human embryonic kidney (HEK293) cells were transfected with RFP-MG53 or RFP and then electroporated with fields of varying strength. The amount of membrane damage was measured by assessing the amount of lactate dehydrogenase (LDH) that leaks into the extracellular media out of holes in the plasma membrane produced by electroporation. The more damage that occurs to the membrane, the higher the reading on the LDH assay will be.

[0056] FIG 33. Fluorescent dye entry used to measure membrane damage following electroporation. Human embryonic palatal mesenchymal (HEPM) cells (lx10^6) were placed in a spinning cuvette of a PTI fluorescence system. FMI-43 day was added outside of the cells and displayed minimal fluorescence with an excitation of 479 nm and an emission of 598 nm. When cells were electroporated with a field strength of 50 V/cm or 100 V/cm there was a dose dependent increase in fluorescence detected. Electroporation does not produce auto-fluorescence in cells where the dye is not present (control).

[0057] FIG 34. Fluorescent dye entry used to measure membrane damage following mechanical damage. Human embryonic palatal mesenchymal (HEPM) cells (lx10^6) were placed in a spinning cuvette of a PTI fluorescence system. FMI-43 day was added outside of the cells and displayed minimal fluorescence with an excitation of 479 nm and an emission of 598 nm. Cells were removed from the cuvette (Pour) sheared with a 28 gauge needle (Shear), leading to an increase in FMI-43 fluorescence. Mechanical shear stress does not produce auto-fluorescence in cells where the dye is not present as a control (No dye).

[0058] FIG 35. Recombinant MG53 protects kidney cells from cell membrane damage, (a) HEK293 cells (8xlO^4) were treated with 10 ug/mL recombinant human MG53 or vehicle control and then electroporated at various field strengths. Extracellular recombinant MG53 can prevent damage from electroporation. (b) MG53 or a vehicle control was added to recombinant LDH to generate standard curves for LDH activity.

[0059] FIG 36. Recombinant MG53 protects gum lining cells from cell membrane damage, (a) HEPM cells (5xlO^4) were treated with 10 ug/mL recombinant human MG53 or vehicle control and then electroporated at various field strengths. Extracellular recombinant MG53 can prevent damage from electroporation. (b) MG53 or a vehicle control was added to recombinant LDH to generate standard curves for LDH activity.
[0060] FIG 37. Recombinant MG53 protects kidney cells from mechanical cell membrane damage. HEK293 cells (8x10^4) were treated with glass microbeads to induce mechanical damage. Different doses of recombinant human MG53 or vehicle control was applied to the samples when glass beads were added to the media. Cells were rotated on an orbital shaker and then the supernatant was analyzed for LDH levels.

[0061] FIG 38. Effects of MG53 are specific to the function of the protein. MG53 proved to be effective at resealing damage in Hela cervical epithelial cells that was produced due to exposure to glass beads. When the recombinant protein is boiled the protein can no longer facilitate membrane resealing.

[0062] FIG 39. Membrane damage to human keratinocytes induced by nitrogen mustard prevented by MG53. Various doses of nitrogen mustard, a skin blistering agent, can produce LDH release from primary human keratinocytes. Inset picture illustrates the effects of exposure to a skin blistering agent.

[0063] FIG 40. Externally applied recombinant MG53 requires phosphatidylserine (PS) binding to reseal damaged membranes. HEK293 cells were treated with recombinant human MG53 or vehicle and then damaged by shaking in the presence of glass microbeads (black bars). Membrane damage is measured by LDH release from cells that is recorded by colormetric assay recorded at 488 nm. Simultaneous treatment of cells with phosphatidylserine (PS) can prevent resealing of plasma membrane. *p < 0.05

[0064] FIG 41. Competition with another phosphatidylserine (PS) binding protein. HEK293 cells were treated with recombinant human MG53 or vehicle and them damaged by shaking in the presence of glass microbeads (black bars). Membrane damage is measured by LDH release from cells that is recorded by colormetric assay recorded at 488 nm. Simultaneous treatment of cells with an excess (5:1) of a phosphatidylserine (PS) binding protein, and Annexin V. *p < 0.05

[0065] FIG 42. Expression of MG53 in human embryonic palatal mesenchymal (HEPM) dental cells. GFP-MG53 localizes properly in these cell types, it also effectively translocates to the plasma membrane following membrane damage by either physical penetration of a microelectrode or treatment with saponin detergent.

[0066] FIG 43. Expression of MG53 in human embryonic palatal mesenchymal (HEPM) dental cells. GFP-MG53 localizes properly in these cell types, it also effectively translocates to the plasma membrane following membrane damage by either physical penetration of a microelectrode or treatment with saponin detergent.
FIG 44. Lipopolysaccharides induce membrane damage in HEPM cells prevented by exposure to MG53. When HEPM cells are treated with LPS (1 mg/mL) for 24 hours LDH release can be observed, suggesting that membrane damage has occurred. Application of MG53 can prevent the normal levels of LDH release from the HEPM cells, while co-incubation with LPS and MG53 shows normal release of LDH from cells.

FIG 45. MG53 translocates to membrane repair sites in gastric cells. Human gastric adenocarcinoma (AGS) cells were transfected with GFP-MG53 and then subjected to mechanical membrane damage by microelectrode needle penetration (top) or treatment with 0.005% saponin to permeabilize the membrane (bottom). Translocation of GFP-MG53 to the injury site (arrow) was monitored by live cell confocal microscopy.

FIG 46. MG53 translocates to membrane repair sites in neural cells. Mouse primary astrocytes were transfected with GFP-MG53 and then subjected to mechanical membrane damage by microelectrode needle penetration (top) or treatment with 0.005% saponin to permeabilize the membrane (bottom). Translocation of GFP-MG53 to the injury site (arrow) was monitored by live cell confocal microscopy.

FIG 47. MG53 translocates to membrane repair sites in airway epithelial cells. Human C38 airway epithelial cells were transfected with GFP-MG53 and then subjected to mechanical membrane damage by microelectrode needle penetration (top) or treatment with 0.005% saponin to permeabilize the membrane (bottom). Translocation of GFP-MG53 to the injury site (arrow) was monitored by live cell confocal microscopy.

FIG 48. External MG53 reseals membrane damage in airway epithelial cells. Human IB3 airway epithelial cells were treated with external recombinant human MG53 or vehicle control and then exposed to mechanical membrane damage by glass beads. Membrane damage is measured by LDH release from cells that is recorded by colorimetric assay recorded at 488 nm. *p < 0.05

FIG 49. MG53 translocates to membrane repair sites in immune cells. Mouse leukaemic monocyte macrophage (RAW 264.7) cells were transfected with GFP-MG53 and then subjected to mechanical membrane damage by microelectrode needle penetration (top) or treatment with 0.005% saponin to permeabilize the membrane (bottom). Translocation of GFP-MG53 to the injury site (arrow) was monitored by live cell confocal microscopy.

FIG 50. a) GFP-C29L mutant expressed in C2C12 cells (left panel) displayed defective movement toward the acute injury site (middle panel), in an extracellular solution that contain nominal free zinc. Addition of 2 µM Zn-HPT, which serves as ionphore for zinc
entry across the plasma membrane, could partially rescue the movement of GFP-C29L toward the acute injury site (right panel). The image presented in the right panel was obtained from a separate C2C12 cell, 50 s after penetration with a microelectrode with 2 μM Zn-HPT present in the extracellular solution. b) GFP-C105S mutant expressed in C2C12 cells (left panel) could not move to the acute injury site following microelectrode penetration (middle panel), in an extracellular solution that contain nominal free zinc. c) GFP-C29L/C105S double mutant expressed in C2C12 cells (left panel) is completely defective in repair of acute membrane damage, under conditions with nominal free zinc (middle panel) or following addition of 2 μM Zn-HPT (right panel).

FIG 51. Summary data for the dependence of C29L, C105S, and C29L/C105S on zinc entry in repair of acute membrane damage in C2C12 cells. Data with other mutants of MG53 are summarized in Table 1.

FIG 52. Illustration demonstrating the inventors' current hypothesis on the mechanism of membrane repair mediated by MG53. While not being limited to any particular theory, experimental evidence indicates that MG53 is likely localized to the inner surface of the plama membrane due to its association with phosphatidylserine-containing vesicles. Under normal conditions MG53 is likely monomeric and sequestered proximal to the membrane surface due to associations with caveolin-3. Following damage to the cellular membrane MG53, which is normally in its reduced form, is exposed to a localized oxidative environment which triggers the formation of disulfide cross-bridges and intermolecular MG53 oligomerization. The oligomerization of MG53 brings phosphatidylserine-containing vesicles together at the damage site.

FIG 53. MG53 deficiency impairs renal function. The Mg53/- mice develop proteinuria as they age (20-week vs 10-week ages), as shown by colloidal blue stained SDS-PAGE of urine (a), and urine protein/creatinine (Up/Uc) levels (b). **P < 0.001. Bovine serum albumin (BSA) was used as a loading control (10 μg and 3 μg). (c) Mg53/- animals display impaired kidney function with an increase in serum creatinine (Scr) compared with littermate wt controls (**P < 0.001). (d) Compared with wt kidney, Mg53/- kidney shows pathology at the inner cortex with pronounced vacuolization (red arrow) and disorganized cisternae (yellow arrow). (e) H&E staining shows widening of the interstitial compartment in the Mg53/- kidney, (f) Transmission electron micrographs reveal disorganized microvilli and brush border at the apical surface of PTE cells derived from the Mg53/- kidney. (g) The
inter-tubular space was -2.5 fold larger in the mg53-/- kidney than that in the wt kidney (averaged from a total of 12 images, P <0.001).

[0077] FIG 54. MG53-mediated membrane repair in proximal tubular epithelial cells. (a) Western blot of lysates (50 μg) from total kidney or isolated cortex and medulla derived from wt (+/+ ) or Mg53 knockout (-/- ) mice. One tenth (5 μg) of wt skeletal muscle lysates was used for comparative purpose. Purified rhMG53 was used as a positive control, (b) MG53 protein is detected in PTE cells, but not in glomeruli isolated from rats. The identities of PTE cells or glomeruli were verified by the expression of E-cadherin or nephrin, respectively, (c) Total tissue lysates (50 μg) from human kidney and bladder were used for immunoblotting with anti-MG53 antibody, (d) GFP-MG53 expressed in Mg53-/- PTE cells translocates to the area of acute mechanical injury following microelectrode penetration, (e) The wt PTE cells survive after acute mechanical injury, whereas the Mg53-/- PTE cells often die within 10 seconds of microelectrode penetration, (f) GFP-MG53 overexpression rescues survival of Mg53-/- PTE cells following microelectrode-induced membrane damage. P values against Mg53-/- group are all < 0.001.

[0078] FIG 55. MG53 deficiency aggravates I/R-induced AKI. H&E and PAS were used to evaluate the pathologial changes of the wt (a) and Mg53-/- mice (b) upon sham treatment (top panels) or I/R induced AKI (bottom panels). Mg53-/- kidneys are more susceptible to I/R-induced injury. Time-dependent urinary protein excretion (c), and SCr levels at 5 days post I/R-induced AKI (d) show significant difference between wt and Mg53-/- mice. *P<0.01, **P<0.001.

[0079] FIG 56. Co-localization of rhMG53 and Annexin V at the plasma membrane of PTE cells after anoxia/reoxygenation (A/R) injury. (a) PTE cells were treated with rhodamine-labeled rhMG53 (0.1 mg/ml) or rhodamine-labeled BSA (0.1 mg/ml, as control). Immunostaining was performed with FITC-AnnexinV for labeling of PS exposed at the plasma membrane. Control PTE cells are negative for staining with rhMG53 or AnnexinV. PTE cells exposed to A/R show positive staining with rhMG53 and AnnexinV (bottom panels). In addition to localization at the plasma membrane (overlapping pattern with AnnexinV), a significant portion of rhMG53 could enter the PTE cells following exposure to A/R. As control, cells incubated with BSA showed neither plasma membrane targeting nor intracellular localization of BSA.

[0080] FIG 57. rhMG53 protein ameliorates I/R-induced AKI in rat model. Kidney function assessed by Ualb/Uc (a) or SCr (b) demonstrates the beneficial effects of rhMG53 in prevention of I/R-induced AKI. (c) IHC staining with anti-KIM-1 reveals reduced kidney
pathology 5-days post VR injury. (d) H&E staining shows rhMG53 treatment led to improvement of kidney histopathology 5 days post I/R-induced AKI. (e) Injury scores based on quantitative analysis of KIM-1 (as shown in (c)) reveal diminished tubular injury in I/R-injured rats that receive rhMG53 (n= 4-9/group, *P<0.01, **P<0.001).

FIG 58. Cisplatin-induced injury of PTE cells leads to co-localization of rhMG53 and Annexin V at the plasma membrane. PTE cells were treated with 50 µg/ml of cisplatin for 3 hours. Rhodamine-labeled rhMG53 (0.1 mg/ml) or rhodamine-labeled BSA (0.1 mg/ml, as control) was then added to the cells. Immunostaining was performed with FITC-AnnexinV for labeling of exposed PS on the plasma membrane. Control PTE cells are negative for staining with rhMG53 or AnnexinV. PTE cells exposed to cisplatin show positive staining with rhMG53 and AnnexinV (bottom panels). Cells incubated with BSA show neither plasma membrane targeting nor intracellular localization of BSA, under control conditions or following cisplatin treatment.

FIG 59. rhMG53 protects against cisplatin-induced AKI in mice. (a) H&E and PAS staining show kidney pathology in wt mice 5 days post cisplatin treatment (30 mg/kg, i.p.). Mice were given one intravenous dose of either rhMG53 (2 mg/kg, left) or vehicle (right) 10 min prior to cisplatin treatment. Kidney histology showed reduced grades of tubular injury after rhMG53 administration. (b) Summary data of urinary protein measurements (Up/Uc) with mice following different combinations of treatment (vehicle, cisplatin, cisplatin+rhMG53). (c) Mice receiving rhMG53 display reduced SCr levels at 5 days post cisplatin treatment. *P<0.01, ** P<0.001. (d) MTT assay show rhMG53 does not alter IC50 for cisplatin-induced cell death in murine pancreatic cancer cells (KPC-Brcal). n=4/group. (e) rhMG53 does not alter the efficacy of cisplatin suppression of tumor growth. KPC-Brcal pancreatic tumor cells were injected subcutaneously into both flanks of nude mice and allowed to grow for 5 days before initiating treatment. Arrows indicate when the mice received injections of cisplatin (6 mg/kg, i.p.) together with rhMG53 (2 mg/kg, i.v.) or saline as vehicle control. Mice demonstrated a similar extent of tumor regression with or without rhMG53 administration (n=10 for each group).

FIG 60. Utrastructural examination of kidneys from wild type and Mg53-/- mice. Transmission electron micrographs of renal tubular (PTE) and glomerulus (Podocyte) derived from 4-month-old mice. Left panels: Compared with wild type kidney (top), Mg53-/- kidney showed abnormal-appearing brush border and intracellular vacuolization (bottom, arrows). Right panels: Glomeruli from the Mg53-/- kidney appear to be structurally intact and
their podocytes are comparable to those of wt mice. Insert - magnified view of podocyte foot processes.

**FIG 61.** Recapitulation of MG53-mediated cell membrane repair in human renal proximal tubular cells (HKC-8). HKC-8 cells were grown in DMEM media supplemented with GlutaMAX, glucose (1 g/L) and sodium pyruvate (110 g/L), 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin in a humidified environment at 37°C and 5% C02. GFP-MG53 or a mutant form of MG53, GFP-C242A was transfected into HKC-8 cells with Lipofectamine LTX reagents (Control). The cells were injured by microelectrode penetration of the cell membrane (Arrows). HKC-8 cells expressing GFP-MG53 (n=10), but not those expressing the mutant form (C242A, n=8) showed accumulation of MG53 at acute injury sites caused by microelectrode penetration (Post injury, Arrows).

**FIG 62.** Abnormal membrane structures in Mg53/- PTE cells. Representative scanning electron micrographs of primary cultured PTE cells derived from wt (left) and Mg53/- (right) mice. Wild type PTE cells show abundant microvilli (a) and organized network of filapodia (b), which are attenuated in numbers and length in the Mg53/- cells (c, d).

**FIG 63.** rhMG53 is concentrated to PTE compartment of I/R-injured rat kidney. (a) To analyze whether rhMG53 could pass through glomerular basal membrane, the glomerular permeability of rhMG53 was accessed using immunoblotting. Various amounts of rhMG53 (mg/kg, denoted on the top) was injected into healthy Sprague Dawley rats and urine excretion collected between the time intervals of 1.5 and 6 hours post-injection. rhMG53 (lane 1) serves as a positive control. Equal protein loadings were verified by Ponceau S staining. (b) Rats subjected to I/R-induced AKI were treated with 2 mg/kg rhMG53 (i.v.). Kidney samples were cryo-preserved 2 hours-post I/R injury. Cryosections of the kidney were stained with H&E and PHA-L (left), or probed with anti-MG53 antibody (middle). Overlay of PHA-L and rhMG53 revealed that rhMG53 concentrated to the PTE region of the I/R-injured kidney (right).

**FIG 64.** Toxicological evaluation of rhMG53 in canine model. rhMG53 (1 mg/kg body weight) was administrated to Beagle dogs through intravenous injection every other day for a total of seven doses. Histological (H & E staining) analyses did not reveal any apparent abnormalities of major vital organs (a), suggesting the animals could tolerate repetitive exposure to rhMG53. ELISA determination of serum levels of rhMG53 show the pharmacokinetic properties for MG53 remained unchanged, at the beginning (first dosing)
and the end of repetitive i.v administration (seventh dosing), both having a half lifetime of ~
1.4 hrs (b).

**Detailed Description**


[0089] The description is related, in part, to the surprising and unexpected discovery of recombinant nucleic acid sequences and related polypeptides (See, SEQ ID NOs.: 1-15), which are capable of facilitating the repair of cell membranes. In particular, the inventors discovered that vesicular fusion during acute membrane repair is driven by mitsugumin53 (MG53) (SEQ ID NOs. 1-15), a tri-partite motif (TRIM) family protein. MG53 expression facilitates intracellular vesicle trafficking to and fusion with the plasma membrane. In particular, as described herein, MG53 is a vital component of reno-protection.

