Tropomyosin 3.1 Association With Actin Stress Fibers is Required for Lens Epithelial to Mesenchymal Transition

Justin Parreno,[1,2] Michael B. Amadeo,[1] Elizabeth H. Kwon,[1] and Velia M. Fowler[1,2]

[1]Department of Molecular Medicine, The Scripps Research Institute, La Jolla, California, United States
[2]Department of Biological Sciences, University of Delaware, Newark, Delaware, United States

Correspondence: Justin Parreno and Velia M. Fowler, Department of Biological Sciences, University of Delaware, 105 The Green, Newark DE 19702, USA; jparreno@udel.edu; vfowler@udel.edu.

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Purpose. Epithelial to mesenchymal transition (EMT) is a cause of anterior and posterior subcapsular cataracts. Central to EMT is the formation of actin stress fibers. Selective targeting of actin stress fiber-associated tropomyosin (Tpm) in epithelial cells may be a means to prevent stress fiber formation and repress lens EMT.

Methods. We identified Tpm isoforms in mouse immortalized lens epithelial cells and epithelial and fiber cells from whole lenses by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) followed Sanger sequencing. We focused on the role of one particular tropomyosin isoform, Tpm3.1, in EMT. To induce EMT, we treated cells or native lenses with TGFβ2. To test the function of Tpm3.1, we exposed cells or whole lenses to a Tpm3.1-specific chemical inhibitor, TR100, as well as investigated lenses from Tpm3.1 knockout mice. We examined stress fiber formation by confocal microscopy and assessed EMT progression by analysis of alpha-smooth muscle actin (αSMA) mRNA (real-time RT-PCR), and protein (Western immunoassay [WES]).

Results. Lens epithelial cells express eight Tpm isoforms. Cell culture studies showed that TGFβ2 treatment results in the upregulation of Tpm3.1, which associates with actin in stress fibers. TR100 prevents stress fiber formation and reduces αSMA in TGFβ2-treated cells. Using an ex vivo lens culture model, TGFβ2 treatment results in stress fiber formation at the basal regions of the epithelial cells. Genetic knockout of Tpm3.1 or treatment of lenses with TR100 prevents basal stress fiber formation and reduces epithelial αSMA levels.

Conclusions. Targeting specific stress fiber associated tropomyosin isoform, Tpm3.1, is a means to repress lens EMT.

Keywords: cataracts, fibrosis, epithelial-mesenchymal transition, actin, lens

The transition of lens epithelial cells into myofibroblasts, through the process of epithelial to mesenchymal transition (EMT), is a cause of anterior subcapsular and posterior capsular cataracts.[1–4] In EMT, myofibroblasts synthesize excessive fibrous matrix and contractile proteins, such as actin and myosin, that assemble into stress fibers, ultimately leading to loss of lens architecture and light occlusion.

The reorganization of actin into stress fibers is central to EMT. Stress fibers are thick bundles of polymerized actin that not only regulate migration, contraction, and matrix remodeling but also influence the expression of myofibroblast molecules, such as alpha-smooth muscle actin (αSMA).[5,6] Thus, targeting actin stress fibers may be a means to repress EMT. Yet, how to target actin stress fibers during lens EMT remains unclear.

The tropomyosins (Tpms) are a family of proteins that bind and stabilize actin filaments (F-actin). The Tpms are highly conserved with over 40 nonredundant mammalian isoforms generated from alternative splicing of 4 Tpm genes: Tpm1, 2, 3, and 4 (Fig. 1A). Each isoform has a unique interaction with actin filaments, as well as a specific ability to coordinate the binding of other proteins to actin. Thus, the specific expression pattern of Tpms within a cell defines F-actin organization.[7]

Several Tpms associate with F-actin in stress fibers.[8–13] In osteosarcoma (U2OS) cells, there are six known stress fiber-associated Tpm isoforms: Tpm1.6, Tpm1.7, Tpm2.1, Tpm3.1, Tpm3.2, and Tpm4.2.[12,13] Each isoform plays a distinct role in regulating F-actin in stress fibers by coordinating interactions with specific F-actin-binding proteins, such as non-muscle myosins, F-actin cross-linking, bundling and severing proteins, or actin assembly nucleating proteins. Elimination of any of the stress fiber-associated Tpms can compromise stress fiber formation.[12]

