TRIM72 is required for effective repair of alveolar epithelial cell wounding

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TRIM72 is required for effective repair of alveolar epithelial cell wounding. In vivo injury of lung cells was introduced by high tidal volume ventilation, and repair-defective cells were labeled with postinjury administration of propidium iodide. Primary alveolar epithelial cells were isolated and membrane wounding and repair were labeled separately. Our results show that absence of TRIM72 increases susceptibility to deformation-induced lung injury whereas TRIM72 overexpression is protective. In vitro cell wounding assay revealed that TRIM72 protects alveolar epithelial cells through promoting repair rather than increasing resistance to injury. The repair function of TRIM72 in lung cells is further linked to caveolin 1. These data suggest an essential role for TRIM72 in repair of alveolar epithelial cells under plasma membrane stress failure.

TRIM72 is a member of the tripartite motif family (TRIM) and is abundantly expressed in striated muscle tissues and has been referred to as “biotrauma” in the context of positive pressure ventilation. In disorders associated with increased surfactant loss, such as acute respiratory distress syndrome (ARDS), the interfacial stress on small airway and alveolar epithelia is greatly increased. Several studies suggest that epithelial and endothelial wounding are integrated parts of the ventilator-associated lung injury (VALI) phenotype. Abnormal epithelial cell repair and cell death contribute to the complex pathology of ARDS, and often trigger downstream proinflammatory stress responses that are referred to as “biotrauma” in the context of positive pressure ventilation.

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Similar to other TRIM family proteins, tripartite motif protein 72 (TRIM72, also referred to as mitsugumin 53) contains a conserved RING, B-box, and a coiled coil motif at the NH2-terminus, with a variable SPRY domain at the COOH-terminus to distinguish TRIMs as specific isoforms. TRIM72 is abundantly expressed in striated muscle tissues and has been studied extensively (52). Lateral plasma membrane lipid trafficking is necessary for preventing membrane stress failure during deformation (66), whereas additional exocytosis of intracellular membranes to the injury site is required for patching of larger wounds (40, 52). In addition, endocytotic membrane retrieval is also critically involved in successful repair by removing plasma membrane disruptions (40). Depolymerization and re-polymerization of the subcortical cytoskeleton precedes and follows these vesicle trafficking processes to facilitate membrane repair and restore normal cellular structure (35). Evidence supports that membrane injury-triggered exocytosis and endocytosis are tightly linked (5) and a handful of membrane repair molecules have been associated with each membrane repair step. For example, dysferlin (34) and annexin A5 (7) are thought to be involved in the exocytotic process (36), whereas caveolin 1 (Cav1) (17) and cdc42 (45) among others are believed to take part in the endocytic process.

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shown to function as a critical component of the “cell membrane repair kit” in these tissues (8, 51). The cell/tissue protective effect of TRIM72 is supported by observations from TRIM72 knockout mice (TRIM72KO) showing increased tissue damage and reduced contractility in older mice subjected to downhill running (8) and that recombinant TRIM72 protein provides therapeutic benefits on skeletal muscle myonecrosis following toxin exposure or vigorous exercise (82). Moreover, it has also been shown that TRIM72 plays a cardioprotective role following ischemia-reperfusion injury (12, 81, 88).

In this report we characterized the expression of TRIM72 in the lung and examined its role in repair of plasma membrane wounds of the lung epithelial cells using in vivo and in vitro assays. Our results show that TRIM72 is required for effective repair of lung resident epithelial cells following deformation injury. In vitro assay further revealed that TRIM72 is involved in the repair process of lung cells following plasma membrane stress failure. In addition, biochemical assays link the physiological function of TRIM72 in lung cells with its interaction with Cav1, a protein enriched in ATI and pulmonary endothelial cells (22), which facilitates repair of cell membrane disruption through the caveolar endocytosis process.

MATERIALS AND METHODS

Construction of inducible TRIM72 overexpressor mice. FLAG-tagged full-length trim72 cDNA (accession no.: AB231474) was cloned into a tet-inducible gene expression vector downstream of a tetracycline (tet)-responsive element (TRE) and mini-cytomegalovirus (PminCMV) fusion promoter (Mutagenex, Hillsborough, NJ). Constitutive expression of reverse tet-controlled transcriptional activator (rtTA) and transcriptional silencer (tTS) was driven by a separate cytomegalovirus (CMV) promoter (P(CMV)) (44). The overexpression plasmid (pTi-flag-trim72) was transfected into HEK293 cells to test inducibility by 2 μg/ml doxycycline (Dox) and then linearized by XholSpeI digestion. Pronuclear injection and oviduct transfer were performed in the Genetically Engineered Mouse Modeling Shared Resource of the Ohio State University (OSU, Columbus, OH). Genotyping primers for the TRIM72 overexpressor mice (TRIM72OE) mice were designed and transgenic copy number standards were made by mixing: copy number × (8,400 bp transgene/3 × 109 bp mammalian genome) × mass of genomic DNA. Two primers (i.e., primer 9 and 3) allowing rough estimation of the transgene copy number were used for detecting presence of the transgene: primer 9 forward 5′-CAAGCT- GTTTGGACCTCTCAT-3′ and reverse 5′-GTACCGCCTGGAC- CTG-3′; primer 3 forward 5′-AGGGCATCGGTAAACATCTG-3′ and reverse 5′-GACGAGCTCCACTTAGACG-3′. Five of 48 pups were identified to bear the transgene through PCR-based assays, among which two founder lines (no. 11 and no. 41) had good germline transmission and were used to establish the TRIM72OE transgenic line.