[0090] Dynamic membrane repair is essential not only for long-term maintenance of cellular integrity but also for recovery from acute cell injury. Repair of the cell membrane requires intracellular vesicular trafficking that is associated with accumulation of vesicles at the plasma membrane. Acute injury of the cellular membrane leads to recruitment of MG53-containing vesicles to patch the membrane at the injury site. Our data indicate that MG53-mediated membrane repair constitutes a vital component of reno-protection under both physiological and pathophysiological conditions. Defects in membrane repair due to the absence of MG53 can lead to increased susceptibility of the kidney to stress-induced injury. We show that MG53 is expressed in the renal proximal tubule (PTE), and PTE cells derived from the Mg53/- kidney are defective in repair of acute membrane injury. Ultra-structural analyses reveal the prominent defects with the Mg53/- kidney reside within the apical surface of PTE cells, where active endocytosis and exocytosis occur under normal physiological conditions. Thus, targeting MG53-mediated repair of injury to PTE cells represent a novel therapeutic approach for prevention of AKI associated with ischemia/reperfusion and nephrotoxin exposure.

[0091] The proximal tubule, especially the S3 segment, is the most affected nephron segment during ischemia or nephrotoxin insult. We find that MG53 expression is enriched in the inner cortex, but not present in the medulla region, further supporting the physiological
function for MG53 in protection against injury to the kidney. MG53 has a molecular size of 53 kilodalton and contains positive charges (pi = 6.2). Previous studies show that proteins with comparable properties can cross the glomerulus, thus providing the feasibility that intravenously administered rhMG53 could access the apical surface of PTE cells. Indeed, we have shown that rhMG53 can be excreted into the urine within a few hours after i.v. administration, in a time frame that follows the pharmacokinetic property for rhMG53 in the serum. Since the amount of MG53 in the urine do not increase proportionally to the amount of the injected rhMG53 as shown in Fig. 63, it is possible that MG53 secreted from PTE cells could also contribute to the appearance of MG53 in the urine. MG53 from either filtration or secretion could both help to repair the injury of tubular epithelial cells. We find that rhMG53 can target to the injured PTE cells, but not to PTE cells when the kidneys are healthy. This suggests that injury to the kidney, instead of putative transport mechanisms, provides a means for rhMG53 to access the apical surface of the renal epithelium. Stress-induced injury to the kidney, either I/R or cisplatin leads to exposure of PS at the apical surface of PTE cells, which serves as an anchoring mechanism for rhMG53 to initiate the repair process for AKI.

[0092] We believe our data support a potential role for rhMG53 in human AKI. Our study with rhMG53 in prevention of cisplatin-induced renal toxicity is particularly exciting, as it represents the first protein therapeutic agent that can selectively protects the kidney function without interfering with the tumor suppressor efficacy for cisplatin. The mechanism of cisplatin in tumor suppression involves blocking DNA synthesis and there is no evidence to suggest that rhMG53 participates in this process, allowing rhMG53 to be used as a potential adjuvant for chemotherapy to bypass the nephrotoxicity of cisplatin. In addition to nephrotoxin-induced kidney injury, cardiothoracic surgery associated AKI is also a significant clinical problem, as ~1/3 of the patients undergoing surgery developing AKI. We showed that intravenous delivery of rhMG53 prior to ischemia reperfusion is effective in preventing the onset of AKI in rodent models. Prophylactic administration of rhMG53 to patients prior to cardiothoracic surgery or chemotherapy would be a novel strategy for prevention of AKI and associated complications. As MG53 is present in the circulation under normal physiologic conditions, the administration of rhMG53 would not likely produce an immune response, and would potentially be a safe biologic reagent for treatment of acute tissue injuries. We have worked out a protocol to scale up purification and production of rhMG53 protein so that it can be used for future clinical studies in human subjects. In a pilot study to test the safety of intravenous administration of rhMG53 in a large animal model, we found that repetitive intravenous doses of rhMG53 had no adverse effects in Beagles (see
Supplementary Fig. 64a). Additionally, serum the half-life for rhMG53 was about 1.4 hours in these dogs (Fig. 64b). This is similar to the half-life in mice. These large animal data provide optimism that rhMG53 will be well-tolerated in human studies. The rapid clearance in large animals is consistent with renal excretion of rhMG53 via glomerular filtration, similar to what we have shown in rodents (Fig. 63), suggesting that rhMG53 will be delivered to the desired site of therapeutic action, the proximal tubule.

Since our discovery of MG53 in 2009, significant progress has been made in advancing the mechanistic action of this gene in the biology of tissue repair, and also in regulation of metabolic syndromes. MG53 belongs to a member of the TRIM-family proteins that contain the conserved RING motif with E3-ligase activity. Several studies have shown that insulin receptor substrate 1 (IRS-1) and focal adhesion kinase (FAK) are E3-ligase substrates for MG53-mediated ubiquitination and degradation. A recent report by Song et al (R. Song et al., Central role of E3 ubiquitin ligase MG53 in insulin resistance and metabolic disorders. Nature 494, 375-379 (2013)) stated that MG53 expression is markedly elevated in animal models of insulin resistance and MG53 overexpression suffices to trigger muscle insulin resistance and metabolic syndrome. However, a separate report by Yi et al (J. S. Yi et al., MG53-induced IRS-1 ubiquitination negatively regulates skeletal myogenesis and insulin signalling. Nat Commun 4, 2354 (2013)) presented no evidence for MG53 upregulation in diabetes, as muscle samples derived from human diabetic patients and mice with insulin resistance show normal expression of MG53, indicating that altered MG53 expression does not serve as a causative factor for the development of metabolic syndrome. That MG53 expression remains unchanged in metabolic syndromes was also presented in early studies by other investigators. While MG53-mediated down-regulation of IRS-1 could contribute to certain extent the dysregulation of glucose metabolism in skeletal muscle, early publications by Tamemoto et al and Terauchi et al (H. Tamemoto et al., Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature 372, 182-186 (1994); Y. Terauchi et al., Development of non-insulin-dependent diabetes mellitus in the double knockout mice with disruption of insulin receptor substrate-1 and beta cell glucokinase genes. Genetic reconstitution of diabetes as a polygenic disease. J Clin Invest 99, 861-866 (1997)) showed that animal models with knockout of IRS-1 show normal glucose tolerance compared with wild type control mice. This further challenges the proposal by Song et al. that MG53-mediated down regulation of IRS-1 serves as a causative factor for the development of metabolic syndromes. Clearly more studies are required to dissect the functional relationship between MG53 and IRS-1 in regulation of metabolic function, as this is fundamental for our
effort in translating the basic findings into clinical application. In principle, protein engineering of MG53 that disrupts the putative E3-ligase activity without affecting its membrane repair function may offer a better approach for rhMG53 in treatment of AKI and chronic renal diseases.

[0094] Many studies remain for complete understanding of the molecular mechanisms for repair of injury to PTE cells. In addition to MG53, other genes may also participate in the assembly of the cell membrane repair machinery, e.g. dysferlin, caveolin3, annexin, NM-IIA, PTRF to name a few. One avenue of future research is to assess whether genetic variations in MG53 expression or its associated repair machinery predispose patients to ischemic or nephrotoxic kidney injury. If so, this could provide a way to screen patients to determine susceptibility of AKI prior to treatments that normally cause AKI.

[0095] The biopolymer compositions encompassed by the invention are collectively and interchangeably referred to herein as "MG53 nucleic acids" or "MG53 polynucleotides" or "nucleic acids encoding membrane repair polypeptides" or "membrane repair protein nucleic acids," and the corresponding encoded polypeptides are referred to as "MG53 polypeptides" or "MG53 proteins" or "membrane repair polypeptides." Unless indicated otherwise, "MG53" is used generally to refer to any MG53 related and/or MG53-derived biopolymers as explicitly, implicitly, or inherently described herein. Also, as used herein, "membrane repair polypeptides" and "polypeptides facilitating membrane repair" are used interchangeably to refer to the polypeptides of the invention and their biological activity.

[0096] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

[0097] In response to external damage and internal degeneration, the cells of the body must repair the membrane surrounding the each individual cell in order to maintain their function and the health of the organism. Defects in the ability of the cell to repair external membranes have been linked to many diseases, such as neurodegenerative diseases (Parkinson's Disease), heart attacks, heart failure and muscular dystrophy. In addition, the muscle weakness and atrophy associated with various diseases, as well as the normal aging process, has been linked to altered membrane repair. Moreover, membrane damage occurs in many other pathologic states outside of chronic disease. Skin aging due to UV exposure,
minor cuts, dermal abrasion, surgical incisions and ulcers in both diabetic and otherwise healthy patients all involve some component of damage to cellular membranes. In order for these cells to repair their membranes in response to acute damage they make use of small packets of membrane that are inside of the cell, referred to as vesicles. These vesicles are normally found within the cell, but upon damage to the cell membrane, these vesicles move to the damage site and form a patch to maintain the cell integrity. Without this essential function, the cell can die and the cumulative effect of this cellular injury can eventually result in dysfunction of the tissue or organ.

[0098] It is contemplated that the present invention provides compositions and methods for treating and/or preventing the detrimental effects of cell damage. As described above, the present description relates to the surprising and unexpected discovery that MG53 constitutes a vital component of reno-protection. The description demonstrates that MG53 surprisingly and unexpectedly repairs injury to renal proximal tubular epithelium (PTE) cells.

[0099] In certain aspects, the description provides compositions comprising MG53 nucleic acids or MG53 polypeptides, and therapeutic compositions comprising effective amounts of the same, which, alone or in combination with other components, can modulate or enhance the process of cell membrane resealing in PTE. In certain embodiments, the MG53 polypeptide has the amino acid sequence of SEQ ID NO. 1. In certain additional embodiments, the MG53 polypeptide is a homolog, fragment, truncation, pseudopeptide, peptide analog, or peptidomimetic of SEQ ID NO:1. In certain aspects, the description includes compounds that can modulate the activity of MG53.

[0100] In an exemplary embodiment, the description provides a pharmaceutical composition for the treatment of a kidney injury, disease or disorder comprising an effective amount of a recombinant MG53 polypeptide or fragment thereof possessing MG53-like activity sufficient to treat or ameliorate a symptom of the kidney injury, disease or disorder, and a pharmaceutically acceptable carrier. In certain embodiments, the kidney injury, disease or disorder is acute kidney injury (AKI). In still certain additional embodiments, the pharmaceutical composition is in a form suitable for intravenous injection. In certain embodiments, the composition can be in unitary dosage form or comprise approximately an entire daily, weekly, or monthly dose.

[0101] In certain additional aspects the description relates to methods for the treatment of kidney cell or tissue injury/damage. In certain exemplary embodiments, the method comprises, for example, the administration of a therapeutic composition as described herein for the prevention and/or treatment of kidney injury. In certain embodiments, the
method comprises administering to a subject in need thereof a composition comprising an effective amount of mitsugumin 53 (MG53), wherein the composition is effective in treating, preventing or ameliorating kidney injury. In certain embodiments, the kidney injury is acute kidney injury. In certain embodiments, the acute kidney injury includes at least one of surgery-related AKI, contrast media-induced AKI, drug or chemotherapy-induced AKI, toxin-induced AKI, dialysis, ischemia/reperfusion-induced AKI, sepsis-induced AKI, nephrotoxin exposure or a combination thereof.

In certain embodiments, the method includes a step whereby the subject is co-administered a drug, e.g., chemotherapeutic agent (in the same or different formulation), and an effective amount of MG53. In certain embodiments, the chemotherapeutic agent is cisplatin.

In any of the aspects or embodiments described herein, the composition further comprises a pharmaceutically acceptable carrier or excipient.

In any of the aspects or embodiments described herein, the subject can be a human or a non-human primate.

In an additional aspect, the description provides a method of enhancing PTE cell membrane repair comprising treating a PTE cell having a damaged cell membrane with a composition comprising an effective amount of mitsugumin 53 (MG53), wherein the composition is effective in enhancing PTE cell membrane repair.

In any of the aspects or embodiments described herein, the MG53 polypeptide can be a recombinant MG53 polypeptide or fragment thereof possessing membrane repair activity.

In any of the aspects or embodiments as described herein, the MG53 polypeptide or fragment thereof possessing MG53 activity is recombinant human MG53 (rhMG53) polypeptide.

In any of the aspects or embodiments as described herein, the MG53 polypeptide comprises or consists of the amino acid sequence of SEQ ID NO:1.

In any of the aspects or embodiments, the MG53 polypeptide is a recombinantly produced polypeptide. In certain embodiments, the MG53 polypeptide comprises or consists of the amino acid sequence of SEQ ID NO:1. In certain embodiments, the MG53 polypeptide, e.g., SEQ ID NO:1, further includes at least one of a signal peptide, e.g., a secretory signal sequence, a fusion protein, a protein tag, a conservative amino acid modification, a non-conservative amino acid change or a combination thereof.
In another embodiment, the invention encompasses a synthetic or recombinant nucleic acid encoding an MG53 polypeptide as set forth in SEQ ID NOs: 1, 3, 5, 7, 8, 9-15, and/or a homolog, or fragment thereof, wherein the polypeptide facilitates cell membrane repair.

As described herein, an effective amount, pharmaceutically effective dose, therapeutically effective amount, or pharmaceutically effective amount is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state or pathological condition. The effective amount depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors which those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 1000 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer. In addition, effective amounts of the compositions of the invention encompass those amounts utilized in the examples to facilitate the intended or desired biological effect.

In certain embodiments, the effective amount of an MG53 polypeptide is administered at least once per day, per week, per month, every two months, every three months, every six months or every year. In certain embodiments, the effective amount of an MG53 polypeptide is administered in unitary dosage form, i.e., one or more units comprising the effective amount.

As described herein, in one aspect, the description provides a composition comprising an effective amount of an mitsugumin 53 (MG53) polypeptide for use in a method of treating or preventing kidney injury comprising administering the composition to a subject in need therof, wherein the composition is effective in ameliorating the effects or symptoms of kidney injury.

In any of the aspects or embodiments described herein, the kidney injury is acute kidney injury (AKI).

In any of the aspects or embodiments described herein, the MG53 polypeptide is a recombinant MG53 polypeptide possessing proximal tubule epithelium (PTE) membrane repair activity. In any of the aspects or embodiments described herein, the MG53 polypeptide is recombinant human MG53 (rhMG53) polypeptide.

In any of the aspects or embodiments described herein, the kidney injury is at least one of surgery-related kidney injury, contrast media-induced AKI, drug or...
chemotherapy-induced AKI, toxin-induced AKI, dialysis, ischemia/reperfusion-induced AKI, sepsis-induced AKI, or a combination thereof.

[00117] In any of the aspects or embodiments described herein, the method includes co-administering a chemotherapeutic agent to the subject.

[00118] In any of the aspects or embodiments described herein, the chemotherapeutic agent is cisplatin.

[00119] In any of the aspects or embodiments described herein, the subject is human.

[00120] In any of the aspects or embodiments described herein, the composition is in a form suitable for intravenous injection.

[00121] In any of the aspects or embodiments described herein, the composition is in unitary dosage form.

[00122] In an additional aspect, the description provides a composition comprising an effective amount of mitsugumin 53 (MG53) polypeptide for use in a method of enhancing proximal tubule epithelium (PTE) membrane repair comprising treating a PTE cell having a damaged cell membrane with the composition, wherein the composition is effective in enhancing PTE cell membrane repair.

[00123] In any of the aspects or embodiments described herein, the PTE cell membrane damage is drug or toxin-induced or ischemia/reperfusion-induced.

[00124] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects. The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the
concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[00125] In certain aspects, the description relates to compositions useful as therapeutics for treating and prevention of diseases and disorders related to cellular and/or tissue damage. Therapeutic compositions of the invention comprise MG53 polypeptides, and nucleic acids encoding MG53 polypeptides, for example, the protein of SEQ ID NO. 1 and MG53 polypeptide mutants, homologs, fragments, truncations, pseudopeptides, peptide analogs, and peptidomimetics, as well as compounds that can modulate the activity of MG5.

[00126] In an exemplary embodiment, the description provides a pharmaceutical composition for the treatment of a kidney injury, disease or disorder comprising an amount of a recombinant MG53 polypeptide or fragment thereof possessing MG53-like activity sufficient to treat or ameliorate a symptom of the kidney injury, disease or disorder, and a pharmaceutically acceptable carrier. In certain embodiments, the kidney injury, disease or disorder is acute kidney injury (AKI). In still certain additional embodiments, the pharmaceutical composition is in a form suitable for intravenous injection. In certain embodiments, the composition can be in unitary dosage form or comprise approximately an entire daily, weekly, or monthly dose.

[00127] In any of the methods described herein, the nucleic acids or polypeptides of the invention may be delivered or administered in any pharmaceutically acceptable form, and in any pharmaceutically acceptable route as described in further detail below. For example, compositions comprising nucleic acids and/or polypeptides of the invention can be delivered systemically or administered directly to a cell or tissue for the treatment and/or prevention of cell membrane damage. In certain additional embodiments, the nucleic acids and/or polypeptides of the invention comprise a carrier moiety that improves bioavailability, increases the drug half-life, targets the therapeutic to a particular cell or tissue type or combination thereof.

[00128] In an additional aspect, the invention relates to compositions comprising an effective amount of a polypeptide of the invention and a carrier, and optionally, in combination with at least one other agent, e.g., bioactive agent or agent that modulates synergistically, MG53. In certain embodiments, the agent acts synergistically, via direct or indirect interaction with the polypeptide of the invention, to facilitate cell membrane repair. For example, agents such as phosphotidylserine, zinc, oxidizing agents, and plant extracts can
modulate the membrane repair activity of the polypeptides of the invention. Therefore, in additional embodiments, any of the membrane repair polypeptide-containing compositions encompassed by the invention may also comprise, in combination, an effective amount of at least one of a phospholipid; a zinc containing agent; an oxidizing agent; a plant extract or a combination thereof. In certain embodiments the phospholipid is phosphatidylserine. In additional embodiments, the zinc containing agent is a zinc ionophore, for example, Zn-1-hydroxypyridine-2-thine (Zn-HPT). In other embodiments, the oxidizing agent is thimerosal. In additional embodiments, the plant extract is notoginsing extract.

[00129] In certain additional aspects, the invention relates to a composition comprising an isolated or recombinant MG53 polypeptide in combination with a pharmaceutically acceptable carrier. The present invention also relates to the surprising and unexpected finding that polypeptides of the invention can patch the membrane in many different cell types and tissues. Without being bound by any particular theory, it is believed that the repair mechanism is mediated by the formation of polypeptide oligomers, e.g., dimers, through the coiled-coil domain in the protein, which contains a leucine zipper protein-protein interaction motif.