Previous studies in the lens have implied a role for Tpm1 and Tpm2 genes in cataract formation[14,15]; however, the specific isoform(s) involved remains elusive. The study of specific Tpm isoforms can be challenging. Tpm antibodies are generated using peptides corresponding to specific exons, which are highly conserved between the various Tpm isoforms.[16] Thus, antibodies are not specific to one Tpm isoform, but rather to a specific exon. Previous investigations into the role of Tpms in cataractogenesis used the commercially available Tpm antibody, TM311,[14,15] which recognizes an epitope in exon 1a of Tpm1 and Tpm2.[16] Thus, TM311

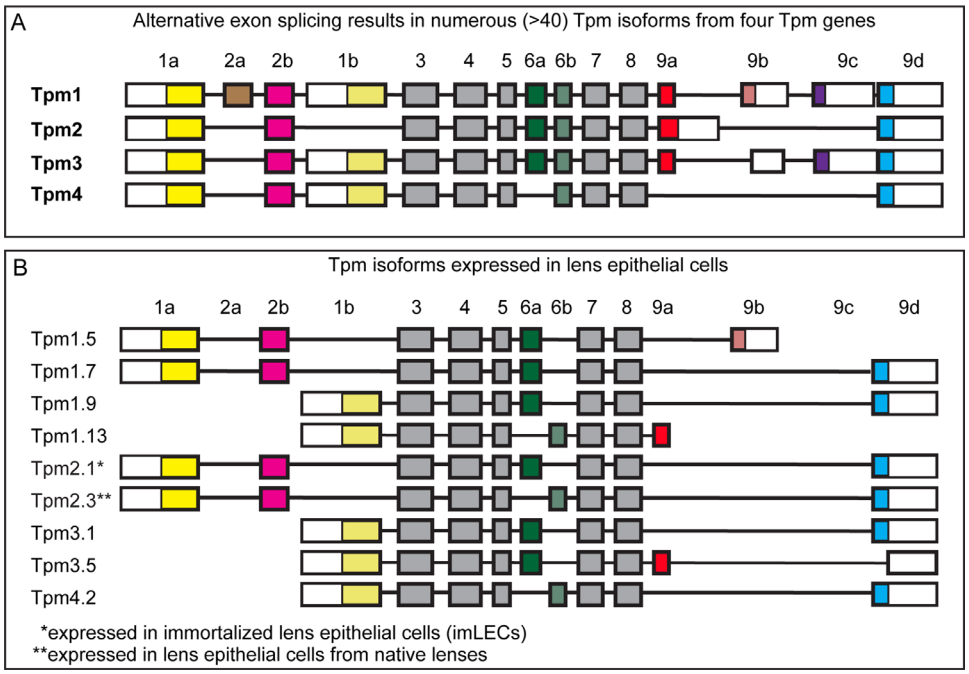

Figure 1. Diagram of Tpm isoforms. (**A**) Alternative exon splicing of four Tpm genes can result in the generation of over 40 Tpm isoforms.[7] In this diagram, grey exons are constitutively expressed by the indicated Tpm gene, colored exons are known to be alternatively spliced, and white boxes indicate noncoding transcript regions.[53] (**B**) Diagram of Tpm isoforms found to be expressed in mouse lens epithelial cells.

recognizes several Tpm isoforms: Tpm1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.10, 2.1, 2.2, and 2.3. Additional studies are required to identify which Tpm isoforms are present in lens epithelial cells and which specific isoforms contribute to lens EMT.