Animals. The TRIM72KO mice were constructed by targeted disruption of trim72/mg53 gene expression through insertion of a neomycin cassette at exon 1 and homologous recombination as previously described (8). The Aqp5-Cre-IRESDsRed knockin mice (ACID) were generated through placing a Cre-IRES-DsRed cassette into the exon 1 of the Aqp5 gene for ATI-specific expression of Cre recombinase as described previously (27). The ACID mice were then crossed to ROSAmT/mG reporter mice (stock no. 007576, Jax Laboratory), which ubiquitously express a membrane-targeted tdTomato (mT) that is flanked by loxp sites, resulting in loss of mT expression to allow for expression of the downstream membrane-targeted EGFP cassette (mG) in ACID:mT/mG double heterozygous mice (27). Importantly, although the ACID knockin allele includes IRES-DsRed, there is no expression of DsRed from this allele, likely due to a mutation. Cav1KO mice were obtained from the Jackson Laboratory (stock no. 004585, Bar Harbor, ME). Genotype of the TRIM72KO mice was confirmed by PCR using the following primers: forward: 5′-CCTTCGGCTCAGAACTGCTTG-3′ and reverse: 5′-CAGCAGTCCCCACCTGCTTACC-3′; the null allele generates a 1,250-bp fragment and the wild-type allele produces a 480-bp fragment. The homozygous mice produce both fragments. Cav1KO mice were genotyped following instruction provided by the Jackson Laboratory.

Mice were housed in the sterile ventilated facility of the University Laboratory Animal Resources of OSU under standard husbandry. TRIM72KO and TRIM72OE mice were crossed to 129/C56BL/6J wild type (WT) mice for more than five generations to minimize genetic background discrepancy. Both male and female mice, ~2-6 mo of age, were used for experiments. All experiments were approved by the Institutional Animal Care and Use Committee of OSU.

Cell culture and transfection. HEK293 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) until 80% confluence. Cells were transfected with TRIM72-HA and GFP-Cav1 by use of Xfect transfection reagent (Clontech, Mountain View, CA) for communoprecipitation experiments, or transfected with empty red fluorescent protein vector:empty green fluorescent protein vector (RFP-GFP), GFP-TRIM72-RFP, or GFP-Cav1:TRIM72-RFP for imaging on an Infinity 3 HAWK 2D-Array Live Cell Imaging Confocal Microscope (VisiTech International, Charlotte, NC) in the Campus Microscopy & Imaging Facility core facility of OSU.

Primary cell isolation. We have previously established a protocol to isolate primary rat ATI (79) and type II alveolar epithelial cells (ATII cells) (68) with purity ranging from ~82 to 97% for ATI cells on the basis of T1α/Cav1 immunostaining and cell morphology. Briefly, rat lungs were lavaged to remove alveolar macrophages and digested with 1 mg/ml elastase (Worthington, Lakewood, NJ). Cell suspension was filtered through 100-μm mesh and incubated on IgG-coated petri dishes for 1 h at 37°C to remove leukocytes (panning). Unattached cells were collected and incubated with 5 μg/ml mouse anti-rat T1α antibody (DSHB, Iowa City, IA) for 45 min at 4°C on a rotator, followed by incubation with Dynabeads pan-mouse IgG kit (Life Technologies, Grand Island, NY) in 0.5% BSA for 30 min to isolate ATI cells. ATIs were separated from the beads by the release buffer supplied with the kit. Cells bound to the magnetic beads were collected as ATI cell. Multiple washing and releasing steps were repeated for increased cell purities. Cell purity was estimated by using Cav1 Western blot on freshly isolated primary ATI and ATII cells. We detected Cav1 expression in ATII cells isolated from three rats but it is absent in two of three ATII cell preparations (Fig. 2A). The presence of Cav1 in one of the three ATII preparations may be due to contamination with endothelial cells, which is enriched in caveolae (19). Experiments were repeated using cells isolated from three rats and cell pellets were immediately lysed for Western blot.