[00130] In certain additional embodiments, the therapeutic compositions of the invention further comprise, in combination with a membrane repair polypeptide of the invention, one or more additional ingredients, including a phospholipid; a zinc containing agent; an oxidizing agent; a plant extract or a combination thereof, which have a synergistic effect on the membrane repair of the polypeptides of the invention. In additional embodiments, the therapeutic of the invention may comprise one or more biologically active ingredients such as, Analgesics, Antacids, Antianxiety Drugs, Antiarrhythmics, Antibacterials, Antibiotics, Anticoagulants and Thrombolytics, Anticonvulsants, Antidepressants, Antidiarrheals, Antiemetics, Antifungals, Antihistamines, Antihypertensives, Anti-Inflammatories, Antineoplastics, Antipsychotics, Antipyretics, Antivirals, Barbiturates, Beta-Blockers, Bronchodilators, Cold Cures, Corticosteroids, Cough Suppressants, Cytotoxics, Decongestants, Diuretics, Expectorants, Hormones, Hypoglycemics (Oral), Immunosuppressives, Laxatives, Muscle Relaxants, Sedatives, Sex Hormones, Sleeping Drugs, Tranquilizer, Vitamins or a combination thereof.

[00131] Also included in the invention are substantially purified membrane repair polypeptides, for example, MG53 polypeptides (SEQ ID NOS: 1, 3, 5, 7, 8, and 9-15). In certain embodiments, the membrane repair polypeptides, e.g., MG53 polypeptides, include an
amino acid sequence that is substantially identical to the amino acid sequence of a human MG53 polypeptide (SEQ ID NO.:1).

In another aspect, the invention includes pharmaceutical compositions that include therapeutically- or prophylactically-effective amounts of a therapeutic and a pharmaceutically-acceptable carrier. The therapeutic can be a nucleic acid, e.g., a MG53 nucleic acid, for example, a peptide nucleic acid, a cDNA, or RNA, such as for example, a small inhibitory RNA; a membrane repair polypeptide for example, MG53; or an antibody specific for a MG53 polypeptide. In a further aspect, the invention includes, in one or more containers, a therapeutically- or prophylactically-effective amount of this pharmaceutical composition.

In a further aspect, the invention includes a method of producing a polypeptide by culturing a cell that includes an endogenous or exogenously expressed nucleic acid encoding a membrane repair polypeptide, for example a MG53 nucleic acid, under conditions allowing for expression of the polypeptide encoded by the DNA. If desired, the polypeptide can then be recovered.

The therapeutic composition of the invention comprises, in certain embodiments, for example, an MG53 polypeptide, peptide analog, pseudopeptide or peptidomimetic based thereon; a small molecule modulator of a membrane repair polypeptide, MG53 or a membrane repair polypeptide or MG53 protein-protein interaction; or a MG53-specific antibody or biologically-active derivatives or fragments thereof. As described herein, MG53 mediates the repair of damage to cellular membranes. Therefore, targeting the expression and/or activity of these nucleic acids, polypeptides, and homologs thereof will allow for a novel treatment of various acute and chronic diseases and conditions related to tissue repair.

The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, e.g., acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A further object of the present invention is to provide a kit comprising a suitable container, the therapeutic of the invention in a pharmaceutically acceptable form disposed therein, and instructions for its use.

As described in detail below, and as would be readily appreciated by those skilled in the art, the recombinant membrane repair polypeptides can be produced in prokaryotic cells or eukaryotic cells, for example, mammalian cells and then secreted into the
extracellular solution through protein engineering, an approach that should produce large quantities of functional protein.

[00138] The term "host cell" includes a cell that might be used to carry a heterologous nucleic acid, or expresses a peptide or protein encoded by a heterologous nucleic acid. A host cell can contain genes that are not found within the native (non-recombinant) form of the cell, genes found in the native form of the cell where the genes are modified and re-introduced into the cell by artificial means, or a nucleic acid endogenous to the cell that has been artificially modified without removing the nucleic acid from the cell. A host cell may be eukaryotic or prokaryotic. General growth conditions necessary for the culture of bacteria can be found in texts such as BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY, Vol. 1, N. R. Krieg, ed., Williams and Wilkins, Baltimore/London (1984). A "host cell" can also be one in which the endogenous genes or promoters or both have been modified to produce one or more of the polypeptide components of the complex of the invention.

[00139] "Derivatives" are compositions formed from the native compounds either directly, by modification, or by partial substitution.

[00140] "Analogs" are nucleic acid sequences or amino acid sequences that have a structure similar to, but not identical to, the native compound.

[00141] Furthermore, one of ordinary skill will recognize that "conservative mutations" also include the substitution, deletion or addition of nucleic acids that alter, add or delete a single amino acid or a small number of amino acids in a coding sequence where the nucleic acid alterations result in the substitution of a chemically similar amino acid. Amino acids that may serve as conservative substitutions for each other include the following: Basic: Arginine (R), Lysine (K), Histidine (H); Acidic: Aspartic acid (D), Glutamic acid (E), Asparagine (N), Glutamine (Q); hydrophilic: Glycine (G), Alanine (A), Valine (V), Leucine (L), Isoleucine (I); Hydrophobic: Phenylalanine (F), Tyrosine (Y), Tryptophan (W); Sulfur-containing: Methionine (M), Cysteine (C). In addition, sequences that differ by conservative variations are generally homologous.


In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. For suitable expression systems for both prokaryotic and eukaryotic cells see, e.g., Chapters 16 and 17 of Sambrook, et al., MOLECULAR CLONING: A LABORATORY MANUAL. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

A polynucleotide can be a DNA molecule, a cDNA molecule, genomic DNA molecule, or an RNA molecule. A polynucleotide as DNA or RNA can include a sequence wherein T (thymidine) can also be U (uracil). If a nucleotide at a certain position of a polynucleotide is capable of forming a Watson-Crick pairing with a nucleotide at the same position in an anti-parallel DNA or RNA strand, then the polynucleotide and the DNA or RNA molecule are complementary to each other at that position. The polynucleotide and the DNA or RNA molecule are substantially complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides that can hybridize with each other in order to effect the desired process.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. By "transformation" is meant a permanent or transient genetic change induced in a cell following incorporation of new DNA (i.e., DNA exogenous to the cell).

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert, et al., 1987. Genes Dev. 1: 268-277), lymphoid-specific promoters (Calame and Eaton, 1988. Adv. Immunol. 43: 235-275), in particular promoters of T cell receptors (Winoto and Baltimore, 1989. EMBO J. 8: 729-733) and immunoglobulins (Banerji, et al., 1983. Cell 33: 729-740; Queen and Baltimore, 1983. Cell 33: 741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle, 1989. Proc. Natl. Acad. Sci. USA 86: 5473-5477), pancreas-specific promoters (Edlund, et al., 1985. Science 230: 912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Pat. No. 4,873,316 and European Application

[00147] In any of the embodiments, the nucleic acids encoding a membrane repair polypeptide, MG53, membrane repair polypeptide binding protein, MG53 binding protein, membrane repair polypeptide receptor, and/or MG53 receptor can be present as: one or more naked DNAs; one or more nucleic acids disposed in an appropriate expression vector and maintained episomally; one or more nucleic acids incorporated into the host cell's genome; a modified version of an endogenous gene encoding the components of the complex; one or more nucleic acids in combination with one or more regulatory nucleic acid sequences; or combinations thereof. The nucleic acid may optionally comprise a linker peptide or fusion protein component, for example, His-Tag, FLAG-Tag, Maltose Binding Protein (MBP)-Tag, fluorescent protein, GST, TAT, an antibody portion, a signal peptide, and the like, at the 5’ end, the 3’ end, or at any location within the ORF.

[00148] Where the host is prokaryotic, such as E. coli, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the CaCl₂ method by procedures well known in the art. Alternatively, MgCl₂, RbCl, liposome, or liposome-protein conjugate can be used. Transformation can also be performed after forming a protoplast of the host cell or by electroporation. These examples are not limiting on the present invention; numerous techniques exist for transfecting host cells that are well known by those of skill in the art and which are contemplated as being within the scope of the present invention.

[00149] When the host is a eukaryote, such methods of transfection with DNA include calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors, as well as others known in the art, may be used. The eukaryotic cell may be a yeast cell (e.g., Saccharomyces cerevisiae) or may be a mammalian cell, including a human cell. For long-term, high-yield production of recombinant proteins, stable expression is preferred.

[00150] Formulations

[00151] A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, e.g., systemic administration, into a cell or subject, preferably a human. By "systemic administration" is meant in vivo systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Suitable forms, in part, depend upon the use or the route of entry, for
example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (i.e., a cell to which the negatively charged polymer is desired to be delivered to). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms which prevent the composition or formulation from exerting its effect.

In any aspect of the invention, the therapeutic composition of the invention can be in any pharmaceutically acceptable form and administered by any pharmaceutically acceptable route, for example, the therapeutic composition can be administered as an oral dosage, either single daily dose or unitary dosage form. By pharmaceutically acceptable formulation is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity.

Non-limiting examples of agents suitable for formulation with the MG53 molecules as described herein include: PEG conjugated, phospholipid conjugated, lipophilic moieties, phosphorothioates, P-glycoprotein inhibitors (such as Pluronic P85) which can enhance entry of drugs into various tissues, for example the CNS (Jolliet-Riant and Tillement, 1999, Fundam. Clin. Pharmacol., 13, 16-26); biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery after implantation (Emerich, DF et al, 1999, Cell Transplant, 8, 47-58) Alkermes, Inc. Cambridge, Mass.; and loaded nanoparticles, such as those made of polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (Prog Neuropsychopharmacol Biol Psychiatry, 23, 941-949, 1999). Other non-limiting examples of delivery strategies, including CNS delivery of nucleic acid molecules include material described in Boado et al., 1998, J. Pharm. Sci., 87, 1308-1315; Tyler et al, 1999, FEBS Lett., 421, 280-284; Pardridge et al., 1995, PNAS USA., 92, 5592-5596; Boado, 1995, Adv. Drug Delivery Rev., 15, 73-107; Aldrian-Herrada et al., 1998, Nucleic Acids Res., 26, 4910-4916; and Tyler et al., 1999, PNAS USA., 96, 7053-7058. All these references are hereby incorporated herein by reference. Pharmaceutically acceptable carriers and excipients and methods of administration will be readily apparent to those of skill in the art, and include compositions and methods as described in the USP-NF 2008 (United States Pharmacopeia/National Formulary), which is incorporated herein by reference in its entirety.

The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other
untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

[00155] Administration routes which lead to systemic absorption include, without limitations: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation which can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful.

[00156] The active compounds will generally be formulated for parenteral administration, e.g., formulated for injection via the intravenous, intraarticular, intrathecal, intramuscular, sub-cutaneous, intra-lesional, or even intraperitoneal routes. The preparation of an aqueous composition that contains a cancer marker antibody, conjugate, inhibitor or other agent as an active component or ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectibles, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

[00157] The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). Nucleic acid molecules of the invention can also comprise covalently attached PEG molecules of various molecular weights. These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic et al. Chem. Rev. 1995, 95, 2601-2627; Ishiwata et al., Chem. Pharm. Bull. 1995, 43, 1005-1011). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes,
based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen. All of these references are incorporated by reference herein.

[00158] The present invention also includes compositions prepared for storage or administration which include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985) hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

[00159] Sustained-release preparations can be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing MG53, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), poly(lactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods.

[00160] Preparations for administration of the therapeutic of the invention include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's intravenous vehicles including fluid and nutrient replenishers, electrolyte replenishers, and the like. Preservatives and other additives may be added such as, for example, antimicrobial agents, anti-oxidants, chelating agents and inert gases and the like.

[00161] The compounds, nucleic acid molecules, polypeptides, and antibodies (also referred to herein as "active compounds") of the invention, and derivatives, fragments, analogs and homologs thereof, can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein,
or antibody and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, finger's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (i.e., topical), transmucosal, intraperitoneal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor™, (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example,
glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[00164] For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups, or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl- p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring, and sweetening agents as appropriate. Preparations for oral administration may be suitably formulated to give controlled release of the active compound. For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner. For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethylene, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for
use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch. The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing, and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use. The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides. In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[00165] In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

[00166] It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly
dependent on the unique characteristics of the active compound and the particular therapeutic
effect to be achieved, and the limitations inherent in the art of compounding such an active
compound for the treatment of individuals.

[00167] Also disclosed according to the present invention is a kit or system utilizing
any one of the methods, selection strategies, materials, or components described herein.
Exemplary kits according to the present disclosure will optionally, additionally include
instructions for performing methods or assays, packaging materials, one or more containers
which contain an assay, a device or system components, or the like.

[00168] Additional objects and advantages of the present invention will be appreciated
by one of ordinary skill in the art in light of the current description and examples of the
preferred embodiments, and are expressly included within the scope of the present invention.

[00169] Examples

[00170] Discovery of MG53, a muscle specific TRIM family protein. MG53 was
isolated using a previously established an immuno-proteomic approach that allows
identification of novel proteins involved in myogenesis, Ca^{2+} signaling and maintenance of
membrane integrity in striated muscle cells. Briefly, this approach uses a monoclonal
antibody library containing -6500 clones that was generated from mice immunized with
triad-enriched membranes from rabbit skeletal muscle. Antibodies of interest were selected
based on the z-line staining patterns of striated muscle sections observed under an
immunofluorescence microscope. The target-proteins were purified through antibody-affinity
column, and partial amino acid sequences of the purified proteins were obtained. Based on
the partial amino acid sequence, the complete cDNA coding for the target gene was isolated
from a skeletal muscle cDNA library. Homologous gene screening was then used to search
for the presence of different isoforms of the identified genes in other excitable tissues.
Finally, transgenic or knockout mouse models were generated to study the in vivo
physiological function of genes of interest.

[00171] Screening of this immuno-proteomic library for muscle specific proteins led to
the identification of an antigen recognized by mAb5259 with a molecular size of 53
kilodaltons (kDa) specifically with striated muscle tissues (Fig. 3B). The protein, "MG53",
was partially purified from rabbit skeletal muscle by a mAb5259 immunoaffinity column and
subjected to amino acid sequencing. Skeletal muscle cDNA library screening and genomic
database searches identified the predicted amino acid sequences for MG53 and the
corresponding mg53 gene on the human 16p11.2 locus. Nothern blotting for the mg53 mRNA
confirmed specific expression with skeletal and cardiac muscle (Fig. 3C). Domain homology
analysis revealed that MG53 contains the prototypical tri-partite motifs that include a Ring, B-box and Coiled-Coil (RBCC) moieties, as well as a SPRY domain at the carboxyl-terminus (Figs. 1, 2, and 3A). The SPRY domain is a conserved sequence first observed in the ryanodine receptor Ca$^{2+}$ release channel in the sarcoplasmic reticulum of excitable cells. Of the approximately 60 TRIM family members so far identified in various mammalian genomes, 15 members carry a similar SPRY domain following the RBCC domain, and MG53 shows a conserved primary structure with these TRIM sub-family proteins.

MG53 mediates vesicle trafficking in muscle cells. Although there is no membrane-spanning segment or lipid-modification motif in its primary structure, MG53 appears to be primarily restricted to membrane structures in skeletal muscle. Immunohistochemical analysis revealed specific labeling for MG53 in the sarcolemma membrane and intracellular vesicles (Fig. 3D). MG53 is a muscle-specific protein that contains TRIM and SPRY motifs. In previous studies we have established a monoclonal antibody (mAb) library that targets proteins associated with the triad junction in skeletal muscle. Screening of this immuno-proteomic library for muscle specific proteins led to the identification of an antigen named MG53 with a molecular size of 53 kilodaltons (kDa), which was recognized by mAb5259. MG53 was partially purified from rabbit skeletal muscle by an immunoaffinity column conjugated with mAb5259, and subjected to amino acid sequencing. Based on the obtained partial amino acid sequences, cDNAs encoding MG53 were isolated from rabbit and mouse skeletal muscle libraries. Genomic library search identified the corresponding MG53 gene on the human 16p11.2 locus. The predicted amino acid sequences for MG53 in several species are shown in Fig. 1.

Domain homology analysis revealed that MG53 contains the prototypical TRIM signature sequence of RBCC plus a SPRY domain at the carboxyl-terminus, and thus belongs to the TRIM/RBCC family (Fig. 1). Of the approximately 60 TRIM family members so far identified in the mammalian genomes, 15 members carry a similar SPRY domain following the RBCC domain, and MG53 shows a conserved primary structure with these TRIM sub-family proteins (Fig. 2). However, surprisingly and unexpectedly our studies indicate that MG53 is the only TRIM family protein of those in Fig. 2 that demonstrate membrane repair function.

Western blot assay confirms the muscle-specific expression of MG53 in mouse tissues (Fig. 3B). Although there is no membrane-spanning segment or lipid-modification motif in its primary structure, MG53 appears to be primarily restricted to membrane structures in skeletal muscle. Immunohistochemical analysis with mAb5259
showed specific labeling for MG53 in the sarcolemmal and TT membranes in transverse sections of skeletal muscle fibers (Fig. 3C). Moreover, transverse sections revealed localized concentration of MG53 near the sarcolemmal membrane, with a broader staining pattern than is typically observed for integral membrane proteins of the sarcolemma. Thus, MG53 is a muscle specific TRIM family protein that displays a unique subcellular distribution pattern for a TRIM family protein.

MG53 mediates acute membrane repair in skeletal muscle fibers following cellular injury. To further define the physiological function of MG53 in muscle membrane repair, a mouse model null for MG53 was generated. The mg53/- mice are viable up to 11 month of age under unstressed conditions. In vivo stress tests revealed severe defects in membrane repair function of the mg53/- muscle. As shown in Fig. 4C, membrane injury induced by down-hill running exercise revealed severely compromised contractile function of the soleus muscle from the mg53/- mice. Without the strenuous exercise, mg53/- soleus muscles displayed some difficulty in recovery of contractile function after ex vivo fatigue stimulation, compared with the wild type (wt) controls (not shown). These differences can be drastically exaggerated following exercise-induced damages at 8-10 month of age. Clearly, more severe damage could be found with the mg53/- muscle, where weaker and fluctuating contractile function was observed in comparison with the wt muscle (Fig. 4D).