In this study, we clarify the specific isoforms expressed by lens epithelial cells by systematically performing semi-quantitative, reverse transcription-polymerase chain reaction (RT-PCR) analysis of full-length mRNA from Tpms1 to 4, followed by sequencing the RT-PCR products. This strategy previously allowed us to determine specific Tpms expressed in whole lenses.[17] By using this strategy, we identified eight Tpm isoforms enriched in mouse lens epithelial cells in culture and in whole lenses. We showed that Tpm3.1 is the most highly upregulated isoform upon TGFβ2–stimulation of mouse lens epithelial cells. Reduction of Tpm3.1 activity with a small molecule chemical inhibitor, or by genetic deletion revealed that Tpm3.1 plays a critical role in stress fiber formation during EMT in the mouse lens. Our strategy to identify and inhibit the stress fiber associated Tpms may be broadly applicable in targeting stress fibers to repress EMT in cataracts.

Materials and Methods

Cell Culture

Immortalized mouse lens epithelial cells (imLECs)[18] were a gift from Dr. Xiaohua Gong (University of California Berkeley). The imLECs were derived from mouse lens epithelial cells, dissected from 10-day-old mice. The lens epithelial cells were immortalized by infection with recombinant retrovirus expressing oncogenes E6E7 and *H-Ras*V12. These cells retain high-proliferation ability for more than 20 passages. ImLECs were routinely passaged in growth media, which consisted of Dulbecco's Modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, PA, USA) supplemented with 10% fetal bovine serum (FBS; GenClone, Genesee Scientific, San Diego, CA, USA) and 1% penicillin-streptomycin, at 37°C and 5% CO_2. Cells were cultured until 70% to 90% confluency, at which point cells were dissociated from culture vessels using 0.05% Trypsin-EDTA (Thermo Fisher Scientific) and reseeded onto new culture vessels for subsequent passaging. The imLEC were maintained up to 15 passages.

Cells were seeded at an approximate density of 2.5 $\times$ 10^4 cells/cm^2 onto either tissue culture polystyrene or glass-bottomed dishes in complete media for experiments. After 24 hours, the cells were placed in serum-reduced media (DMEM supplemented with 0.5% FBS). After an overnight incubation, the media was replaced with fresh serum-reduced media supplemented with 5 ng/mL TGFβ2 (Thermo Fisher Scientific; PHG9114) in the presence or absence of Tpm3.1 inhibitor, TR100. The concentration of 5 ng/mL TGFβ2 was used as it was determined to be optimal for the upregulation of myofibroblast markers, αSMA, and alpha-1 type I collagen (col1a1) mRNA, in the imLECS (data not shown).

Cell Viability Assays

Following 24 hours of TR100 treatment, cell viability was measured. Adherent imLECs were detached from culture dishes using 0.05% Trypsin-EDTA and then centrifuged. After pelleting, the cells were resuspended in fresh media, and an aliquot was mixed in a 1:1 ratio with Trypan blue. Cell counts were conducted on a TC20 automated cell counter (Bio-Rad). Cells treated with <5 µM of TR100 remained adherent. However, the treatment with higher concentrations (>5 µm) of TR100 led to cell detachment. To count floating cells, the media that contained the cells was

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harvested and centrifuged. Pelleted cells were resuspended in fresh media, and an aliquot was mixed in a 1:1 ratio with Trypan blue. Approximately 94.1% and 100% were floating following treatment with 10 µM or 20 µM TR100, respectively. The vast majority of floating cells (>87.4%) were not viable (Trypan blue positive). Total percent viability was calculated by dividing the sum of live cells in floating and adherent fractions by the total amount of (live and dead) cells in both the floating and adherent fractions.

Cell viability was also assessed visually by staining cells cultured on glass dishes with 1µM of Calcein-AM (BioVision, Milpitas, CA, USA) and TO-PRO3 (Thermo Fisher Scientific) to determine live and dead cells, respectively. Cells were stained for 20 minutes, followed by 3 washes in phosphate-buffered saline (PBS), and then imaged using a Zeiss LSM 880 confocal fluorescence microscope (20× 0.8 NA objective lens, 1× zoom).