The method to harvest primary murine epithelial cell was modified from previous protocol (57). Murine lungs were lavaged and digested in 1.8 U/ml dispase (Life Technologies). Cell suspension was filtered through 100-μm mesh and panned on IgG-coated dish. Unattached cells were incubated with rat anti-mouse CD31 antibody (Biolegend, San Diego, CA) bound to anti-rat IgG Dynabeads (Life Technologies) to remove endothelial cells (42). Cells not bound to CD31 were cultured as mixed alveolar epithelial cells in F-12K culture medium containing 10% FBS and 1% P/S until confluence for in vitro wound- and repair assays.

RT-PCR. Total mRNA was isolated from lung tissues by use of TRIzol reagent (Life Technologies) and converted to cDNA by using M-MLV Reverse Transcriptase (Life Technologies). PCR amplification of trim72 gene was conducted with use of forward primer 5′-CTGGACATCAGGTGGAG-3′ and reverse primer 5′-CAGGCCAAGTTCATGAGA-3′, product size of 741 bp. This sequence is conserved among mouse, rat, and human on the basis of gene alignment.
Western blot. Whole lung tissues from mouse and rat were collected for Western blot. Total denatured protein samples were separated on SDS polyacrylamide gel and transferred onto polyvinylidene fluoride membranes. The following primary antibodies were used: custom polyclonal rabbit anti-TRIM72, monoclonal mouse anti-TRIM72, monoclonal mouse anti-T1α (DSHB), polyclonal rabbit anti-caveolin-1 (Cell Signaling, Danvers, MA), polyclonal rabbit anti-Prosurfactant Protein C (EMD Millipore, Billerica, MA), monoclonal rabbit anti-dysferlin (Abcam, Cambridge, MA), monoclonal mouse anti-Flag (Sigma-Aldrich, St. Louis, MO), monoclonal rabbit anti-HA (Cell Signaling), polyclonal rabbit anti-GFP (Life Technologies) and monoclonal mouse anti-β-actin antibodies (Sigma-Aldrich). Gray values of specific bands were quantified by use of Image J software (NIH, Bethesda, MD).

Immunocytochemistry. Lungs were fixed in 4% paraformaldehyde at transpulmonary pressure of 20 cm H₂O for 30 min at 4°C, WT lung blocks were embedded in paraffin and ACID:mT/mG lung blocks were embedded in optimal cutting temperature medium as previously described (61). Immunostaining was performed on lung slices with use of the above antibodies for TRIM72, T1α, and proSP-C.

In situ lung injury assay. Expression of the transgene in TRIM72OE mice was induced by intraperitoneal injection of 50 mg/kg body wt 9-tert-butyl-doxycycline for 5 days prior to experiments. WT, TRIM72KO, and TRIM72OE mice were anesthetized with ketamine/xylazine, paralyzed with 0.3 mg/kg pancuronium, and mechanically ventilated with room air, at a rate of 150 breaths/min, a tidal volume of 561 nm (PI signal) on an Infinity 3 ventilator (SCIREQ, Tempe, AZ). After 30 min of injurious mechanical ventilation the mice were killed by exsanguination, their chests were opened, and 1 mg/ml propidium iodide (PI) in heparinized PBS was injected into the pulmonary circulation. Lungs and heart were then excised en bloc and subpleural alveoli were imaged at 488 nm (alveolar autofluorescence) and 561 nm (PI signal) on an Infinity 3 confocal microscope (VisiTech International). The number of red-fluorescent PI-positive alveolar resident cells was measured and reported as a fraction of the number of alveoli in each image field, thereby defining the cell injury index.

In vitro cell wounding and repair assay. Primary lung epithelial cell monolayer was subjected to mechanical injury by glass microwebs (54, 55) (500 μm, Sigma-Aldrich) through 5 min gentle agitation in the presence of 0.2 mg/ml 10,000 MW FITC-dextran (Life Technologies). The dish was immediately washed and 0.5 μg/ml PI (Life Technologies) was added and incubated for 2 min. Confocal images were collected at wavelengths of 488 and 561 nm on an Infinity 3 Live Cell Imaging Confocal Microscope with a ×40 objective (VisiTech International). Three to six dishes of primary cells were isolated from WT and TRIM72KO mice, and an average of seven to nine images per dish were taken for quantification of cell wounding and repair. The number of wounded cells was counted as total number of FITC-dextran-labeled plus PI-labeled cells on each image, and the percentage of repaired cells was calculated as the percentage of PI-labeled cells among all wounded cells.

Statistics. All data were analyzed by use of Origin 6.0 (OriginLab, Northampton, MA). Data were presented as means ± SE, and group means were compared with a Student’s t-test for unpaired observations. Statistical significance was assumed at \( P < 0.05 \).