Injection of Evans blue dye into the intraperitoneal space of mice directly monitors sarcolemmal membrane integrity after down-hill exercise-induced muscle damage. As shown in Fig. 4E, muscle fibers isolated from the mg53/- mice showed significantly more Evans blue staining than the wt muscle, revealing extensive degree of exercise-induced muscle damage. This was confirmed by H/E staining that illustrated increased dystrophy in the mg53/- muscle that was increased in aged mg53/- mice compared to young mg53/- mice (Fig. 4A). Quantitative assay of total absorbance of Evans blue extracted from muscle bundles provided direct support for the increased muscle damage in the mg53/- mice after down-hill running (Fig. 4F).

Consistent with the role of MG53 in membrane repair, elevated concentrations of MG53 was observed at the site of injury with immunostaining of individual flexor digitorum brevis (FDB) muscle fibers that were damaged during isolation (Fig. 5A). These membrane patches would frequently co-localize with staining for dysferlin. We directly evaluated the MG53-mediated membrane repair function through measurement of FM-143 fluorescent dye entry after laser-induced membrane damage to individual FDB muscle fibers. The wt muscle fibers possessed intrinsic membrane repair function and were fairly resistant
to laser-induced damage of the sarcolemmal membrane, as they displayed effective exclusion of the FM-143 fluorescent dye (Fig. 5B). Significant entry of FM-143 fluorescent dye into the mg53-/ FDB muscle fibers could be observed following laser-induced damage (Fig. 5C). The time-dependent accumulation of FM-143 inside the FDB muscle fibers following laser damage of the sarcolemmal membrane provides direct support for a defective membrane repair function of the mg53-/ muscle (Fig. 5D).

Expression of MG53 is essential to maintain normal cardiac membrane integrity. Defects in in mg53-/ mice are not limited to skeletal muscle fibers. During injection of Evans blue dye -50% of the mg53-l- mice would die within 16 hours of injection compared to none of the wild type animals injected. Postmortem examination of mg53-/ hearts revealed extensive labeling of cardiac muscle fibers with Evans blue, even in absence of exercise stress (Fig. 6). We also found that exercise would greatly exacerbate the extent of Evans blue staining in mg53-/ hearts.

Loss of MG53 increases susceptibility to cardiac ischemia reperfusion injury (Fig. 7). Hearts from wild type (WT) and mg53-/- (mg53KO) mice were isolated and perfused on a Langendorff apparatus. Global ischemia was induced for 30 minutes by cessation of perfusate flow. The damage produced in the heart following restoration of perfusate flow (time 0) was measured by enzymatic assays for (a) creatine kinase (CK) or (b) lactate dehydrogenase (LDH). Hearts from mg53-/- mice (dashed lines) show more damage than WT (solid lines). Data is presented as mean+S.D. for each listed time point.

Vesicle fusion with the plasma membrane is required for membrane repair and previous studies indicate a role for dysferlin in maintenance of skeletal muscle membrane integrity. Our findings indicate that MG53 is capable of driving the trafficking of vesicles to the plasma membrane, perhaps to mediate the repair process following membrane disruption. Acute cellular injury generated by physical penetration of the plasma membrane with a microelectrode leads to rapid recruitment of GFP-MG53 vesicles toward the injury site (Fig. 8A). When more severe damage that results in fracture of the cell occurs, the repair site is densely labeled with GFP-MG53 (Fig. 8B). In addition, this acute membrane repair also was observed in mature C2C12 myotubes. This data indicates that MG53-mediated vesicle trafficking play an active role in acute repair of cell membrane.

The function of MG53 appears to be essential to allow membrane patching and survival of muscle cells following injury. When myoblasts are isolated from wild type and mg53 knockout (mg53-/-) mice and differentiated to form myotubes, the mg53-/-myotubes cannot recover from mechanical injury induced by microelectrode penetration (Fig. 8). This
indicates that MG53 is essential for membrane resealing and cell survival in muscle, as the wild type myotubes can reseal their membrane and survive injury while mg53<sup>-/-</sup> myotubes cannot (Fig. 8C). This confirms the functional role of MG53 in membrane resealing, and indicated that MG53 is essential for membrane resealing in striated muscle.

[00182] Role of TRIM and SPRY motifs in MG53 function. Structure/function assessment of the domains of MG53 (Fig. 9) revealed a remarkable polarity of GFP fusion to MG53 in the intracellular distribution of MG53. In particular, fusion of GFP to the carboxyl-terminal end of MG53 alters the ability of MG53 to partition to the vesicular compartment and to target to the sarcolemmal membrane. To further test the function of the TRIM and SPRY domains in facilitating the membrane-fusion function of MG53, a series of deletion mutants coupled to GFP (Fig. 9A) were generated.

[00183] To analyze the subcellular localization of these mutant constructs of MG53, confocal microscopic imaging was applied to C2C12 myoblasts following transient expression. As shown in Fig. 9B (right panels), GFP-TRIM or TRIM-GFP were predominantly localized to intracellular vesicles without apparent labeling of the sarcolemmal membrane. This result suggests that the SPRY domain, which is absent from GFP-TRIM or TRIM-GFP, is necessary for targeting of MG53 to the sarcolemmal membrane. The fact that MG53-GFP exhibited a predominantly cytosolic distribution (Fig. 9B, left panel), further supports the role of SPRY in targeting MG53 to the cell surface membrane.

[00184] Interestingly, although GFP-SPRY or SPRY-GFP displayed a predominantly cytosolic pattern of distribution, they are clearly excluded from intracellular vesicles (Fig. 9B, middle panels). The cytosolic distribution pattern coupled with the exclusion of localization at intracellular vesicles of GFP-SPRY and SPRY-GFP likely reflects the role of TRIM. Presumably, the TRIM motif can mediate the adherence of MG53 to intracellular vesicles (Fig. 9B, right panels). The SPRY domain is insufficient to target to the sarcolemma by itself, therefore the TRIM domain must be present in tandem with the SPRY domain for proper trafficking of MG53 to the sarcolemmal membrane.

[00185] MG53 cab interact with caveolin-3 (Cav-3). In addition, our co-immunoprecipitation data shows that caveolin-3 interacts with the TRIM motif of MG53 (Fig. 9C). Thus, it is possible that the functional interaction between MG53 and caveolin-3 may underlie some of the cellular factors contributing to the diffuse pattern of GFP-SPRY and SPRY-GFP in C2C12 myoblasts. Overall, the regulated distribution of MG53 to the cell surface and intracellular compartments would likely result from coordinated action between the TRIM and SPRY domains. This requirement for both TRIM and SPRY for proper MG53
subcellular localization also has apparent functional significance, as none of these deletion mutants display the filapodia-like structures or the robust vesicle budding events observed from overexpression of full-length MG53.

MG53 can fully function in non-muscle cell types. Analysis of MG53 function in myogenic C2C12 cells and in isolated skeletal muscle fibers reveals an essential role for MG53 in vesicle trafficking and membrane repair in striated muscle. Considering that membrane repair is an essential to maintain cellular homeostasis, it is likely that similar repair mechanisms in other non-muscle cell types could use similar molecular machinery to facilitate this process. To test this possibility, several of the previous experiments conducted with C2C12 myogenic cells were replicated with non-muscle Chinese hamster ovary (CHO) cells. In these cells, a very similar phenotype to that seen in the C2C12 cells was found. First, GFP-MG53 could produce filapodia-like protrusions of the plasma membrane and localize to both intracellular vesicles and to the plasma membrane. Second, MG53 deletion proteins behaved in an identical fashion to that seen in C2C12 cells. Finally, caveolin-3 can also control the activity of MG53 expressed in CHO cells. As a result, these studies indicate that MG53 acts through a conserved molecular mechanism that is present in other cell types besides muscle.

MG53 can interact with Kinesin family member 11 (Kif11). Cell lysates were isolated from HEK293 cells stably expressing FLAG-tagged versions of either RFP (mRFP), RFP-MG53 (MG53) or C29L mutant RFP-MG53 (C29L). Extracts were co-
immunoprecipitated with anti-FLAG antibody and then run on a SDS-PAGE gel. Commassie staining revealed specific bands that we co-IP by this approach. One prominent band was for Kif11 (arrowhead) (Fig. 11a). Mass spectroscopy was used to identify particular bands from these gels. This representative mass spectroscopy tracing shows that MG53 can pull down Kif11 from cell lysates (Fig. 11b).

MG53 can interact with COP9 complex homolog subunit 6 (CSN6). HEK293 cells were transiently transfected with HA-tagged human MG53 and myc-tagged CSN6 and then used for co-immunoprecipitation (IP) using antibodies against the recombinant tags (Fig. 12). The presence of the protein following pull down was confirmed using Western immunoblots (IB). In some cases, a proteasome inhibitor, MG132, was also added to maintain protein stability during protein overexpression. We find that MG53 can pull down CSN6 and that CSN6 can also pull down MG53. This provides evidence that these two proteins can interact within the cell. Lanes 1= HA-hMG53+hCSN6 +DMSO, Lanes 2= HA-hMG53+hCSN6 +MG132, Lanes 3= HA-mMG53+hCSN6 +DMSO, Lanes 4= HA-mMG53+hCSN6 +MG132.

MG53 can interact with myelin basic protein or periaxin. Schematic diagrams of methods for biochemical isolation of vesicle fractions from either wild type (WT) or mg53/- (KO) skeletal muscle (Fig. 13a). Fractions isolated with methods presented in a were run on with 15% (left) of gradient (right) SDS-PAGE gels. Brilliant Blue (CBB) staining revealed specific bands that we differentially present in WT or KO muscle (Fig. 13b). Two prominent bands were identified as myelin basic protein or periaxin (arrows) by mass spectroscopy.

MG53 interacts with cellular membranes through an association with phosphatidylserine to mediate vesicular trafficking. When GFP-MG53 is expressed in these mg53(-/-) myotubes, the protein will properly localize to the plasma membrane and intracellular vesicles (Fig. 14A, top). When these mg53(-/-) myotubes are injured the GFP-MG53 can localize to the injury site. (Fig. 14A, bottom). Lipid profiling(22) revealed that the purified recombinant MG53 could interact with phosphatidylserine (PS), lipids that preferentially appear at the inner leaflet of the plasma membrane and the cytoplasmic face of intracellular vesicles. PEVStrip lipid dot blot analysis reveals recombinant MG53 (1 μg/ml) specifically binds phosphatidylserine (PS) and not other membrane lipids, including sphingosine-1-P, phosphatidic acid, phosphotidylcholine, phosphatidylethanolamine and various phosphoinositol metabolites (Fig. 14B). Using Annexin-V-GFP, we observed rapid
labeling of Annexin-V-GFP at the C2C12 myoblast injury site. Annexin-V-GFP (a molecule with well defined ability to bind PS) transfected into C2C12 myoblasts displays minimal translocation following cell wounding with a microelectrode (left), while co-expression of Annexin-V-GFP with RFP-MG53 (right) results in accelerated accumulation of Annexin-V-GFP. (Fig. 14C). The accumulation of Annexin-V-GFP was accelerated by co-expression of RFP-MG53 (0.93±0.21 AF/F_0 control; 2.9±0.63 AF/F_0 +MG53), consistent with a role for MG53 in mediating repairosome formation at the injury site. Entry of extracellular Ca^{2+} through the damaged plasma membrane allowed Annexin-V binding to PS, leading to its transition from a soluble pattern before cell injury to distinct localization to plasma membrane and intracellular vesicles (Fig. 14D). Removal of Ca^{2+} from the extracellular solution disrupted the labeling of PS by Annexin-V-GFP at the injury site, translocation of RFP-MG53 to the injury site was maintained (Fig. 14E).

[Cvs242] allows MG53 to acts as a sensor of cellular redox state and reseal cellular membranes. Thimerosal oxidizes sulphhydryl groups at cysteine residues, which provided a mutagenesis target to identify specific amino acids that underlie oxidation-mediated oligomerization of MG53. Multiple conserved cysteine residues were mutated into alanines. One particular mutation, C242A, resulted in complete loss of MG53 oligomerization property (Fig. 15A). This mutation maintained membrane targeting, but completely disrupted its ability to facilitate the membrane repair process (Fig. 15E); i.e., no accumulation of C242A was observed at the injury site. A respective conserved cysteine mutant, C313A, maintained oligomerization pattern under oxidized conditions and displayed similar translocation and membrane-repair function as the wild type GFP-MG53 (Fig. 16). Under a reduced extracellular environment (+DTT), translocation of GFP-MG53 toward the injury site was largely disrupted. The addition of an oxidizing agent (Thimerosal) into the extracellular solution results in an increased translocation of GFP-MG53 to injury sites on the cell membrane. The experiments were conducted in C2C12 cells. MG53 with a C242A mutation (GFP-C242A) cannot translocate to injury sites on the plasma membrane. Since a different conserved cysteine mutant, C313A, maintained oligomerization pattern under oxidized conditions and displayed similar translocation and membrane-repair function as the wild type GFP-MG53. Thus, the oxidation of Cys242 likely induces oligomerization of MG53, providing a nucleation site for repairosome formation at injury sites. These experiments were conducted with C2C12 cells. Modulation of the extracellular redox state can affect the reseling of isolated muscle fiber membranes as the addition of DTT to the
extracellular solution prevents membrane resealing, as measured by an increase in entry of FM-143 dye applied outside of the cell. Thus, the oxidation of Cys242 likely induces oligomerization of MG53, providing a nucleation site for repairsome formation at injury sites. [00193] MG53-mediated repairsome formation and restoration of acute sarcolemma membrane damage is shown in Fig. 17. The entry of FM4-64, a red-shifted variant of FM1-43, was used as an index of membrane repair capacity in mg53/- myotubes transfected with GFP-MG53 and GFP-C242A. Following UV-bleaching of the green fluorescence, rapid translocation of GFP-MG53 took place at the injury site, whereas GFP-C242A remained static due to its defective oligomerization properties (Fig. 16E, 17A). Significantly less entry of FM4-64 was observed in cells transfected with GFP-MG53 compared with GFP-C242A, suggesting that the mutant was not able to restore membrane integrity following injury (Fig. 17B). This data provides direct support that MG53 translocation to sites of injury results in membrane resealing. Oligomerization of MG53 appears to be an essential step in repairsome formation, as the GFP-C242A mutant expressed in wt skeletal muscle displayed a dominant negative function over the native MG53 (Fig. 17C). Compared with GFP-MG53, overexpression of GFP-C242A in adult wt muscle fibers inhibited sarcolemmal membrane repair function (Fig. 17C).

[00194] Several TRIM-family proteins with diverse cellular functions that contain conserved Ring-finger motifs have been shown to display E3-ligase activity. To test whether MG53 can catalyze ubiquitination in vitro, we prepared a recombinant maltose-binding protein (MBP) fusion protein for MG53. MBP-MG53 was incubated with ATP, ubiquitin, E1 and E2 enzymes, and subjected to immunoblotting with the anti-MBP antibody. High molecular-mass ladders derived from ubiquitination were observed when MBP-MG53 was incubated with Ubc4 or UbcH5 as E2 (Fig. 18a and data not shown). The exclusion of either ubiquitin, E1 or E2 (UbcH5) from the assay abolished the appearance of such ladders, confirming that the modification acquired by MBP-MG53 was indeed auto-ubiquitination. When a conserved cysteine residue (Cys-29) in the Ring-finger motif of MG53 was replaced with leucine (C29L), the intrinsic E3-ligase activity of MG53 was significantly reduced (Fig. 18b). Thus, MG53 is a Ring-finger type ubiquitin ligase that couples with Ubc4/5 sub-family of E2 enzymes.

[00195] To test the functional impact of the C29L mutation in MG53, we compared the subcellular distribution of GFP-MG53 and GFP-C29L expressed in C2C12 myotubes. Strikingly, the unique membrane-partition and vesicular tethering of GFP-MG53 was lost in GFP-C29L, with the mutant protein displaying predominantly a cytosolic pattern in C2C12
myotubes (Fig. 18d, left). Western blot demonstrated that the full-length GFP-MG53 and GFP-C29L proteins were present in the differentiated C2C12 myotubes (Fig. 18c), thus it is unlikely that degradation of these fusion proteins contributes to the different subcellular distribution of GFP-C29L and GFP-MG53 observed in Fig. 18d. Furthermore, similar phenomena were observed with transient expression of these fusion proteins into primary cultured skeletal myotubes derived from the mg53−/− neonates, where targeting of GFP-MG53 to sarcolemmal membrane and intracellular vesicles were attenuated for the GFP-C29L mutant (Fig. 18d, right). Similar to the adult mg53−/− muscle fibers that displayed defective membrane repair function (shown previously), the primary cultured mg53−/− myotubes were also defective in membrane repair compared with the wt control, thus providing a homologous reconstruction system to test the cellular function of MG53.

Further studies show that alterations to membrane-trafficking and membrane-partition properties of GFP-C29L lead to defective membrane repair function of MG53. Following acute membrane damage, rapid accumulation of GFP-MG53 is observed in C2C12 myoblasts, whereas GFP-C29L appeared to be immobile and ineffective in repair of membrane injury (Fig. 18e, left). Similar defects with GFP-C29L were also observed in C2C12 myotubes (Fig. 18e, middle). Moreover, while GFP-MG53 could translocate to the plasma membrane following injury in primary cultured mg53−/− myotubes, GFP-C29L expressed in these cells remained generally unresponsive to acute cell injury (Fig. 18e, right). Together, these results show that loss of E3-ligase activity associated with C29L mutation likely caused defective trafficking of MG53, underlying the defective membrane repair function of MG53.

The Ring domain of MG53 also contains a zinc finger motif, which is known to bind Zn to facilitate enzymatic action in numerous proteins. To test if Zn could contribute to the MG53 function in membrane repair, we tested the effect of removing Zn from the extracellular solution before wounding C2C12 myoblasts expressing GFP-MG53. We found that chelating Zn with N,N,N,N-tetraakis(2-pyridyl-methyl)ethylenediamine (TPEN) could prevent the translocation of GFP-MG53 to the site of microelectrode penetration (Fig. 19A), indicating that Zn was necessary for MG53 function. Addition of a Zn ionophore, Zn-1-hydroxyypyridine-2-thine (Zn-HPT), could induce the translocation of GFP-MG53 in C2C12 cells (Fig. 19B), suggesting that additional Zn can induce increased MG53 function. This observation was confirmed in wild type FDB muscle fibers, as addition of Zn-HPT to these cells could reduce the amount of FM-1-43 dye that can enter the muscle fiber following injury induced by a UV laser (Fig. 19C). These results indicate that the presence of Zn is
vital for the function of MG53, and suggest that provide additional Zn can increase the function of MG53 in membrane resealing. The implication of these results is that function of recombinant MG53 protein used for a therapeutic application could be increased by the addition of Zn into the formulation for the MG53 protein.