Mice and Lens Dissections

Wild-type and *Tpm3/*Δ*exon9d*$^{-/-}$ mice between the ages of 8 and 10 weeks in the C57BL/6J background were used for experiments. The *Tpm3/*Δ*exon9d*$^{-/-}$ mouse strain has been previously described[19] and was a generous gift from Dr. Peter Gunning (University of New South Wales). Mice were backcrossed to wild-type C57BL/6J to generate congenic strains. All animal procedures were conducted in adherence to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and performed in accordance with approved animal protocols from The Scripps Research Institute and the University of Delaware.

Following euthanasia, eyes were enucleated, and lenses were dissected as previously described.[20] Lenses were immediately placed in medium 199 (Thermo Fisher Scientific) supplemented with 1% penicillin-streptomycin. EMT was stimulated by placing the lenses in 1 mL of medium 199 containing 5 ng/mL TGFβ2 and incubating at 37°C in a CO_2 incubator for 2 to 4 days, as indicated in the figures.

RT-PCR Gene Expression Analysis and Sequencing

To isolate RNA from imLECs, cells within one well of a six-well plate were homogenized in 1 mL of TRIzol reagent (Thermo Fisher Scientific) and stored at −80°C until further processing. To isolate RNA from lens epithelium, lens epithelium was manually dissected from the fiber cells. To achieve this, lens capsules were punctured at the lens equator using fine forceps. With the second set of fine forceps, the lens capsules were peeled from the fiber cell mass. At least six pooled lens capsules, which contained mostly epithelial cells, were immersed in 300 µL of TRIzol. In a separate tube, lens fiber masses from two lenses were placed in 300 µL of TRIzol.

RNA from tissue or cells were then processed according to the manufacturer's instructions. Reverse transcription was performed on equal amounts of total RNA for each sample using Superscript III First-Strand Synthesis System for RT-PCR Kit (Thermo Fisher Scientific).

Semi-quantitative RT-PCR was performed using Hot-Start Taq Blue Mastermix (Thomas Scientific, Swedesboro, NJ, USA) on equivalent amounts of cDNA in a 20 µL reaction and using previously validated mouse Tpm primers.[17] Equal volumes of PCR product were loaded for electrophoresis on a 1% agarose gel. Under ultraviolet illumination, PCR bands were cut from gels. Products were purified using a gel extraction kit (QIAquick; Qiagen, Germantown, MD, USA) and submitted for Sanger sequencing (Genewiz, La Jolla, CA, USA).

Relative real-time RT-PCR was performed using PowerSYBR Green PCR Master Mix (Thermo Fisher Scientific) in a 25 µL reaction according to manufacturer's directions, using a CFX-96 real-time system (Bio-Rad). Real-time PCR primers were developed using National Center for Biotechnology Information (NCBI) primer-blast. Primers for mouse αSMA (forward, 5'-TCACCATTGGAAACGAACGC-3'; reverse, 5'-CCCCTGACAGGACGTTGTTA-3') and Gapdh (forward, 5'-TTCGAGAGTCAGCCGCATTT-3'; reverse, 5'-ATCCGTTGACTCCGACCTTC-3') were based on NCBI reference sequences NM_007392.3 and NM_001289726.1, respectively.

Total Protein Extraction and WES Capillary-Based Immunoassay

Cells were washed once in PBS (1.1 mM KH_2PO_4, 155 mM NaCl, 3.0 mM Na_2HPO_4-$7H_20$; pH 7.4) and then harvested from culture by scraping into fresh PBS. Cells were pelleted by centrifugation at 800 × g for 5 minutes and protein was extracted by incubating pellets in 1× RIPA buffer (20 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM Na_2 EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3 VO_4, and 1 mg/mL leupeptin) with Protease Inhibitor Cocktail (1:100; P8430; Sigma-Aldrich, Saint Louis, MO, USA) for 30 minutes on ice. To extract protein from lens epithelium, lens epithelia were first mechanically separated from fibers as above. Each lens epithelium was placed in 10 µL of cold 1× RIPA buffer with Protease Inhibitor Cocktail. Extracts were incubated on ice for 30 minutes and then briefly sonicated.