RESULTS

TRIM72 expression is present in the lung tissue. A previous study showed that TRIM72 has very limited tissue expression by Northern blot, with the most predominant expression in striated muscles (8). Here we screened mammalian lung tissues for the presence of this potentially cytoprotective molecule. Murine whole lung tissues from WT and TRIM72KO mice were used for Western blots with a polyclonal rabbit anti-mouse TRIM72 antibody. We detected TRIM72 immunosignals in WT lung tissue but not in the TRIM72KO tissue (Fig. 1A). The TRIM72 band was detected in human whole lung homogenates by using a monoclonal mouse anti-human TRIM72 antibody (top band, Fig. 1B). The lower band is determined to be nonspecific on the basis of comparison with other TRIM72 antibodies (not shown). Notably, TRIM72 expression in the lung was approximately sixfold lower than that in the skeletal muscle, since sixfold greater sample loading was needed to detect a similar level of protein (Fig. 1, A and B), possibly accounting for the negative TRIM72 detection in the previous study (8). The trim72 mRNA expression was detected by using primers against a conserved nucleotide sequence among mouse, rat, and human (see MATERIALS AND METHODS). We detected specific trim72 sequence in lung tissues from these species but not in the lung from TRIM72KO mice (Fig. 1C). It is intriguing that the expression of a membrane repair molecule is limited to certain tissues (8), since the membrane wounding and repair process is observed in a wide variety of cells and tissues (59). One interesting hypothesis is that TRIM72 is expressed in response to cyclic mechanical stress, pointing to future directions of study.

TRIM72 is detected in alveolar epithelial cells. The lung alveoli are composed of multiple cell types of different origins including epithelial cells, endothelial cells, smooth muscle cells, neuronal cells, fibroblast cells, immune cells, etc. Among these, ATI cells are major targets of cell wounding following deforming stress because of their size and abundance in alveolar spaces and as such play an important role in the pathogenesis of ARDS (56, 65, 78). We therefore isolated ATI and...
ATII cells from rat lungs using an established protocol (15, 24). Cav1 is enriched in ATII but is not expressed in ATII cells and thus is used to estimate purity of the primary cell isolation (22, 79). As shown in Fig. 2A, TRIM72 expression was detected in both ATI and ATII cells, with higher expression level in ATII cells. To date, TRIM72 has been shown to interact with dysferlin (10), caveolin 3 (Cav3) (10), polymerase I and transcript release factor (89), and nonmuscle myosin IIA (NMIIA) (48), as well as to bind to plasma lipid components phosphatidylycerine (8) and cholesterol (89) in the context of its membrane repair function in skeletal muscle. We found that dysferlin, the mutation of which causes several types of muscular dystrophy (10, 37), is also expressed in alveolar epithelial cells (Fig. 2A). This may be one of the factors that shapes the cell specificity of the TRIM72-mediated membrane repair process. Since it has been shown that TRIM72 is required for dysferlin-mediated membrane repair patch formation (10), it would be interesting to examine whether dysferlin is required for TRIM72-mediated membrane repair in the lung.

We further confirmed the in situ localization of TRIM72 by comparing the immunostaining pattern of TRIM72 with cellular markers that label ATI or ATII cells specifically with genetic or immunohistochemical methods on sections of murine lungs. ACID:mT/mG lungs express GFP specifically in the thin and long ATI cells lining the alveoli (Fig. 2B) (27). Immunostaining of TRIM72 on ACID:mT/mG lung sections revealed overlapping patterns of TRIM72 expression (magenta) with GFP-positive ATI cells, as well as with cuboidal ATII cells localized at the alveolar corner that are negative for GFP (Fig. 2C). Furthermore, coimmunostaining of TRIM72 with another ATI cell-specific marker, T1α (50), and an ATII cell-specific marker, surfactant-associated protein C (SP-C) (29), on WT lung sections showed that TRIM72 (green or red respectively) stains both the T1α-positive (red, Fig. 2D) ATI cells and SP-C-positive ATII cells (green, Fig. 2E), in keeping with our findings from dissociated rat alveolar epithelial cells (Fig. 2A).

Susceptibility of the lung to deforming stress is dependent on TRIM72 expression. In the next set of studies, we investigated the role of TRIM72 in injury and repair of lung cells using genetically modified transgenic mouse models. TRIM72KO mice were constructed previously (8). To investigate the gain of function for TRIM72, we established a tet-on-inducible TRIM72 overexpressor mouse line (TRIM72OE) (Fig. 3, A and B, see MATERIALS AND METHODS). Expression of FLAG-tagged full-length mouse trim72 was driven by a tet-responsive element promoter (TRE). Constitutive expression of a rtTA and a tet-controlled tTS were driven by a separate CMV promoter (44). The transgene cassette showed good inducibility in response to Dox in cultured HEK293 cells (Fig. 3C). Robust exogenous TRIM72 expression can be detected by both TRIM72 and FLAG antibody in TRIM72OE induced by 5 days of 9-tert-butyl-doxycycline intraperitoneal injection, an analog of Dox lacking antibiotic activity but more potent as an inducer of the tet-on system (90) (Fig. 3D).