[00198] Protective effect of zinc on membrane repair is lost in mg53-/− skeletal muscle. To access membrane repair capacity, individual flexor digitorum brevis (FDB) muscle fibers were isolated from wild type (WT) mice (3-6 months) (Fig. 20a). A strong UV laser was applied to the FDB fiber that caused local damage to the muscle (arrow). Entry of FM1-43 fluorescent dye (2.5 μM) was used as an indicator for the measurement of membrane repair capacity. The images were taken 200 s following UV irradiation (control). Application of 2 μM zinc-ionophore (l-hydroxypyridine-2-thione) (+Zn-HPT) led to increased membrane repair capacity as reflected by the decreased amount of FM1-43 dye entry following UV-damage. Addition of 40 μM TPEN (Tetrakis-2-pyridylmethylenediamine), a specific buffer for zinc ions, led to compromised membrane repair capacity, as reflected by the significant increase in FM1-43 dye entry following UV-damage (+TPEN). FDB muscle fibers isolated from the mg53-/− mice (3-6 months) exhibited defective membrane repair function, as shown by the elevated amount of FM1-43 dye entry following identical treatment of UV-damage (control) (Fig. 20b). Unlike the WT muscle fibers, the membrane repair capacity observed with mg53-/− muscle fibers did not show dependence with changes in zinc movement across the plasma membrane, e.g. addition of Zn-HPT did not produce protective effect on membrane repair (+Zn-HPT), and buffering of extracellular zinc with TPEN did not produce more entry of FM1-43 dye following UV-damage (+TPEN). Summary data for panel a and b (Fig. 20c). The additional data with Ca-EDTA (100 μM), a reagent that buffers zinc without altering extracellular Ca concentration, also caused compromised membrane repair capacity in WT muscle (left). Treatment with Ca-EDTA did not produce any significant changes in membrane repair capacity in mg53-/− muscle. Overall, these data suggest that zinc-entry across the plasma membrane plays an important role in repair of acute UV-laser induced damage to the WT skeletal muscle. The protective effect of zinc on membrane repair is lost in mg53-/− muscle fibers, indicating that MG53 presumably functions as the receptor or target for zinc during the acute membrane repair process. Schematic diagram of zinc-binding motifs in MG53. The amino-terminus of MG53 contains two putative zinc-binding motifs: one located at the RING motif (a.a. 1-56, human cDNA), and the other located at the B-box
motif (a.a. 86-117, human cDNA). The specific amino acids that participate in zinc-binding are indicated (Fig. 20d).

[00199] Extracellular zinc entry is essential for MG53-mediated vesicle translocation to acute membrane injury sites. To follow the process of intracellular vesicle translocation associated with repair of acute membrane damage, GFP-MG53 fusion protein was expressed in C2C12 myoblast cells (Fig. 21a). GFP-MG53 displayed localization at the intracellular vesicles and the plasma membrane under resting condition (left). Acute injury of the cell generated by penetration of a microelectrode caused rapid translocation of MG53-containing vesicles at the injury site (arrow, right panel). Incubation of the C2C12 cell with 40 μM Ca-EDTA prevented translocation of GFP-MG53 containing vesicles at the acute injury site (Fig. 21b). Addition of 20 μM TPEN to the extracellular solution also completely abolished the translocation of GFP-MG53 containing vesicles toward the mechanical injury site (Fig. 21c). C2C12 cells transiently transfected with GFP-MG53 was incubated with 20 μM Zn-HPT. Under control condition (0 min), GFP-MG53 was distributed in the cytosol, as well as intracellular vesicles (Fig. 21d). Prolonged incubation with Zn-HPT caused redistribution of GFP-MG53 toward the cell surface membrane and the intracellular membrane compartments (15 min). Summary data with Ca-EDTA and TPEN on GFP-MG53 mediated membrane repair in C2C12 myoblast cells (Fig. 21e). The results show that chelation of extracellular zinc with either Ca-EDTA or TPEN produced significant defects in repair of acute damage to the cell.

[00200] Zn-binding to RING and B-box motifs of MG53 is critical for membrane repair. Toward understanding the molecular mechanisms underlying the role of zinc-binding to MG53 in repair of acute damage to cell membrane, we generated several site-specific mutations in the RING and B-box motifs of MG53. These mutant constructs were transiently expressed in C2C12 myoblast cells (Fig. 22). 24 hours after transfection, the cells were harvested and the expression of the various GFP-MG53 mutants was assayed by Western blot with specific antibody against MG53. In the absence of DTT (left panel), with the exception of C242A mutant, all other constructs exhibited oligomeric patterns (marked dimer), indicating that disulfide-cross link of MG53 was maintained with these mutant constructs. With the addition of 10 mM DTT, all mutant constructs displayed monomeric forms of -75 kD (predicted molecular size of GFP-MG53).

[00201] The GFP-C29L mutant expressed in C2C12 cells displayed defective movement toward the acute injury site, in an extracellular solution that contain nominal free
zinc (Fig. 50a). Addition of 2 µM Zn-HPT, which serves as ionophore for zinc entry across the plasma membrane, could partially rescue the movement of GFP-C29L toward the acute injury site. GFP-C105S mutant expressed in C2C12 cells could not move to the acute injury site following microelectrode penetration, in an extracellular solution that contain nominal free zinc. Similar to GFP-C29L, the addition of 2 µM Zn-HPT could lead to partial rescue of the membrane repair capacity of the GFP-C105S mutant (e.g. movement toward the injury site, (Fig. 50b, right panel). GFP-C29L/C105S double mutant expressed in C2C12 cells is completely defective in repair of acute membrane damage, under conditions with nominal free zinc or following addition of 2 µM Zn-HPT (Fig. 50c). Single mutation of the RING motif (C29L), or the B-box motif (C105S) led to significant defects in membrane repair capacity in an extracellular solution with nominal free zinc (Fig. 51). The addition of Zn-HPT ionophore could partially restore the membrane repair capacity of these single cysteine mutants. The membrane repair function of the C29L/C105S double mutant is completely lost, and is independent of the movement of zinc across the plasma membrane. Data with other mutants of MG53 are summarized in Table 1. Overall, these results suggest that zinc-binding to the RING and B-box motifs of MG53 plays an important role in the intracellular vesicle translocation process associated with repair of membrane damage.

Table 1. Characteristics of MG53 mutants.

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Oligomerization</th>
<th>Zinc-binding</th>
<th>Membrane Repair (- Zn-HPT)</th>
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<td>WT</td>
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<tr>
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MG53 can bind Zn through a RING motif. MG53 contains a canonical TRIM domain that contains a Zn binding motif (Ring) and a Bbox motif (Fig. 23a). Bacterial culture was lysed by sonication, centrifuged and bind to Amylose resin in column buffer containing 100μM zinc for overnight at 4 degree (Fig. 23b). Then the resin was washed by zinc free column buffer following by 50ml of zinc free column buffer with 0.3mM maltose. Protein levels and stability were confirmed by SDS-PAGE gel as shown. Lane 1 (Marker), Lane 2 (mMG53), Lane 3 (mC29L - MG53 mutant), Lane 4 (mC29L/C105S double mutant DM clonel) Lane 5 (mC29L/C105S double mutant DM clone2), Lane 6 (10mg/ml BSA), Lane 7 (5mg/ml BSA), Lane 8 (2.5mg/ml BSA), Lane 9 (1mg/ml BSA). The proteins on beads were first tested for the presence of free zinc in the solution (from 0.01 to 0.1 μM or ND depending on the preparation) (Fig. 23c). The beads ( aliquote) were stained with a zinc-specific probe TSQ and fluorescence was observed under the fluorescent microscope and relative fluorescence intensity taken. Then the proteins were denatured at 56 C for 5 min, vortexed, centrifuged, and the measurements were taken again from the solution. The assay uses TSQ (Mol Probe) and an atomic standard solution of zinc (Sigma) for calibration. Chart indicates the amount of Zn binding to recombinant wild type (WT) MG53, C29L mutant (C29L) and double mutant (DM). Both mutants are located in the Ring motif of the TRIM domain. Data presented as mean+S.D. *P<0.05, **P<0.001 compared to wt; n=4-5.

Disruption of Zn-binding to MG53 correlates with defects in MG53-mediated repair of acute membrane damage. To directly monitor the participation of zinc in membrane repair, FDB muscle fibers isolated from the wild type mice were loaded with 2 μM TSQ, a specific fluorescent indicator for zinc in the intracellular solution (lower panels) (Fig. 24). A strong UV-laser was used to cause local damage to the FDB muscle fiber, as reflected by the accumulation of FM4-64 fluorescent day at the local injury site (top panels). Notice that significant elevation of TSQ fluorescence (and therefore more zinc) was observed at the acute injury site.

Production of recombinant MG53 protein by secretion from cultured cells. Our previous methodology used E. coli bacteria to produce recombinant protein at levels for experimental use. These bench-level preparations provided the initial reagent for our biochemical and in vitro cell culture assays. To improve the yield and purity of the hMG53 preparation, we plan to optimize the purification protocol by adding an additional step to the Ni-column that involves immuno-affinity chromatography. Since our initial monoclonal antibody generated against the rabbit MG53 protein does not bind human MG53, we have
recently generated a hybridoma that produces mAb against hMG53. This monoclonal antibody (mAb 4A3F6F2) is highly effective at detecting human (and mouse) MG53 protein on a Western blot (Fig. 25). This antibody should be very useful in generating an immune-affinity column to improve our protein purification. Protein purified from E. coli has two potential disadvantages, one is the possibility of contamination with endotoxin from bacteria, and the other is the chance that the lack of post-translational modifications, such as glycosylation and phosphorylation, in prokaryotes may prevent the recombinant protein from fully functioning. Thus, we have developed additional methodology for the production of recombinant MG53 from cultured mammalian cells. To achieve this, we have also adapted the methodology used for the generation of other commercial protein therapeutics, such as humanized monoclonal antibody for cancer treatment, which involve purification of secreted protein from culture media from engineered CHO cells. A signal-peptide at the amino-terminus of hGM53 allows export of the recombinant MG53 as a secretory protein. Western blot showed that abundant MG53 protein could be purified from conditioned media with CHO cells that are transiently transfected with the engineered hMG53 cDNA. This methodology was generally accepted by FDA. We are in the process of establishing stable CHO cell lines that express MG53 as a secretory product, to generate recombinant MG53 for broad use in treatment to protect against damage to the skin, heart and muscle cells.

Expression of recombinant MG53 can be performed in eukaryotic or prokaryotic cells. Figure 26 illustrates that recombinant MG53 can be expressed in either eukaryotic or prokaryotic systems. Briefly, recombinant MG53 is expressed in Sf9 cells as a fusion protein containing both a TAT peptide portion and a six-histidine tag (6-HIS tag). This histidine tag can be used to isolate and purify recombinant protein using filtration chromatography techniques well known in the art. Panel (A) shows the Coomassie blue stained gel of recombinant human MG53 protein (arrow) fractions isolated from Sf9 cells with a Ni-NTA column. Input= cell extract, FT= flow through, M= marker, E= elution number. (B) Coomassie blue stained gel of recombinant human TAT-MG53 (arrow) isolated from Sf9 cells. The Coomassie blue stained gel in (C) represents recombinant mouse TAT-MG53 (arrow) expressed and isolated from E.coli.

Figure 27 Illustrates that a signal-peptide at the amino-terminus of hMG53 allows export of the recombinant MG53 as a secretory protein. Western blot shows that abundant MG53 protein could be purified from conditioned media with CHO cells that are transiently transfected with the engineered hMG53 cDNA.
Co-immuno-precipitation (Co-IP) experiments in HEK293 cells transfected with a Flag-MG53 fusion protein construct and a series of HA-MG53 fusion protein mutants. Co-IP was performed with an anti-Flag antibody on whole cell extracts followed by Western blot with an anti-HA antibody (Fig. 28a). The anti-Flag antibody can pull down wild type MG53 and all conserved cysteine residue MG53 mutants tagged with HA, indicating that MG53 proteins associate to form dimers and that this association is not dependent on oxidation of the cysteine residues. Co-IP experiments show that formation of MG53 dimers requires the presence of the coiled-coil domain (Fig. 28a). HEK293 cells were co-transfected with a HA-MG53 fusion protein construct and a series of GFP-MG53 fusion protein mutants, including a construct containing only the coiled-coil domain of MG53 linked to GFP (GFP-CC). An anti-HA antibody was used to Co-IP from whole cell extracts and the resulting proteins were analyzed by Western blot with an anti-GFP antibody.

Heterologous expression of MG53 in a human cell line results in membrane repair in response to acute injury. Figure 29 demonstrates that recombinant MG53 can be expressed in a heterologous expression system and retain its ability to repair cell membrane damage without the expression of additional proteins. Specifically, MG53 was cloned into an expression vectors as a fusion protein with red fluorescent protein (RFP). The fusion protein was expressed in a human embryonic kidney cell line (HEK293 fibroblast cell line) and the cell’s ability to repair membrane damage was compared to cells expressing only RFP. Panel (a) demonstrates that cell lines stably expressing an RFP (red fluorescent protein) control protein show a cytosolic expression pattern. However, in HEK293 cells expressing RFP only (Fig. 29a); injury with a microelectrode results in no translocation of RFP to the injury site (arrow). Some bleaching of RFP fluorescence occurs from excessive entry of extracellular buffer (*). In contrast, HEK293 cells that are stably expressing RFP-MG53 (Fig. 29c) show localization to intracellular vesicles. Microelectrode injury of HEK293 cells expressing RFP-MG53 (Fig. 29d) results in massive translocation of MG53 to the injury site (arrow) in less than 90 seconds. This result demonstrates that recombinant MG53 can be useful for repairing cellular and/or tissue damage in any cellular environment. Although recombinant MG53 is able to repair injury to cellular membranes when expressed in a heterologous system the invention is not so limited. In certain embodiments, the invention encompasses methods of co-expression of MG53 and caveolin-3 in order to promote membrane repair in order to treat or prevent tissue damage. In another embodiment, the present invention relates to a therapeutic composition comprising a TAT-MG53 polypeptide and a TAT-caveolin-3
polypeptide; or a MG53 and a caveolin-3 polypeptide either with or without another protein tag linked to either.

An active ingredient from notoginseng can facilitate MG53-membrane repair function. GFP-MG53 was expressed in C2C12 cells and then these cells were perfused with an alcohol extract from notoginseng. As can be seen in Fig. 30, application of this active ingredient can rapidly induce MG53 translocation to the plasma membrane within 2 min after perfusion. This rapid response was not observed with the carrier control, suggesting that notoginseng can potentially energize MG53 membrane repair function. Based on this observation, we reasoned that a therapeutic approach with the combination of MG53 and notoginseng may provide additive protective effects in preventing both inflammation and membrane damage to the cells, and thus can improve the function of MG53 when applied in tandem with recombinant MG53 protein or when applied alone as a supplement of pharmaceutical.

Notoginseng (Panax notoginseng) is an important component of traditional Chinese herbal medicines that has been widely used for treatment of a number of different disorders. Such herbal medicines frequently use the plant's root that is harvested after the fruit has ripened. The natural habitat of this herb is in southwest Asia, mainly in Yunnan Province of China. The importance in traditional medicine is emphasized in Materia Medica by Li Shi-zhen (1518-1593 AD), which refers to notoginseng as "more valuable than gold." The long history of notoginseng use at therapeutic doses emphasizes the proven safety and efficacy of this herbal compound.

Notoginseng has been particularly prized in wound treatment due to its ability to control both internal and external hemorrhaging (J Nat Med. 2006 60: 135). Notoginseng extracts can reduce bleeding time and improves hemostasis more effectively than placebo controls. Other studies have shown beneficial effects on cardiovascular system by decreasing blood pressure, improving blood supply and providing protection against shock. Many studies have also shown that notoginseng acts as a broad inhibitor of inflammation in a number of tissues. Due to these findings, notoginseng is increasingly the focus of research in complementary and alternative medicine (CAM). We have tested the effect of notoginseng on the membrane repair function of MG53. GFP-MG53 was expressed in C2C12 cells and then these cells were perfused with an alcohol extract from notoginseng. As can be seen in Fig. 30, application of this active ingredient can rapidly induce MG53 translocation to the plasma membrane within 2 min after perfusion. This rapid response was not observed with the carrier control, suggesting that notoginseng can potentially energize MG53 membrane repair
function. Based on this observation, we reasoned that a therapeutic approach with the combination of MG53 and notoginseng may provide additive protective effects in preventing both inflammation and membrane damage to the cells, and thus can improve the function of MG53 when applied in tandem with recombinant MG53 protein or when applied alone as a supplement of pharmaceutical.

[00213] Patching of plasma membrane by external application of MG53. The therapeutic use of recombinant MG53 as a tissue repair reagent is demonstrated in Fig. 31. Our previous experiments shown that MG53 expressed within a cell can increase resistance to cellular damage, however we have not shown that protein applied externally can reseal the plasma membrane following damage. To establish if this is the case, we isolated RFP-MG53 (a MG53 fusion protein that contains a red fluorescent protein) expressed in HEK293 cells and applied this protein extract to the external media surrounding C2C12 myoblasts in culture. Cells were mechanically wounded with a microelectrode while the localization of the fusion protein was observed by confocal microscopy. There was clear accumulation of RFP-MG53 at injury sites where resealing took place (Fig. 31). These results indicate that MG53 protein can be applied externally to cells and remain effective at resealing the damaged membrane. This finding has significant consequences for the application of MG53 as a therapeutic protein. By simply providing the protein outside the cell at the time of damage, MG53 can facilitate resealing of the membrane and prevent cellular damage. Such an approach will significantly simplify the formulation of MG53 into an effective therapeutic compound.

[00214] Genetic overexpression of MG53 can prevent membrane damage. Human embryonic kidney (HEK293) cells were transfected with RFP-MG53 or RFP and then electroporated with fields of varying strength (Fig. 32). The amount of membrane damage was measured by assessing the amount of lactate dehydrogenase (LDH) that leaks into the extracellular media out of holes in the plasma membrane produced by electroporation. The more damage that occurs to the membrane, the higher the reading on the LDH assay will be. We observed that HEK293 cells transfected with RFP-MG53 can reseal their membranes more effectively following electro-poration and prevent the leak of LDH into the extracellular solution. Thus, expression of exogenous MG53 in non-muscle cells can increase the capacity for cell membrane repair in such non-muscle cells.