Protein was quantified using bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific). Equal amounts of protein were prepared for capillary-based (Western [WES]) immunoassay using a 12-230kDa Separation module kit according to manufacturer's instructions (Protein Simple, San Jose, CA, USA). The concentrations of protein extract and antibodies used in experiments were in the dynamic range of protein level detection and were determined through empirical optimization. To detect αSMA protein levels, 1 mg/mL of protein extract was loaded onto WES plates and probed with mouse anti-αSMA antibody (1:50, clone 14A, ab7817; Abcam, Cambridge, MA, USA). To detect Tpm3.1 protein levels, 2 mg/mL of protein extract was loaded onto WES plates and probed using anti-tropomyosin-5 (1:300, clone 2G10.2, MAB2256; Sigma-Aldrich). The total protein of samples loaded into capillaries was detected using a Total Protein Separation module kit (Protein Simple). Protein expression of specific molecules was normalized to total protein. WES data are presented in electropherogram format and as virtual blots, which are scan intensity traces converted to image densities to resemble a standard Western blot of an SDS gel.

Triton X-100 Fractionation of imLECs

Triton X-100 soluble and insoluble proteins were separated as previously described with slight modifications.[21,22] Briefly, imLECs cultured in 6-well dishes were washed once in PBS and then incubated in 72 µL/well of Triton-extraction

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buffer (0.1% Triton X-100, 100 mM NaCl, 3 mM $MgCL_2$, 300 mM sucrose, 1 mM EGTA, 10 mM PIPES, and pH 6.8) with 1:100 Protease Inhibitor Cocktail for 2 minutes at room temperature. The Triton-extracted soluble portion was then pipetted into a 1.5 mL Eppendorf tube. Approximately 8 µL of cold 10× RIPA was then added to the solution. The remaining Triton-insoluble imLEC fractions were isolated by directly scraping into 80 µL of cold 1× RIPA buffer with 1:100 Protease Inhibitor Cocktail and pipetted into a separate 1.5 mL Eppendorf tube. Equal volumes were prepared for WES immunoassay, as above.

Immunostaining of imLECs

Cells cultured on glass-bottom dishes (World Precision Instruments, Sarasota, FL, USA) were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 1× PBS at room temperature for 10 minutes. Samples were then washed in PBS and kept at 4°C until further processing. For immunostaining, cells were permeabilized for 30 minutes in permeabilization/blocking solution (PBS containing 0.3% Triton, 0.3% bovine serum albumin, and 3% goat serum). Following permeabilization, cells were incubated with mouse anti-tropomyosin-5 antibody (1:200; clone 2G10.2; Millipore), which recognizes Tpm3.1 and Tpm3.2, or with mouse anti-αSMA antibody (1:200; clone 1A4; Abcam). Following overnight incubation at 4°C, cells were washed 3 times in PBS, 5 minutes per wash, and then incubated at room temperature for 1 hour in secondary antibody solution. Secondary antibody solution contained Alexa 488-conjugated anti-mouse antibody (1:200; Thermo Fisher Scientific), rhodamine-phalloidin (1:50; Thermo Fisher Scientific), and Hoechst 33342 (1:500; Thermo Fisher Scientific). Cells were washed and then mounted using ProLong gold anti-fade reagent (Thermo Fisher Scientific).