Previous studies (30, 69) established a protocol to label injured alveolar lung cells in situ with the membrane-impermeable fluorescent marker PI following transient high tidal volume ventilation. We adopted this method to mechanically ventilate and injure the WT, TRIM72KO, and TRIM72OE lung. PI was injected into the pulmonary circulation of the mice after withdrawal of the injurious ventilation to irreversibly label damaged alveolar resident cells as described under MATERIALS AND METHODS. Following 30 min of high tidal volume ventilation at positive end-expiratory pressure of 3 cmH2O, confocal images of subpleural alveoli revealed significantly more inj-

Fig. 2. TRIM72 is detected in type I and type II alveolar epithelial cells (ATI and ATII cells, respectively). A: Western blots of TRIM72, dysferlin, and caveolin 1 (Cav1) were conducted by using ATI and ATII cells isolated from 3 rat lungs in 3 separate experiments (exp#1–exp#3). ATI cells were isolated using monoclonal mouse anti-rat T1α antibody bound to pan-mouse IgG Dynabeads (see MATERIALS AND METHODS) and released from Dynabeads for immediate cell lysis and Western blot. Exp#3.2 is an additional ATI cell release step that was only performed in exp#3. Cells not bound to T1α antibody were collected as ATII cells and used for Western blot immediately; 30 μg cell lysate was loaded and 10 μg rat lung homogenate was included as a positive control. B: Immunostaining of proSP-C (magenta) on ACID:mT/mG lung slides where ATI cells express GFP (green). C: Immunostaining of TRIM72 (magenta) on ACID:mT/mG lung slides. D: Coimmunostaining of TRIM72 (green) and T1α (red) on WT lung slides. E: Coimmunostaining of TRIM72 (red) and proSP-C (green) on WT lung slides. Images are captured at ×40 on an Olympus FV1000 confocal microscope. Arrows, TRIM72 in ATI cells; arrowheads, TRIM72 in ATII cells; asterisk, alveolar macrophages.
jured alveolar resident cells in ventilated TRIM72KO lungs than in WT lungs (0.63 ± 0.12 vs. 0.32 ± 0.07, P < 0.05) (Fig. 4, A and B). As postulated, we observed significant reduction in the number of irreversibly wounded and presumably necrotic alveolar pneumocytes (0.07 ± 0.01, P < 0.05 compared with WT) in the TRIM72KO lungs after high tidal volume ventilation (Fig. 4, A and B). This indicates that TRIM72 plays an essential role in repair of the lung cells and that overexpression of TRIM72 provides a protective effect for lung cells from fatal injury resulting from deformation stress. We did not observe histological signs of severe lung injury such as hyaline membrane formation, whereas alveolar hemorrhage, mild thickness of alveolar septa, and neutrophil recruitment can be sporadically seen in the ventilated groups (Fig. 4C), indicating that transient deformating stress is sufficient to injure lung resident cells, which is an early event of lung injury prior to systematic signs of severe lung injury (30, 69).

**TRIM72 promotes plasma membrane repair and survival of alveolar epithelial cells.** Membrane protective molecules can potentially improve resealing of already disrupted plasma membrane or prevent membrane disruption from occurring under deformation stress, both leading to increased cell integrity and cell survival. Distinguishing these possibilities is important for the understanding of the mechanisms of cell