[00215] Fluorescent dye entry can be used to measure membrane damage following electroporation. Human embryonic palatal mesenchymal (HEPM) cells (1x10^6) were placed in a spinning cuvette of a PTI fluorescence system (Fig. 33). FM1-43 day was added outside
of the cells and displayed minimal fluorescence with an excitation of 479 nm and an emission of 598 nm. When cells were electroporated with a field strength of 50 V/cm or 100 V/cm there was a dose dependent increase in fluorescence detected. Electroporation does not produce auto-fluorescence in cells where the dye is not present (control).

Fluorescent dye entry can be used to measure membrane damage following mechanical damage. Human embryonic palatal mesenchymal (HEPM) cells (1x10^6) were placed in a spinning cuvette of a PTI fluorescence system (Fig. 34). FM1-43 day was added outside of the cells and displayed minimal fluorescence with an excitation of 479 nm and an emission of 598 nm. Cells were removed from the cuvette (Pour) sheared with a 28 gauge needle (Shear), leading to an increase in FM1-43 fluorescence. Mechanical shear stress does not produce auto-fluorescence in cells where the dye is not present (control).

Recombinant MG53 can protect kidney cells from cell membrane damage. (a) HEK293 cells (8x10^4) were treated with 10 ug/mL recombinant human MG53 or vehicle control and then electroporated at various field strengths (Fig. 35). Extracellular recombinant MG53 can prevent damage from electroporation. (b) MG53 or a vehicle control was added to recombinant LDH to generate standard curves for LDH activity. Since MG53 does not affect the LDH reactions the LDH assay is valid for measuring membrane damage under these conditions.

Recombinant MG53 can protect gum lining cells from cell membrane damage. (a) HEPM cells (5x10^4) were treated with 10 ug/mL recombinant human MG53 or vehicle control and then electroporated at various field strengths (Fig. 36). Extracellular recombinant MG53 can prevent damage from electroporation. (b) MG53 or a vehicle control was added to recombinant LDH to generate standard curves for LDH activity. Since MG53 does not affect the LDH reactions the LDH assay is valid for measuring membrane damage under these conditions.

Recombinant MG53 can protect kidney cells from mechanical cell membrane damage. HEK293 cells (8x10^4) were treated with glass microbeads to induce mechanical damage (Fig. 37). Different doses of recombinant human MG53 or vehicle control was applied to the samples when glass beads were added to the media. Cells were rotated on an orbital shaker and then the supernatant was analyzed for LDH levels. We find that MG53 can prevent mechanical membrane damage and that 10 ug/mL is likely a saturating dose of the protein.

Effects of MG53 are specific to the function of the protein. MG53 proved to be effective at resealing damage in Hela cervical epithelial cells that was produced due to
exposure to glass beads (Fig. 38). When the recombinant protein is boiled the protein can no
longer facilitate membrane resealing. This indicates that the resealing activity is specific to
the function of the MG53 protein that depends on the proper conformation of the protein.

Membrane damage to human keratinocytes induced by nitrogen mustard can
be prevented by MG53. Various doses of nitrogen mustard, a skin blistering agent, can
produce LDH release from primary human keratinocytes (Fig. 39). Some of this damage can
be prevented by the application of recombinant protein after exposure and removal of the
nitrogen mustard. Inset picture illustrates the effects of exposure to a skin blistering agent.

Externally applied recombinant MG53 requires phosphatidylycerine (PS)
binding to reseal damaged membranes. HEK293 cells were treated with recombinant human
MG53 or vehicle and them damaged by shaking in the presence of glass microbeads (black
bars) (Fig. 40). Membrane damage is measured by LDH release from cells that is recorded by
colormetric assay recorded at 488 nm. Simultaneous treatment of cells with
phosphatidylycerine (PS) can prevent resealing of plasma membrane. Thus, MG53 must be
able to bind exposed PS on damaged cells in order to facilitate membrane repair. * p < 0.05

Competition with another phosphatidylycerine (PS) binding protein reveals
externally applied recombinant MG53 requires PS binding to reseal damaged membranes.
HEK293 cells were treated with recombinant human MG53 or vehicle and them damaged by
shaking in the presence of glass microbeads (black bars) (Fig. 41). Membrane damage is
measured by LDH release from cells that is recorded by colormetric assay recorded at 488
nm. Simultaneous treatment of cells with an excess (5:1) of a phosphatidylycerine (PS) binding
protein, Annexin V, can prevent resealing of plasma membrane. Thus, MG53 must be able to
bind exposed PS on damaged cells in order to facilitate membrane repair. * p < 0.05

MG53 can patch the plasma membrane in many different human cell types and
prevent cell death. To test if exogenous MG53 can recapitulate membrane resealing function
in non-muscle cell types, we used either adenovirus or liposome-based transfection methods
to express GFP-MG53 in a number of different cell types. In all cell types tested, MG53
performed in a similar fashion as that seen in muscle cells. Here we illustrate these effects in
immortalized human cell lines, including HEK293 (not shown) and human embryonic palatal
mesenchymal (HEPM) dental cells (Fig. 43), as well as primary cultures of human
keratinocytes (Fig. 42). Not only does GFP-MG53 localize properly in these cell types, it
also effectively translocates to the plasma membrane following membrane damage by either
physical penetration of a microelectrode or treatment with saponin detergent. The function of
MG53 appears to be essential to allow membrane patching and survival of muscle cells
following injury. Thus, providing MG53 in different cell types can recapitulate MG53 function in membrane resealing, indicating that MG53 has therapeutic potential for many different tissues beyond the musculoskeletal and cardiovascular system.

Lipopolysaccarides can induce membrane damage in HEPM cells that can be prevented by exposure to MG53. When HEPM cells are treated with LPS (1 mg/mL) for 24 hours LDH release can be observed, suggesting that membrane damage has occurred (Fig. 44). Application of MG53 can prevent the normal levels of LDH release from the HEPM cells, while co-incubation with LPS and MG53 shows normal release of LDH from cells. This suggests that MG53 can prevent damage to HEPM cells produced by LPS.

MG53 can translocate to membrane repair sites in gastric cells. Human gastric adenocarcinoma (AGS) cells were transfected with GFP-MG53 and then subjected to mechanical membrane damage by microelectrode needle penetration (top) or treatment with 0.005% saponin to permeabilize the membrane (bottom) (Fig. 45). Translocation of GFP-MG53 to the injury site (arrow) was monitored by live cell confocal microscopy. In both cases, cell membrane damage resulted in the translocation of MG53 to the plasma membrane.

MG53 can translocate to membrane repair sites in neural cells. Mouse primary astrocytes were transfected with GFP-MG53 and then subjected to mechanical membrane damage by microelectrode needle penetration (top) or treatment with 0.005% saponin to permeabilize the membrane (bottom) (Fig. 46). Translocation of GFP-MG53 to the injury site (arrow) was monitored by live cell confocal microscopy. In both cases, cell membrane damage resulted in the translocation of MG53 to the plasma membrane.

MG53 can translocate to membrane repair sites in airway epithelial cells. Human C38 airway epithelial cells were transfected with GFP-MG53 and then subjected to mechanical membrane damage by microelectrode needle penetration (top) or treatment with 0.005% saponin to permeabilize the membrane (bottom) (Fig. 47). Translocation of GFP-MG53 to the injury site (arrow) was monitored by live cell confocal microscopy. In both cases, cell membrane damage resulted in the translocation of MG53 to the plasma membrane.

External MG53 can reseal to membrane damage in airway epithelial cells. Human IB3 airway epithelial cells were treated with external recombinant human MG53 or vehicle control and then exposed to mechanical membrane damage by glass beads (Fig. 48). Membrane damage is measured by LDH release from cells that is recorded by colormetric assay recorded at 488 nm. MG53 could prevent cell membrane damage due to mechanical damage. * p < 0.05
MG53 can translocate to membrane repair sites in immune cells. Mouse leukaemic monocyte macrophage (RAW 264.7) cells were transfected with GFP-MG53 and then subjected to mechanical membrane damage by microelectrode needle penetration (top) or treatment with 0.005% saponin to permeabilize the membrane (bottom) (Fig. 49). Translocation of GFP-MG53 to the injury site (arrow) was monitored by live cell confocal microscopy. In both cases, cell membrane damage resulted in the translocation of MG53 to the plasma membrane.

Figure 50 illustrates the inventors' current hypothesis on the mechanism of membrane repair mediated by MG53. While not being limited to any particular theory, experimental evidence indicates that MG53 is likely localized to the inner surface of the plasma membrane due to its association with phosphatidylserine-containing vesicles. Under normal conditions MG53 is likely monomeric and sequestered proximal to the membrane surface due to associations with other proteins. Following damage to the cellular membrane MG53, which is normally in its reduced form, is exposed to a localized oxidative environment which triggers the formation of disulfide cross-bridges and intermolecular MG53 oligomerization. The oligomerization of MG53 brings phosphatidylserine-containing vesicles together at the damage site.

These studies demonstrate that MG53 is a critical component of the cell membrane repair machinery, as illustrated by the significant deficiency in membrane repair function of the mg53/- muscle. The response of MG53-mediated membrane patching is rapid, occurring on the order of seconds after injury, therefore MG53 appears to mediate acute repair processes. For MG53 to function in membrane repair it must oligomerize, a process that depends on oxidation of the protein rather than entry of extracellular Ca\(^{2+}\). Extracellular Ca\(^{2+}\) likely acts to facilitate the fusion of the vesicles after they have moved to the plasma membrane through the oxidation-activated translocation of MG53. Through interaction with PS, MG53 oligomerization provides a nucleation site for recruitment of intracellular vesicles to the injury site (Fig. 50). This two step process is essential for the maintenance of cellular integrity. Thus, modulation of the extracellular oxidation state surrounding a cell or the Ca\(^{2+}\) available for membrane resealing would potentially constitute a methodology for improving the membrane repair capacity of a cell.

MG53 knockout mice exhibit renal pathological phenotypes While Mg53-/- mice were viable and behaved normally at young age (until 10-weeks), proteinuria was observed at 20-weeks after birth (Fig. 53a). The Mg53-/- mice displayed a higher urine protein to creatinine ratio (Up/Uc) than the wild type (wt) littermates under basal conditions...
(Fig. 53b). Additionally, the serum creatinine (SCr) level was significantly elevated in the Mg53-/− mice (Fig. 53c). We also screened the urine of the Mg53-/− mice, and did not find significant hematuria, leukocyturia, glycosuria, and proteinuria. These data suggest that the Mg53-/− mice did not display the typical Fanconi syndrome (22).

Compared with wt kidney, the Mg53-/− kidney showed pathology at the inner cortex with pronounced vacuolization and disorganized cisternae (Fig. 53d). Hematoxylin-Eosin (H&E) staining showed widening of the interstitial compartment in the Mg53-/− kidney (Fig. 53e). On average, the inter-tubular space was -2.5 fold larger in the Mg53-/− kidney than that in the wt kidney (Fig. 53g). Transmission electron micrographs revealed disorganized microvilli and brush border at the apical surface of PTE cells derived from the Mg53-/− kidney (Fig. 53f) suggesting possible defects in PTE cells.

Pronounced pathologic findings were observed in the junction area between the inner cortex and outer medulla, where PTE cells displayed disorganized mitochondria, abnormal appearing brush border, and a frequent appearance of vacuoles near the basolateral membrane (see Supplementary Fig. 60). These defective structures were observed in -20% of the PTE cells examined from the Mg53-/− mice, but rarely seen in the wt mouse kidney. After conducting ultra-structural analyses of the Mg53-/−-glomeruli, no defects in podocytes such as foot process fusion or glomerular basement membrane detachment, were found (Fig. 60). Thus, genetic ablation of Mg53 led to renal tubulointerstitial defects without affecting glomeruli.

**MG53 is expressed in proximal tubule cells and mediates membrane repair** The renal pathology exhibited by the Mg53-/− mice led us to investigate whether MG53 is expressed in the kidney. We performed quantitative immunoblotting and found that MG53 was present in the kidney lysates of wt mice but not in those of the Mg53-/− mice, at a level roughly -1/40 of the expression in muscle tissues (Fig. 54a). Further tissue fractionation showed that the inner cortex is the main site of MG53 expression, as MG53 was nearly undetectable in the medullary region (Fig. 54a). Using primary cultured PTE cells derived from mice, and isolated glomeruli and PTE from rats, we found that MG53 was specifically expressed in PTE cells, but not in glomeruli (Fig. 54b). The cell type-specific expression of MG53 was confirmed by immunoblotting using E-cadherin as a PTE marker, and nephrin as a glomerular cell marker. We also performed western blot with human tissues and found MG53 could be detected in the kidney but not in the bladder (Fig. 54c).

PTE cells derived from Mg53-/− neonates were transfected with GFP-MG53, a fusion construct with green fluorescent protein (GFP) added to the amino-terminal end of
MG53, in order to investigate the extent of MG53’s participation in the repair of membrane injury. As shown in Fig. 54d, GFP-MG53 in PTE cells localized to the cytosol, intracellular vesicles, and plasma membrane, a subcellular distribution similar to that observed in striated muscles (C. Cai, et al., MG53 nucleates assembly of cell membrane repair machinery. Nat Cell Biol 11, 56-64 (2009)). In response to injury caused by penetration of a micro-electrode into the plasma membrane, GFP-MG53 rapidly translocated toward the acute injury site (Fig. 54d). This GFP-MG53 translocation to membrane injury sites in PTE cells is similar to that observed in C2C12, HEK293 and other cell types (C. Cai, et al., MG53 nucleates assembly of cell membrane repair machinery. Nat Cell Biol 11, 56-64 (2009); N. Weisleder et al., Recombinant MG53 protein modulates therapeutic cell membrane repair in treatment of muscular dystrophy. Sci Transl Med 4, 139ral85 (2012)).

MG53 translocation to the site of cell membrane injury was recapitulated in human PTE cells. HKC-8 cells are an immortalized human renal proximal tubular cell line established by Racusen et al (L. C. Racusen et al., Cell lines with extended in vitro growth potential from human renal proximal tubule: characterization, response to inducers, and comparison with established cell lines. J Lab Clin Med 129, 318-329 (1997)), and have been widely used as a model system for kidney research. Using HKC-8 cells, we found that GFP-MG53 moved to sites of acute injury induced by mechanical perturbation of the plasma membrane (see Fig. 61). Furthermore, transfected GFP-C242A, a mutant form of MG53 that cannot oligomerize in response to environmental redox changes following acute cellular injury (C. Cai, et al., MG53 nucleates assembly of cell membrane repair machinery. Nat Cell Biol 11, 56-64 (2009)), failed to form a repair patch in mechanically-injured HKC-8 cells. These studies demonstrate that MG53 facilitates nucleation of membrane repair machinery to sites of acute cellular injury in human kidney cells in a redox-dependent fashion, similar to what we have shown for murine kidney tubular cells.

A striking phenomenon was observed when the susceptibility to mechanical injury of PTE cells derived from wt and Mg53/- mice was compared. As shown in Fig. 54e, wt PTE cells were able to survive micro-electrode penetration (top), whereas Mg53/- PTE cells always died within 10 seconds of injury (bottom). On average, -93% of wt cells survived, but the majority of Mg53/- cells rapidly died. This outcome reflects defective membrane repair capacity, as restoration of MG53 by gene transfection increased survival of the Mg53/- PTE cells (Fig. 54f).

Another interesting observation was related to changes in morphology of the Mg53/- PTE cells examined under scanning electron microscopy. As shown in
Supplementary Fig. 62, PTE cells derived from wt mice show characteristic microvilli at the apical surface and filapodia that extend from the plasma membrane (Fig. 62a and b), whereas the Mg53/- PTE cells show significantly fewer microvilli and disorganized filapodial structures (Fig. 62c and d).

[00241] Mg53/- mice exhibit exacerbated kidney injury under ischemia/reperfusion To test if MG53-mediated repair of injury to PTE cells contributes to maintenance of renal function under physiologic and pathophysiologic conditions, we compared the response of the Mg53/- mice and wt littermates to ischemia/reperfusion (I/R)-induced AKI (Fig. 55). H&E and PAS staining revealed that Mg53/- mice develop kidney pathology under basal conditions (sham operation with mice at 11-weeks of age) (Fig. 55b). Animals subjected to I/R injury to the kidney (25 min ischemia) showed exaggerated tubular injury, as demonstrated by the increase in hyaline casts in tubular lumens and loss of nuclei, suggestive of acute tubular necrosis (ATN) (Fig. 55b). The Up/Uc was significantly increased in the Mg53/- mice at each time point after I/R injury to the kidney (Fig. 55c), and SCr measurements showed compromised kidney function at 5 days post I/R injury (Fig. 55d).

In summary, the Mg53/- kidney showed widening of inter-tubular space under basal conditions (with sham surgery), and I/R treatment led to significantly elevated ATN phenotypes. These data show that MG53 deficiency leads to aggravation of I/R-induced AKI.

[00242] rhMG53 protein recognizes injury sites on PTE cells to facilitate renoprotection We previously showed that rhMG53 protein protects various cell types against cell membrane disruption when applied to the extracellular environment (N. Weisleder et al., Recombinant MG53 protein modulates therapeutic cell membrane repair in treatment of muscular dystrophy. Sci Transl Med 4, 139ral85 (2012)). Moreover, administration of rhMG53 provided dose-dependent protection against injury to muscle cells and ameliorated the pathology associated with muscle dystrophy (N. Weisleder et al., Recombinant MG53 protein modulates therapeutic cell membrane repair in treatment of muscular dystrophy. Sci Transl Med 4, 139ral85 (2012)). The association of rhMG53 with sites of membrane disruption requires recognition of lipid-based signals, and our previous findings indicated that MG53 can bind phosphatidylserine (PS) (N. Weisleder et al., Recombinant MG53 protein modulates therapeutic cell membrane repair in treatment of muscular dystrophy. Sci Transl Med 4, 139ral85 (2012)), a phospholipid usually sequestered in the inner leaflet of the plasma membrane that may be exposed to the extracellular environment following injury. Therefore, the association of rhMG53 with exposed PS at the
tissue injury site might provide an anchoring mechanism for the tissue protective function of rhMG53.