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**Fig. 3. Construction of inducible TRIM72 overexpressor mice.** A: schematic diagram of the transgene construct (pTi-flag-trim72) and enzyme digestion method prior to pronuclear injection. Expression of FLAG-tagged TRIM72 is driven by a modified tet-responsive element (TRE) fused with a mini cytomegalovirus (CMV) promoter (PminCMV). Expression of a dual reverse tetracycline-controlled transactivator (rtTA) and transcriptional suppressor (tTS) cassette connected through an internal ribosome entry site (IRES) is driven by a separate CMV promoter (PCMV). XhoI/SpeI double digestion is performed to remove prokaryotic vector backbone including pUC vector origin of replication (pUC ori) and ampicillin resistance cassette (AmpR) and to linearize the transgene construct. B: screening of offspring for presence of transgene. Five of 48 offspring containing flag-trim72 in their genome were identified through PCR-based methods. Lines no. 11 and no. 41 (founders) were chosen for establishing the TRIM72OE line (left). Genotyping primers were tested for specificity and sensitivity (right). Primers 9 and 3 can semiquantitatively detect presence of transgene (0, 1, 10, and 50 copies of transgene mixed with genomic DNA). C: testing of transgene construct for inducibility. pTi-flag-trim72 plasmid was transfected into HEK293 cells. 2 μg/ml doxycycline (Dox) induced robust TRIM72 expression in transfected cells (pTi-flag-trim72/Dox). Minimal leakage was observed in transfected cells without Dox treatment (pTi-flag-trim72/Dox). D: expression of flag-trim72 was induced in mice by intraperitoneal injection of 50 mg/kg bw 9-tert-butyl-doxycycline (9-tert Dox) for 5 days. Lung tissues from the induced mice were homogenized for detection of transgene expression by anti-TRIM72 and anti-FLAG antibodies. β-Actin was used as a loading control.
wounding and repair and also helps to judge the efficacy of membrane protective measures in preventive vs. postinjury treatment. To determine through which process TRIM72 protects the alveolar resident cells, we isolated primary alveolar epithelial cells from WT and TRIM72KO mice (Fig. 5A). Cell monolayers in culture were injured by glass bead agitation (54, 55) in the presence of a FITC-conjugated macromolecule, FITC-dextran. PI was added to the cell culture supernatant after the beads were removed and plasma membrane integrity had been reestablished. This method (4) enables discrimination between 1) cells that are capable of resealing and trapping FITC-dextran in their cytosol while excluding PI, “repaired cells” (green); 2) uninjured cells, not stained, and 3) mortally wounded cells that accumulate PI (red)flu. In agreement with our in vivo finding, WT cells were significantly more likely to repair membrane defects caused by glass bead agitation compared with TRIM72KO cells (57.0 ± 3.0 vs. 20.5 ± 2.8%, P < 0.05, Fig. 5, B and C). Importantly, the WT and TRIM72KO groups had the same number of total wounded cells upon glass bead injury (sum of PI+ and FITC-dextran+ cells) (22 ± 3 and 22 ± 2 per field, respectively; Fig. 5D), suggesting that the presence or absence of TRIM72 made no difference in susceptibility to mechanical injury in vitro. Thus TRIM72-mediated membrane resealing is the major process by which TRIM72 promotes cell integrity and viability under mechanical stress.

TRIM72 interacts with caveolar protein caveolin 1. Although the exact molecular events during membrane resealing are not clear, coordination of a membrane repair molecule team is required for successful repair (51). Cav1, the predominant caveolar protein in the lung (41), has been generally regarded as a membrane repair molecule (17). Here we examine the possible link between TRIM72 and Cav1, the major type of caveolar protein present in ATI, endothelial cells, and fibroblasts of the lungs (41). As shown in Fig. 6A, we detected a significant reduction in Cav1 expression in the lung tissues from the TRIM72KO mice compared with the WT lungs. In Cav1KO lung tissue, the expression of TRIM72 was also reduced to 33.2% that of the WT lung (Fig. 6, A and B). We speculate that the transcriptional regulation processes of these two proteins are linked. In addition, we performed coimmunoprecipitation assays using HA-tagged TRIM72 and GFP-tagged Cav1. Our results suggest that a direct physical interaction in cells between TRIM72 and Cav1 may be of physiological significance (Fig. 6C).

To further determine the dynamic interaction between TRIM72 and Cav1 in living cells, we cotransfected fluorescence protein-tagged Cav1 and TRIM72 into HEK293 cells. As shown in Fig. 6D (top left), GFP or RFP alone distributed evenly throughout the cytosol. However, exogenous TRIM72 localized predominantly near or on the cell membrane and was associated with enhanced “dendrite” formation through unclear mechanisms (9) (Fig. 6D, top, middle, and right). On dishes cotransfected with Cav1 and TRIM72, cells that were only positive for Cav1-GFP (Fig. 6D, bottom, white arrows) showed Cav1-GFP distribution in a punctate pattern at the plasma membrane as well as in intracellular organelles, presumably Golgi apparatus (16, 73). Interestingly, in cells labeled with both TRIM72 and Cav1 (Fig. 6D, bottom, red arrows), Cav1 expression at the plasma membrane was greatly enhanced, supporting an endogenous interaction of Trim72 and Cav1 inside the cell. This observation builds on previous results showing retention of TRIM72 in the Golgi network in C2C12
myogenic cell line by overexpressing P104L Cav3 mutant (10). We speculate that TRIM72 facilitates membrane targeting of Cav1 on the basis of ectopic expression results (Fig. 6D). We believe this interaction is functionally significant and that it speaks to a role of TRIM72 in caveolar endocytosis for repair of alveolar cells. However, it is unlikely that Cav1 is another substrate for the E3 ligase activity of TRIM72 (63, 74) since synchronized expression of these two was seen in the lung tissue (Fig. 6, A and B).

**DISCUSSION**

The main finding in this report is the identification of TRIM72 in lung epithelial cells (Fig. 1) and the characterization of its role as a facilitator of membrane repair in them. We show that TRIM72KO mice subjected to high tidal volume ventilation exhibit a greater number of dying or dead alveolar resident cells than the WT, while TRIM72OE mice are relatively resistant to injurious ventilation (Fig. 4). Comparison of wounded and repaired alveolar epithelial cells in monolayer cultures suggests that TRIM72 is essential for the membrane repair process in alveolar epithelial cells (Fig. 5). In addition, we show that TRIM72 interacts with Cav1 and enhances the membrane distribution of Cav1 (Fig. 6).

Early investigations showed that although lung epithelium and endothelium have considerable compensatory capacity in response to volume change of the lung, cell breakage does occur at higher lung parenchymal strains (18, 28). Loss of cellular function and integrity of the lung epithelium and endothelium are associated with increased vascular permeability, compromised alveolar fluid clearance, and initiation of proinflammatory signaling cascades, contributing to clinical pathologies of ARDS (1, 49, 72). Despite the recognition of the clinical relevance of biophysical lung injury mechanisms, the molecular mechanisms of lung cell wounding and repair, especially at single cell level, are largely unknown.

Previous studies show that in skeletal muscle cells, breakage of plasma membrane causes quick accumulation of TRIM72 at the injury site, accompanied by reduced extracellular dye penetration into the cell, suggesting formation of a repair patch by TRIM72 (8, 9). In this study, using PI in a in vivo lung injury model (76), we showed that trim72 ablation led to increased death of lung cells following injurious ventilation while trim72 overexpression had a protective effect (Fig. 4), suggesting that TRIM72 protects lung cells from deformation induced membrane stress failure. Importantly, in vitro cell wounding assays show that trim72 ablation caused significant
Fig. 6. TRIM72 interacts with caveolar protein Cav1. A: Western blot of TRIM72 and caveolin 1 (Cav1) in lung tissues harvested from WT, TRIM72KO, and Cav1KO mice by using polyclonal rabbit antibodies against TRIM72 and Cav1; 30 μg protein was loaded per lane and β-actin was used as a loading control. B: quantification of Cav1 and TRIM72 expression in WT, TRIM72KO, and Cav1KO lungs. Gray values of target proteins were normalized to β-actin, n = 3–4, *P < 0.05 compared with WT. C: coimmunoprecipitation of TRIM72-HA and GFP-Cav1. HEK293 cells were transfected with TRIM72-HA and GFP-Cav1 by use of Xfect transfection reagent and harvested after 72 h. GFP-Cav1 and TRIM72-HA in immunocomplex can be captured by either monoclonal antibody to HA (left) or polyclonal antibody to GFP (right). D: fluorescent images of HEK293 cells cotransfected with: empty GFP and RFP vector (GFP:RFP), GFP-TRIM72 and RFP vector (GFP-TRIM72:RFP), and GFP-Cav1 and TRIM72-RFP (GFP-Cav1:TRIM72-RFP). At 48 h after transfection, images were taken on Visitech Infinity 3 confocal microscope at wavelengths of 488 and 561 nm at ×40. In the GFP-Cav1:TRIM72-RFP group, white arrows are GFP-Cav1-positive cells and red arrows are GFP-Cav1 and TRIM72-RFP double-positive cells. G:R: green/red fluorescent overlay image; G:DIC, green/DIC overlay image; R:DIC, red/DIC overlay image.
defects in membrane resealing without altering the cell’s resistance to deformation stress (Fig. 5). These results support the idea that prevention of membrane stress failure and membrane repair following injury may proceed by two distinct mechanisms and that TRIM72 protects lung epithelial cells from fatal injury mainly by facilitating active repair. Nevertheless, we did not observe macroscopic pulmonary phenotypes at resting condition in trim72 defective mice for up to 12 mo of age. An attractive theory is that aberrant membrane repair, as in the case of trim27 ablation, may lead to escalating microscopic pulmonary fibrosis at the local area (6), which needs to be investigated by careful characterization of lung fibrosis and morphometric techniques.