When rhMG53 was applied to cultured renal epithelial cells, it targeted acute injury sites on the cell membrane and facilitated repair of membrane damage in response to anoxia-reoxygenation. As shown in Fig. 56, co-localization of rhMG53 and Annexin V at the plasma membrane of PTE cells was observed after anoxia/reoxygenation (A/R) injury (Fig. 56, bottom panels). Uninjured PTE cells were negative for staining with rhMG53 or Annexin V (Fig. 56, top panels). As control, PTE cells incubated with BSA showed neither plasma membrane targeting nor intracellular localization of BSA (Fig. 56, middle panels).

rhMG53 protects ischemia/reperfusion (I/R)-induced AKI Based on studies with the cultured renal epithelial cells, we tested whether rhMG53 was effective in protection against I/R-induced AKI in animal models. We first investigated the glomerular permeability of rhMG53 to reach PTE cells in healthy Sprague-Dawley rats with a semi-quantitative immunoblotting method (Fig. 63a). Various amount of rhMG53 was administered to rats through tail vein injection, and excretion of rhMG53 into urine was measured at 1.5-6 hours post intravenous (i.v.) administration. Clearly, rhMG53 could be detected in the urine, suggesting that rhMG53 is permeable across the healthy glomeruli. For I/R-induced AKI, rats were subjected to 35 min ischemia to the left kidney with a non-occlusive vessel clamp on the renal pedicle and contralateral nephrectomy. Two doses of rhMG53 (2 mg/kg) were applied intravenously, one prior to renal artery clamping and one after release of renal artery clamping. By immunohistochemistry (IHC), anti-MG53 antibody detected rhMG53 distribution in a discrete peri-lumen tubular pattern at 2 hours after I/R-induced kidney injury (Fig. 63b). rhMG53 staining was undetectable in kidneys from BSA-treated or sham-operated rats. Extended studies within various renal compartments revealed that rhMG53 was mainly concentrated at the junction of inner cortex and outer medulla with a rich distribution in the proximal tubules, and was not detected in glomeruli. These results suggest that I/R-induces recruitment of rhMG53 to the injured renal epithelia.

Reduced albuminuria (U_alb/creato) was observed in rhMG53-treated rats when compared with the vehicle-treated group at different times after I/R injury (Fig. 57a). Significant reduction of SCr levels was also observed in rats that received rhMG53 treatment (Fig. 57b). Kidney injury molecule-1 (KIM-1) is a phosphatidyserine (PS) receptor expressed on renal PTE cells that has been widely used as a biomarker for kidney injury (T. Ichimura et al., Kidney injury molecule-1 is a phosphatidyserine receptor that confers a phagocytic phenotype on epithelial cells. J Clin Invest 118, 1657-1668 (2008); Q. Lin et al.,
Kidney injury molecule-1 expression in IgA nephropathy and its correlation with hypoxia and tubulointerstitial inflammation. Am J Physiol Renal Physiol 306, F885-895 (2014). IHC staining of KIM-1 revealed that i.v. administration of rhMG53 led to reduction of KIM-1 positive PTE cells at 5 days post I/R-induced kidney injury, when compared with vehicle controls (Fig. 57c). rhMG53-mediated improvement in kidney pathology was also evident in histopathology analysis of H&E staining (Fig. 57d). Based on KIM-1 staining (Fig. 57e), we quantified the degree of tubular injury in Fig. 57e. Clearly, administration of rhMG53 ameliorates I/R-induced AKI in the rats. There is no apparent toxicity from rhMG53 as revealed by the healthy kidneys of the sham-treated rats (Fig. 57c, 57d). Together, these results support the reno-protective effect of rhMG53 against the development of I/R-induced AKI.

[00246] rhMG53 protects cisplatin-induced AKI without affecting its tumor suppressor function To test if rhMG53-PS interaction contributes to the reno-protective effect of MG53 in AKI, we used nephrotoxin-induced AKI as another animal model. Cisplatin is a widely used chemotherapeutic reagent for treatment of cancers, and is also known as a nephrotoxin because of its detrimental effect on kidney function in cancer patients (R. Safirstein et al., Cisplatin nephrotoxicity. Am J Kidney Dis 8, 356-367 (1986); K. U. Wensing et al., Saving ears and kidneys from cisplatin. Anticancer Res 33, 4183-4188 (2013)). While the mechanism of action for cisplatin in tumor suppression involves intercalation and disruption of DNA synthesizing activities (R. P. Miller et al., Mechanisms of Cisplatin nephrotoxicity. Toxins (Basel) 2, 2490-2518 (2010); U. M. Ohndorf et al., Basis for recognition of cisplatinmodified DNA by high-mobility-group proteins. Nature 399, 708-712 (1999)), several studies have shown that a high-affinity interaction between PS and cisplatin appears to contribute to certain aspects of its nephrotoxicity (G. Speelmans et al., Cisplatin complexes with phosphatidylserine in membranes. Biochemistry 36, 10545-10550 (1997); M. Jensen et al., Cisplatin interaction with phosphatidylserine bilayer studied by solid-state NMR spectroscopy. J Biol Inorg Chem 15, 213-223 (2010)).

[00247] Cisplatin-induced perturbation of PS distribution may share some common feature with I/R-induced injury to the cell membrane, where increased oxidative stress and lipid peroxidation may lead to breakdown of the plasma membrane and appearance of PS at the extracellular space (J. H. Kramer et al., Lipid peroxidation-derived free radical production and postischemic myocardial reperfusion injury. Ann N Y Acad Sci 723, 180-196 (1994)). Using cultured PTE cells, we found that treatment with cisplatin led to exposure of PS at the cell surface membrane, as evidenced by immunostaining with FITC-Annexin V. In addition,
colocalization of rhMG53 and AnnexinV could be observed at the cisplatin-induced injury sites on PTE cells (Fig. 58). While control PTE cells are negative for staining with rhMG53 or Annexin V (Fig. 58, top panels), cells exposed to cisplatin showed positive staining with rhMG53 and Annexin V (Fig. 58, bottom panels). In addition to localization at the plasma membrane (overlapping pattern with Annexin V), rhMG53 also enters the PTE cells following exposure to cisplatin. Cells incubated with BSA showed neither plasma membrane targeting nor intracellular localization of BSA, under control conditions or following cisplatin treatment (Fig. 58, middle panels). These data support the concept that PS functions as an important lipid signal for the tissue protective function of rhMG53.

Following a well-established protocol of murine nephrotoxin-induced AKI (32), we treated C57BL/6 mice with 30 mg/kg cisplatin via intraperitoneal (i.p.) injection. This treatment led to the development of AKI, as evidenced by the appearance of pathological features of ATN in cisplatin-treated animals (Fig. 59a). To test if rhMG53 can protect against cisplatin-induced AKI, the animals were treated with i.v. injection of rhMG53 10 min prior to i.p. delivery of cisplatin. As shown in Fig. 59b and 59c, significant improvements in Up/Uc and SCr were observed in animals that received rhMG53 treatment, indicating that rhMG53 is effective in preventing the nephrotoxic effect of cisplatin. These results support targeting repair of PTE injury as a potential mechanism for alleviating the nephrotoxic effect of cisplatin.

To assess whether rhMG53 interferes with the efficacy of cisplatin to induce death in cancer cells, the following two studies were performed. Using cultured murine pancreatic tumor cells (KPC-Brcal) (R. Shakya et al., Hypomethylating therapy in an aggressive stroma-rich model of pancreatic carcinoma. Cancer Res 73, 885-896 (2013); R. Shakya et al., BRCA1 tumor suppression depends on BRCT phosphoprotein binding, but not its E3 ligase activity. Science 334, 525-528 (2011)) in a MTT assay, we found that incubation with 50 μg/ml rhMG53 did not affect the ic50 of cisplatin-induced cancer cell death at 48 hours after drug treatment (Fig. 59d). We then used a pancreatic cancer allograft model to test if co-injection of rhMG53 alters the tumor suppression function of cisplatin. As shown in Fig. 59e, cisplatin (6 mg/kg, i.p.) effectively suppressed growth of KPC-Brcal tumor cells implanted into nude mice. There was no difference in the pattern of tumor growth in animals receiving co-treatment with rhMG53 and cisplatin. Thus, the reno-protective function of rhMG53 did not appear to interfere with the efficacy of cisplatin to treat tumor cells.

Exemplary Methods
Identification and cloning of MG53 - The preparation and screening of a mAb library for microsomal proteins of rabbit skeletal muscle were described previously. The preparation of mAb5259 (IgGI subclass) and immunoaffinity purification was carried out as described previously(21). Purified MG53 was subjected to amino acid sequence analysis and all sequences determined were encoded in the rabbit MG53 cDNA (data not shown). Homology searches in the databases found mouse and human MG53 using the rabbit partial amino acid sequences. An exon region of the mouse MG53 gene was amplified from mouse genomic DNA, and rabbit and mouse skeletal muscle libraries were screened using the 32P-labeled exon fragment to yield full-length cDNAs.

Immunohistochemical and Immunostaining analysis - Immunochemical analyses using mAb5259 were carried out as described previously. Immuno-electron-microscopy using secondary antibody conjugated with 15 nm gold particles was conducted as described previously.

Cell culture - The C2C12 murine myoblast cell line used for all studies was purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in a humidified environment at 37°C and 5% CO₂ in DMEM medium for C2C12 or Ham's F12 medium for CHO cells supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. In order to induce myotube differentiation, C2C12 myoblasts were grown to confluence and the medium was switched to DMEM containing 2% horse serum, penicillin (100 U/ml), streptomycin (100 μg/ml). For transient transfections, C2C12 myoblasts or CHO cells were plated at 70% confluence in glass-bottom dishes. After 24 hours, cells were transfected with plasmids described above using GeneJammer reagent (Stratagene). Cells were visualized by live cell confocal imaging at 24-48 hours after transfection or at times indicated for individual experiments. In some experiments, C2C12 myoblasts were allowed to differentiate into myotubes for the indicated time before observation.

Plasmids construction - The full-length mouse MG53 cDNA and associated truncation mutants were generated by PCR. For construction of pCMS-MG53, after digestion by the appropriate restriction enzymes, the PCR-amplified cDNA was inserted into pCMS-EGFP vector (Invitrogen) at Nhe I/Xba I sites. For construct the GFP-MG53, GFP-TRIM, GFP-SPRY, MG53-GFP, TRIM-GFP and SPRY-GFP, PCR products were inserted into pEGFP-C1 at the XhoI/XbaI sites, or pEGFP-N1 at the XhoI/KpnI sites.
**Live cell imaging** - To monitor intracellular trafficking of GFP-MG53 either CHO or C2C12 cells were cultured in glass-bottom dishes (Bioptechs Inc.) and transfected with the plasmids described above. Fluorescence images (512x512) were captured at 3.18 s/frame using a BioRad 2100 Radiance laser scanning confocal microscope with a 63X 1.3NA oil immersion objective.

**RNAi assay** - The target sequence for shRNA knockdown of MG53 is at position 622-642 (GAG CTG TCA AGC CTG AAC TCT) in the mouse MG53 cDNA. For caveolin-3, the target sequence is at position 363-380 (GAC ATT CAC TGC AAG GAG ATA). Complementary sense and antisense oligonucleotides were synthesized. To construct the MG53 shRNA and control plasmids, annealed oligonucleotides were inserted into psiRNA-hHIGFPzeo G2 (InvivoGene) at the Acc 651/Hind III restriction enzyme sites. For caveolin-3 shRNA and control plasmids, annealed oligonucleotides were inserted into pRNAiDsRed vector (BD Biosciences) at the EcoR I/BamH I restriction enzyme sites. Each vector has an independent fluorescent protein expression cassette (green or red) to act as markers of cell transfection. All plasmids were confirmed by direct sequencing with flanking primers and the down-regulation of MG53 and caveolin-3 protein expression was examined by Western blot analysis.

**Western blot and Co-immunoprecipitation** - Immunoblots were using standard techniques. Briefly, C2C12 or CHO cells were harvested and lysed with ice-cold modified RIPA buffer (150 mM NaCl, 5 mM EDTA, 1% NP40, 20 mM Tris-HCl, pH 7.5) in the presence of a cocktail of protease inhibitors (Sigma). 20 μg of total protein were separated on a 4-12% SDS-polyacrylamide gel. A standard protocol was used for co-immunoprecipitation studies of MG53 and interacting proteins, e.g., Caveolin-3. In brief, skeletal muscle tissue or C2C12 myotubes were lysed in 0.5 ml modified RIPA buffer. The whole cell lysate (500 μg) was incubated overnight with 5 μg polyclonal anti-MG53 (polyclonal antibody), or anti-caveolin-3 antibody (mAb). As a negative control, 500 μg whole cell lysate was incubated with 5 μg normal rabbit and mouse IgG and processed as described above. The immune complexes were collected on protein G-Sepharose beads by incubating for 2 hours and washed four times with RIPA buffer.

**Reagents, human tissue specimens and cells-** Recombinant human MG53 (rhMG53) protein was purified from E. coli following our published protocol (N. Weisleder et al., Recombinant MG53 protein modulates therapeutic cell membrane repair in treatment of muscular dystrophy. Sci Transl Med 4, 139ra185 (2012)). rhMG53 was stored as lyophilized powder and dissolved in saline solution before use. Anti-E-cadherin antibody was
purchased from Proteintech Group (Chicago, IL), anti-KIM-1 antibody was obtained from R&D Systems (Minneapolis, MN), anti-nephrin antibody was from Santa Cruz Biotechnology (Dallas, TX), anti-P-actin was from Sigma. FITC-AnnexinV was purchased from BD Bioscience. Human kidney and bladder tissues were obtained from National Disease Research Interchange Biospecimen (NDRI). Immortalized PTE cells from WKY rats were cultured as described before (C. Zeng et al., Activation of D3 dopamine receptor decreases angiotensin II type 1 receptor expression in rat renal proximal tubule cells. Circ Res 99, 494-500 (2006); A. Parenti et al., Activation of MAPKs in proximal tubule cells from spontaneously hypertensive and control Wistar-Kyoto rats. Hypertension 35, 1160-1166 (2000)).

**Animal care and procedures**-Animal handling and surgical procedure were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University and were compliant with guidelines of the American Association for the Accreditation of Laboratory Animal Care. MG53 knockout mice (Mg53-/-) and their wild-type control mice were bred and maintained as previously described (C. Cai, et al., MG53 nucleates assembly of cell membrane repair machinery. Nat Cell Biol 11, 56-64 (2009)). Sprague-Dawley rats (250-300 grams weight) were purchased from Charles River. NCR nude mice (3-5 weeks age) were purchased from Taconic Farms (Albany, NY).

**Mouse model of ischemia/reperfusion (IZR)-induced AKI**- Mice (10 to 14 week age) were anesthetized with isoflurane. The left renal pedicle was exposed using a dorsal lumbotomy incision and the left renal artery underwent unilateral non-occlusive clamping for 25 min at room temperature followed by reperfusion, which was ensured by visual inspection of kidneys. Sham-operated animals had an incision plus 30 min of waiting time without clamping. After ischemia or sham surgery, flank muscle and skin layers were sutured, and 1 ml pre-warmed 0.9% saline solution was subcutaneously administered immediately before closing the incision to prevent dehydration.

**Rat model of I/R-induced AKI**- Rat kidneys were approached through a ventral midline incision. The left renal pedicle was clamped by a non-occlusive vascular clip followed by contralateral nephrectomy. The left renal artery was released for reperfusion after 35 min of ischemia and the kidney was visually checked for color changes. Sham operated rats received a ventral midline incision and suture without ischemia-reperfusion of the kidney. To test whether rhMG53 could prevent I/R-induced AKI in the rat model, rhMG53 (2 mg/kg) was tail-vein administered 10 min before ischemia and 5 minutes prior to
reperfusion. Control animals received an equal volume of saline solution during the I/R procedure.

Mouse model of cisplatin-induced AKI- Mice were administered 30 mg cisplatin/kg body weight (1.0 mg/ml solution in sterile 0.9% saline) or vehicle (0.9% saline) by a single intraperitoneal injection. After cisplatin administration, mice were placed in metabolic cages for 3 successive days for urine sample collection. rhMG53 (2 mg/kg) was delivered through tail-vein injection at 10 min prior to cisplatin administration (i.p.). Kidneys were harvested at the completion of urine collection, bisected and fixed in 10% neutral buffered formalin for immunohistochemistry.

Measurement of renal functions-Urine samples were collected by housing the animals in metabolic cage for 18-20 hours with free access to water and food. For detection of possible hematuria, leukocyturia and glycosuria, urine samples were spotted onto 10SG urine reagent strips (McKesson Medical-Surgical, Richmond, VA) and read on a Siemens Clinitek Status Analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY). The Clinitek was calibrated before use and runs a self-check before each urine sample is read. All urines were read on the same day and by the same operator to ensure consistency. Semi-quantitative urine parameters (protein, pH, specific gravity, glucose) were averaged for each mouse within a group and then group averages for wild type and Mg53/- mice were determined. None of the mice tested positive for urine leukocytes, and only one mutant and one wild type mouse had blood in their urines, but even these two mice did not have blood in every urine sample collected over several days. Urinary protein excretion was measured by the Bradford method using a BioRad Dc protein determination kit (BioRad, Hercules, CA) and SDS-PAGE followed by Colloidal Blue staining. Urine creatinine (Uc) were measured by the Jaffe method using a BioQuant kit (BQ Kits Inc, San Diego, CA). Urine albumin concentration was quantified using an ELISA kit (Bethyl Laboratory, Montgomery, TX).

Blood Chemistry-Blood samples were obtained by saphenous vein puncture and at the time when mice were euthanized. Serum creatinine (SCr) levels were measured by an HPLC-based method at the Yale University Mouse Metabolic Phenotyping Center Analytic Core.

Immunoblotting-Crude extracts from dissected cortex or medullary regions of the mouse kidney were washed twice with ice-cold PBS and lysed in RIPA buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% SDS and 0.5% deoxycholate), supplemented with a cocktail of protease inhibitors (Sigma, St. Louis, MO, USA) and phosphatase inhibitors (Thermo Scientific, USA). 50 µg of kidney lysates were separated by
10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (PVDF) (Millipore). The blots were washed with Tris-buffered saline Tween-20 (TBST), blocked with 5% milk in TBST for 1 hour, and incubated with custom-made monoclonal anti-MG53 antibody. ImmunobLOTS were visualized with an ECL plus kit (Pierce).

For assaying cell-type specific expression of MG53 in kidney, proximal tubular epithelia (PTE) and glomeruli were isolated from adult rat kidney, using the method of differential sieving (H. M. Wilson, K. N. Stewart, Glomerular epithelial and mesangial cell culture and characterization. Methods Mol Biol 806, 187-201 (2012)). Identities of cell origins were confirmed with E-cadherin as a PTE cell marker, and nephrin as a glomerular cell marker.