We also identified a physical and physiological interaction between TRIM72 and Cav1 (Fig. 6), analogous to the association between TRIM72 and Cav3 in skeletal muscle (10). Caveolins are the major types of membrane proteins in caveolae that mediate receptor-independent endocytosis (84). There are three known isoforms of caveolins. Cav1 and Cav2 often coexpress in many tissues including the lung (41) whereas Cav3 is skeletal muscle specific (84). The role of caveolins in tissue injury has been recognized, and it was shown that mutation of Cav3 led to multiple forms of myopathy (32, 58). Drab et al. (25) and Razani et al. (70) generated Cav1-/- mice and observed major histological abnormalities in lung tissue. Specifically, the Cav1-/- lungs had thickened alveolar walls filled with extracellular fibrillar deposits, irregular alveolar space, and hypercellularity. Corrotte et al. (17) showed that Cav1 is essential for repair of plasma membrane wounds introduced by pore-forming toxin in kidney epithelial cells. Wang et al. (80) show that caveolar endocytosis plays a key role in hypertonic solution-mediated facilitation of ATI cell repair. The physical interaction between TRIM72 and caveolin isoforms raises the intriguing possibility that TRIM72 may be involved in caveolar endocytosis, which is a critical event in the repair processes of fibroblasts and epithelia, including ATI cells (17, 80). Our data also show that this interaction led to enhanced membrane distribution of Cav1 in TRIM72 and Cav1 coexpressed cells (Fig. 6D), which builds on previous results showing retention of TRIM72 in the Golgi network in C2C12 myogenic cell line by overexpressing Cav3 mutant (10). It is possible that TRIM72 and Cav1 interaction is required for plasma membrane targeting and hence proper function of these two molecules, which need to be confirmed in follow-up studies.

We note with interest that ATII cells lack caveolae (19), repair poorly ex vivo (35a), and are thought to be protected from deformation injury in situ by virtue of their location in alveolar corners. Thus we speculate that active in situ membrane repair mediated by TRIM72 may occur preferentially in ATI cells owing to the lack of additional repair partners in ATIIIs, whereas cell proliferation is in place to replenish lost ATII cells (20). The presence of TRIM72 in ATII cells raises the possibility that TRIM72 is involved in other physiological processes such as cell differentiation (11) and surfactant secretion (13). Indeed, TRIM proteins have been shown to participate in a variety of biological processes such as host defense (67), acid secretion (64), and glucose metabolism (74). TRIM72, specifically, was recently identified as an E3 ligase for the ubiquitination of insulin receptor substrate 1 (IRS1) (74, 86) and in a separate report for focal adhesion kinase (FAK) (63). Although abnormal ubiquitination of caveolin was indicated in development of myopathies (31), we do not have evidence for whether the E3 ligase activity of TRIM72 plays a significant role in regulating Cav1 expression in the lung or repair of lung cells, since loss of TRIM72 expression was associated with reduced Cav1 expression and vice versa (Fig. 6, A and B).

The limitations of the present study are that: first, our in vivo ventilation protocol is relatively short for the introduction of any macroscopic/physiological lung injuries, i.e., bronchoalveolar protein, edema, and poor oxygenation, etc. Rather, we just established the link between TRIM72 and cytopathological indication of cell wounding, whereas the overall benefits of modulating TRIM72 for the macroscopic pathologies of ARDS need to be further investigated. Second, it is known that both epithelial and endothelial lining cells are injured in ARDS (26) and ATI and ATII cells repair differently in vitro (35a). Here we did not examine the injured cell types in the in vivo lung injury model and used mixed alveolar epithelial cells in the in vitro cell injury model. So, this study simply highlights the protective role of TRIM72 in alveolar epithelial cells because this is an important event for the pathogenesis of VALI. The significance of this study is that, although there have been intensive efforts to unravel the genomic and proteomic responses of lung cells and tissues to deforming stress for development of novel therapies to improve the phenotype of VALI (14, 43, 62, 69), none of these interventions to date has been clinically shown to improve patient outcomes (71). This study opens up new avenues for targeting the repair process as an attractive therapeutic option for its broad indication, targeting of an early event in lung injury, and plausible postinjury efficacy. Such mechanisms may also be relevant for lung cell injury caused by other biological or mechanical insults in ARDS, including inflammation and atelectrauma since plasma membrane stress failure and cell death are commonly indicated in these cell injury modes (75, 85), and thus it deserves subsequent investigation in other lung injury models.

In summary, we have shown that TRIM72 is a part of the cell membrane wound repair proteome and that TRIM72 deficiency states are associated with alveolar epithelial cell death in lungs subjected to hyperventilation. This study revealed that a novel membrane repair molecule TRIM72 is present in the lung and is required for effective repair of alveolar epithelial cells. Our results demonstrating membrane repair mechanisms in lung epithelial cells lay the foundation for attenuating lung injury in acute and chronic lung diseases through targeting membrane repair pathways.

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DISCLOSURES

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

S.C.K., T.K., S.W., M.N., N.N., B.Z., P.F., and X.Z. performed experiments; S.C.K., T.K., S.W., M.N., K.S., H.T., and X.Z. analyzed data; S.N.G., H.T., R.D.H., and X.Z. interpreted results of experiments; all authors edited and reviewed manuscript; R.D.H. and X.Z. oversaw conception and design of research and drafted manuscript; X.Z. approved final version of manuscript.

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