Primary PTE cell isolation from mouse neonates- Primary PTE cells were isolated from kidneys of 2 day-old Mg53-/− neonates and wt littermates, using the protocol of Lieberthal et al with minor modification (W. Lieberthal et al., Rapamycin impairs recovery from acute renal failure: role of cell-cycle arrest and apoptosis of tubular cells. Am J Physiol Renal Physiol 281, F693-706 (2001)). In brief, kidney cortices from mice were retrieved under a dissecting microscope, minced and digested with collagenase for 30 min. at 37°C, and filtered through a 70-µm sieve over a 50-ml conical tube with medium containing DMEM and HamF12 (1:1 ratio) supplemented with transferrin (5 µg/ml), insulin (5 µg/ml) and hydrocortisone (50 µM). The filtered cells were centrifuged and plated onto a matrigel-coated plate (BD Bioscience, San Jose, CA) and cultured for 3 days before experimentation. Aliquots of cells were stained with phaseolus erythroagglutinin lectin (PHA-L) (Sigma Aldrich, St. Louis, MO) to confirm identity of PTEs.

Transfection of GFP-MG53 into PTE cells derived from the Mg53-/− neonates was performed using the Lipofectamine LTX reagent (Life Technologies) per manufacturer's instructions. PTE cells expressing GFP-MG53 was subjected to microelectrode penetration-induced acute injury to the plasma membrane as previously described (C. Cai, et al., MG53 nucleates assembly of cell membrane repair machinery. Nat Cell Biol 11, 56-64 (2009)).

Electron microscopy- Electron microscopy was performed by the Ohio State University Campus Microscopy and Imaging Facility (CMIF). Renal tissues were sliced in blocks of 1 mm cubes and fixed at room temperature with a fixative solution containing 2.5% glutaraldehyde, 1% formaldehyde, 100 mM sodium phosphate (pH 7.2). These slices were rinsed with 0.1 M Na cacodylate (pH 7.4) and post-fixed for 1 hour in 2% osmium tetroxide. After dehydration, the samples were embedded in epoxy resin, and ultra-thin sections (60
nm) were counterstained with uranyl acetate and lead citrate. Transmission electron microscopy images were obtained with an FEI Tecnai G2 Spirit Transmission Electron Microscope (FEI Hillsboro, Oregon) equipped with a macro-fire monochrome progressive scan CCD camera (Optronics, Goleta, CA). Sections of PTE and glomeruli pathology were analyzed.

Scanning electron microscopic images of PTE cells were used to analyze the morphological changes of the cells derived from wild-type and Mg53-/− mice. Cells grown on matrigel-coated glass coverslips were fixed with 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate, and then post fixed in 1% osmium tetroxide for 1 hr. After dehydration, the cells were sputter-coated with palladium and viewed under a FEI Nova NanoSEM 400 scanning electron microscope (FEI, Hillsboro, Oregon).

Histopathology and immunohistochemical staining- Experimental animals were euthanized 2 hrs after tail vein injection of rhMG53. Systemic perfusion of the animals was immediately conducted with heparin-supplemented PBS (pH 7.4, 200 ml), followed by 2% paraformaldehyde in 200 ml PBS using a peristaltic infusion pump (50 ml/min). Kidneys were harvested, embedded in OCT-Tissue Tek medium (Sakura, Northbrook, IL) and snap-frozen by immersion in an isopentane bath at -80°C. Six-micron-thick cryosections were mounted on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and fixed in freshly prepared 4% paraformaldehyde in PBS at 4°C. Immuno-staining was carried out using a custom-made rabbit anti-human MG53 antibody. Alexa 488-conjugated sheep anti-rabbit IgG (Molecular Probes) was used for labeling of kidney sections that were positive for rhMG53 staining, using a digital fluorescence microscope.

Paraffin embedded kidney sections of 4 µm thickness were used for Periodic acid-Schiff (PAS) and hematoxylin and eosin (H&E) staining. Immunohistochemical (IHC) staining with Goat anti-rat KIM-1 antibody was used to quantify the degree of tubular injury following I/R-induced AKI, following the protocol of Schroppel et al (B. Schroppel et al., Tubular expression of KIM-1 does not predict delayed function after transplantation. J Am Soc Nephrol 21, 536-542 (2010)). Quantification of tubular regions positive for KIM-1 staining was analyzed in a double-blinded fashion.

Rat PTE cell culture and anoxia/reoxygenation assays- Immortalized PTE cells from WKY rats were cultured at 37°C in 95% O2, 5% CO2 in DMEM/F-12 (C. Zeng et al., Activation of D3 dopamine receptor decreases angiotensin II type 1 receptor expression in rat renal proximal tubule cells. Circ Res 99, 494-500 (2006); A. Parenti et al., Activation of MAPKs in proximal tubule cells from spontaneously hypertensive and control Wistar-Kyoto

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rats. *Hypertension* 35, 1160-1166 (2000)). To induce anoxia, cells grown on coverslips were placed in an anoxic chamber with 1% oxygen, 5% CO2 and balanced N2 at 37° C for 2 h followed by reoxygenation for 2 h. Exogenous rhMG53 or BSA were conjugated with rhodamine by a dye labeling kit (G-Biosciences, St Louis, US.), and applied to cells immediately after anoxia. After reoxygenation, the cells were used for FITC-annexin V (BD Biosciences) staining and fixed with 4% paraformaldehyde (30 min). Immunofluorescence images were acquired with LSM 780 (Carl Zeiss) confocal microscopy.

**MTT cell viability assay-** Culture of the KPC-Brca1 pancreatic cancer cells were maintained according to Shakya et al (R. Shakya et al., BRCA1 tumor suppression depends on BRCT phosphoprotein binding, but not its E3 ligase activity. *Science* 334, 525-528 (2011); M. Sekine et al., Selective depletion of mouse kidney proximal straight tubule cells causes acute kidney injury. *Transgenic Res* 21, 51-62 (2012)). Cisplatin-induced changes in cell viability were determined with MTT assay. Cells were plated at 1,000-1,500 cells per well in a 96-well plate and then treated with the indicated amounts of cisplatin for 48 hours. Then 20 μl (5 mg/ml in PBS) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma Chemical Co., St. Louis, Mo.) was added to each well. After 4 hours, culture medium was removed and 150 μl DMSO was added to dissolve formazan crystals in each well, and absorbance was recorded at 570 nm.

**Allograft model-** Five week-old NCR nude mice were implanted subcutaneously in both flanks with 2x106 KPC-Brca1 pancreatic cancer cells. After tumors reached 4 to 7 mm in diameter (5 days after implantation), the mice were divided into three groups so that the mean and the variance of the tumor diameters were not significantly different among the groups prior to treatment. Cisplatin-induced suppression of tumor growth was tested through intraperitoneal administration. Immediately prior to cisplatin administration, 2 mg/kg of rhMG53 (or an equal volume of saline solution as control) was delivered through tail-vein injection. Tumor volume was determined from the orthogonal dimensions (length, width) using the formula: tumor volume = (width)2xlengthx3.14/6. According to the IACUC guideline, the animals were terminated when tumors reached 1.5 cm in diameter. At the end of experiment, mice were sacrificed and allografts were removed and analyzed.

**Statistical Analysis-** All data are expressed as mean ± SEM. Comparison within groups was made by student t-test when comparing two experimental groups and by ANOVA for repeated measures. A value of P<0.01 was considered significant.
[00278] It is understood that the detailed examples and embodiments described herein are given by way of example for illustrative purposes only, and are in no way considered to be limiting to the invention. Various modifications or changes in light thereof will be suggested to persons skilled in the art and are included within the spirit and purview of this application and are considered within the scope of the appended claims. For example, the relative quantities of the ingredients may be varied to optimize the desired effects, additional ingredients may be added, and/or similar ingredients may be substituted for one or more of the ingredients described. Additional advantageous features and functionalities associated with the systems, methods, and processes of the present invention will be apparent from the appended claims.
In the Claims:

1. A composition comprising an effective amount of mitsugumin 53 (MG53) polypeptide for use in a method of treating or preventing kidney injury comprising administering the composition to a subject in need thereof, wherein the composition is effective in ameliorating the effects or symptoms of kidney injury.

2. The composition of claim 1, wherein the MG53 polypeptide is a recombinant MG53 polypeptide possessing proximal tubule epithelium (PTE) membrane repair activity.

3. The composition of claim 1 or 2, wherein the MG53 polypeptide is recombinant human MG53 (rhMG53) polypeptide.

4. The composition of any of claims 1-34, wherein the kidney injury is at least one of surgery-related kidney injury, contrast media-induced acute kidney injury (AKI), drug or chemotherapy-induced AKI, toxin-induced AKI, dialysis, ischemia/reperfusion-induced AKI, sepsis-induced AKI, or a combination thereof.
FIG. 1 (continued)
FIG. 6

<table>
<thead>
<tr>
<th>Brightfield</th>
<th>Evans Blue</th>
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<tbody>
<tr>
<td>Wild type</td>
<td></td>
</tr>
<tr>
<td>ng53-/-</td>
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FIG. 7

(a) Normalized CK release (mU/mg heart mass) vs Minutes post ischemia for WT (n=4) and mg53KO (n=3).

(b) Normalized LDH release (mU/mg heart mass) vs Minutes post ischemia for WT (n=4) and mg53KO (n=3).
FIG. 11 a
FIG. 13

**a**

Mice brain from B6G53-KO or WT mice (8-17 week old male if)

- Stored at -80°C
- Hbuffer (100 ml)
- Homogenization for 20 x 3, 10,000 RPM (at-10°C, 3M0Y)
- 10,000g for 20 min (TA-15, Tomiy)
- S GFP

- 50,000g for 20 min (TA-13, Tomiy)
- GFP

- Add KCl to final 0.5 M
- Incubating 2h min
- 10,000g for 10 min (10-13, Beckman)
- GFP

- Wash X2
- Homogenate & spin at 21,000g for 15 min
- Hbuffer (4 ml)
- GFP

- Supernatant
- Concen gradient
- 80,000g for 18 h (SW28, Beckman)

**b**

SDS-PAGE analysis of R1 fraction (CBB staining)

- WT KO
- 15% gel
- Myelin basic protein

- WT KO
- 3-10% gradient gel
- Parasin
FIG. 14 a-c

A

0 s

GFP-MG53

250 s

20 μm

B

PS

C

20 μm

Anx-V-GFP

Anx-V-GFP +MG53
FIG. 16

A

B

C

ΔF/ΔF₀ (GFP)

ΔF/ΔF₀ (FAM-43)

Time (S)

Time (S)

Time (S)
FIG. 17

A

B

C

GFP-MG53  GFP-C242A

0s  100s  0s  100s

20 μm  20 μm

GFP-MG53  GFP-C242A

0s  200s  0s  200s

20 μm  20 μm

ΔF/F₀ (F/M4-64)

Time (S)

0  100  200

GFP-MG53  GFP-C242A

ΔF/F₀ (F/M4-64)

Time (S)

0  100  200

GFP-MG53  GFP-C242A

ΔF/F₀ (F/M4-64)

Time (S)

0  100  200
FIG. 18 a-c
FIG. 18 d-e

d
C2C12 myotube  mg53-/- myotube

WT

C29L

15 μm

e

GFP-MG53
GFP-C242A

C2C12 myoblast  C2C12 myotube  mg53-/- myotube

ΔF/ιF₀

Time (S)

0 20 40 60 80 100 120

0 1 2 3 4 5 6

0 1 2 3 4 5 6

0 20 40 60 80 100 120

0 1 2 3 4 5 6

0 20 40 60 80 100 120

0 1 2 3 4 5 6
FIG. 20 c-d

C

WT

mg53 -/-

\[ \frac{\Delta F}{F_0} \text{ (FM1-43)} \]

Time (S)

\[ \frac{\Delta F}{F_0} \text{ (FM1-43)} \]

Time (S)

<table>
<thead>
<tr>
<th>C39</th>
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<th>C108</th>
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<tr>
<td>H31</td>
<td>14C</td>
<td>85H</td>
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RING

B-box2
FIG. 21 a-b

a

GFP-MG53

0s

80s

b

GFP-MG53

+ Ca-EDTA

10 μm
FIG. 22

a

WT, C29L, H31A, C29L/C105S, C105S, C242A, C85, C86A

150
100
75
50

KD

-DTT

+ DTT

Dimer

GFP-MG53
FIG. 23

a

MG53 Ring Motif:
SCPLCLQLFDAPYTAECGHSFCRACLGRVAGEPAADGTVLCPCC

MG53 Bbox Motif:
CEEHLSDPISYCEQDRALVCGVCASLGHRGH

b

C

Relative Zinc Concentration (µM Zinc/µM of protein)

WT

* C29L

** DM
FIG. 26

Mouse MG53

Human TAT-MG53

Human MG53
FIG. 27

[Diagram showing experimental results with labels for Beads, Elutes, α-Myc, and α-mMG53]
FIG. 28

a Flag-MG53

- Control
- HA-WT
- HA-CS1
- HA-CS3
- HA-CS4
- HA-CS5

IP: α-flag
IB: α-HA-HRP

b HA-MG53

- GFP-WT
- GFP-CC
- GFP-CS1
- GFP-CS2
- GFP-CS3
- GFP-CS4
- GFP-CS5

IP: α-HA(3F10)
IB: α-GFP
FIG. 30

Before notoginseng

Following notoginseng
FIG. 31

Pre-injury  Following injury

RFP-MG53
0s  50s

Bright field

0s  50s
FIG. 33

Counts x10^6 (479:598)

100 V/cm

50 V/cm

Dye

Control (no dye)

Time (s)
FIG. 36

a

LDH (uU/mL)

Electrical field (V/cm^2)

b

Standard curve

OD490

LDH activity (uU/mL)
FIG. 37

LDH Activity (uU/mL)

Recombinant MG53 (μg/mL)

- - - - Control

- - - - Beads
FIG. 40

- NoBeads
- Beads

Optical density (488 nm)

Rec hMG53
PS (3.3 ug/mL)
PS (33 ug/mL)
FIG. 42

Before injury  Post injury  Before saponin  After saponin

Keratinocytes
FIG. 43

<table>
<thead>
<tr>
<th>Before injury</th>
<th>Post injury</th>
<th>Before saponin</th>
<th>After saponin</th>
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<td>HEPM</td>
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</table>
FIG. 44

![Graph illustrating LDH (uU/mL) levels with LPS and MG53 treatment.]

- LPS
  - -
  - +
- MG53
  - -
  - +
  + +

* p < 0.05
** p < 0.005
FIG. 45

Before needle penetration  After needle penetration

Before saponin  After saponin

0 s  50 s  150 s
FIG. 46

Before needle penetration  After needle penetration

Before saponin  After saponin
FIG. 48

![Graph showing optical density at 488 nm for Rec hMG53 with and without beads.](image-url)
FIG. 53 (Continued)

**b**

![Graph showing data for Up/Uc with two groups: wt and Mg53-/-.

**c**

![Graph showing data for SCR (mg/dL) with two groups: wt and Mg53-/-.]
FIG. 53 (Continued)
FIG. 53 (Continued)
FIG. 54

a

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<th>Cortex</th>
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<tr>
<td></td>
<td></td>
<td>-/-</td>
<td>-/-</td>
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**rhMG53 (0.3 ng)**

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<tr>
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<th>MG53</th>
<th>β-actin</th>
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<td>+/+</td>
<td>Strong signal</td>
<td>Strong signal</td>
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<tr>
<td>-/-</td>
<td>Weak signal</td>
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Legend:
- **rhMG53** (recombinant human MG53)
- **MG53**
- **β-actin**
FIG. 54 (Continued)
FIG. 54 (Continued)
FIG. 54 (Continued)
FIG. 54 (Continued)

![Graph showing cell survival percentages for different conditions.

- Wt: 0%
- Mg53-/-: 0%
- GFP-MG53: 83%

Cell survival percentages range from 0% to 100%.](image-url)
FIG. 55 (Continued)
FIG. 55 (Continued)
FIG. 56
FIG. 57 (Continued)
FIG. 57 (Continued)
FIG. 57 (Continued)
FIG. 58

[Image of fluorescence microscopy images showing DAPI, Rhodamine, Annexin V, and Merge for Control, BSA, Cisplatin, and rhMG53 conditions with scale bar indicating 10 μm]
FIG. 59

a  rhMG53  vehicle

H&E

PAS

100 μm
FIG. 59 (Continued)
FIG. 59 (Continued)

Cell Survival

Cisplatin Log [mM]

- 120
- 80
- 40
- 0

- + Saline
- + BSA
- + rhMG53

-4 -3 -2 -1 0
FIG. 59 (Continued)

![Graph showing tumor volume over days for different treatments.](image)
FIG. 61
FIG. 62
FIG. 63

[Diagram showing gel electrophoresis with labeled lanes for rhMG53 and l.v. rhMG53 (mg/kg).]
FIG. 63 (Continued)
FIG. 64 (Continued)
INTERNATIONAL SEARCH REPORT

International application No. PCT/US2015/068016

A. CLASSIFICATION OF SUBJECT MATTER

A61K 38/16(2006.01)i, A61K 38/17(2006.01)i, A61K 39/395(2006.01)i, A61P 13/12(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 38/16; C12N 15/12; C12N 1/21; C12P 21/02; A61K 31/7052; A61K 48/00; C12N 9/00; A61K 38/17; A61K 39/395; A61P 13/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: mitsugumin 53 (MG53), TFJMT2, kidney injury, membrane repair

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category</th>
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<td>US 2012-0309051 Al (MA, JIANJE et al.) 06 December 2012</td>
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<td>[0079]-[0080], [0165], [0298], [0305], [0325].</td>
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<td>A</td>
<td>WEISLEDER, NOAH et al., &quot;Recombinant MG53 protein modulates therapeutic effects in cell line&quot;, Science Translational Medicine, 20 June 2012, Vol. 4, Issue 139.</td>
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<td>Article 139ra85, Author manuscript, pp. 1-20</td>
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<td>See abstract; page 3, line 32 - page 6, line 37.</td>
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<td>US 2013-0123340 Al (MA, JIANJE et al.) 16 May 2013</td>
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<td>See abstract; paragraphs [0009], [0038], [0084].</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search

21 April 2016 (21.04.2016)

Date of mailing of the international search report

21 April 2016 (21.04.2016)

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<td>WO 2014-200705 Al</td>
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