Immunostaining of Native Lens Epithelial Cells

For immunostaining of native lens epithelial cells, whole lenses were placed in individual wells of a 48 well plate and fixed by immersing in 4% paraformaldehyde in PBS at room temperature for 30 minutes. Fixed lenses were then washed three times in PBS (5 minutes per wash). To allow for the penetration of antibody, each lens was immersed in 0.2 mL (0.25%) collagenase A (Roche Diagnostics, Mannheim, Germany) solution (in PBS) in individual wells of a 48 well plate and incubated at 37°C. After 20 minutes of collagenase digestion, lenses were washed three times in PBS (5 minutes per wash) and placed in permeabilization/blocking solution at room temperature for 2 hours. Next, lenses were labeled with primary mouse anti-tropomyosin-5 antibody (1:100; clone 2G10.2) in permeabilization/blocking solution and incubated at 4°C overnight. Lenses were then washed three times in PBS and placed in permeabilization/blocking solution containing Alexa 488-conjugated anti-mouse secondary antibody (1:200), CF640 dye-conjugated to wheat germ agglutinin (WGA; 1:100; Biotium, Fremont, CA, USA), rhodamine-phalloidin (1:20), and Hoechst 33342 (1:500) at room temperature for 2 hours. Lenses were washed three times in PBS for 5 minutes before whole-mount imaging of the lens epithelium by confocal fluorescence microscopy, as previously described.[20]

Confocal Fluorescence Microscopy

Imaging was performed using a Zeiss LSM880 laser-scanning confocal fluorescence microscope (Zeiss, Germany) using a 20× 0.8 NA objective, a 63× 1.4 NA oil objective, or a 100× 1.4 NA oil objective. Z-stack images were acquired with a 0.3 µm step size. The zooms at which images were acquired are specified in the figure legends. Raw images were processed using Zen (Zeiss) software. Image line scan analysis was performed using FIJI software.

Statistical Analysis

Each experiment was replicated on at least three separate occasions. Statistical analysis was performed using GraphPad Prism7 (San Diego, CA, USA). Unpaired *t*-tests were used to detect differences between two groups of data. To determine differences between multiple groups of data, an analysis of variance followed by post hoc testing was performed. A Dunnett's post hoc test was used to determine the differences between several experimental groups against the control. A planned comparisons test with a Sidak's adjustment post hoc was used to measure differences between multiple groups of data.

Results

Tpm Expression in Immortalized Lens Epithelial Cells

To determine Tpm isoform expression in imLECs, we performed semiquantitative RT-PCR using previously validated primer sets.[17] The cDNA products were extracted from agarose gels and sequenced. We discovered that imLECs express eight Tpm isoforms (Fig. 1B and Fig. 2A): Tpm 1.5, 1.7, 1.9, 1.13, 2.1, 3.1, 3.5, and 4.2.

Next, we induced EMT by exposing imLECs to TGFβ2. We chose to treat cells with TGFβ2 as it is present in the aqueous humor,[23] it is increased in the aqueous humor following lens injury and inflammation,[24,25] and it is more potent than TGFβ1 in enhancing EMT.[26] Exposure of imLECs to 5 ng/mL TGFβ2 downregulated Tpm1.9 and Tpm1.13 mRNA levels, upregulated Tpm1.7, 2.1, 3.1, and 4.2 mRNA levels, but did not affect Tpm1.5 and Tpm3.5 mRNA levels (Figs. 2A, 2B).

Tpm1.7, 2.1, 3.1, and 4.2 are associated with actin in stress fibers in U2OS cells[12,13] and elimination of any of these Tpms can compromise stress fiber formation.[12] For the present study, we chose to further examine the role of Tpm3.1 as it is the most highly upregulated Tpm isoform in imLECs by TGFβ2 treatment. Furthermore, using WES immunoassay, we confirmed that TGFβ2 upregulates Tpm3.1 protein level in imLECs (Figs. 2C–E).

Pharmacological Tpm Inhibitor, TR100, Reduces the Assembly of Tpm3.1 Into F-actin Stress Fibers

To investigate the function of Tpm3.1, we exposed imLECs to TR100. TR100 is a small molecule Tpm inhibitor that associates with a binding pocket located in the 9D exon of Tpm3.1 and prevents end-to-end assembly of Tpm, reducing Tpm binding along actin filaments in cells.[27] To determine the optimal concentration of TR100, we assessed cell viability at different TR100 concentrations. Treatment of imLECs with 5 µM or 10 µM TR100 causes cell death. However, treatment with 1 µM or 2 µM of TR100 does not significantly impact imLEC viability (Figs. 3A, 3B). Based on these

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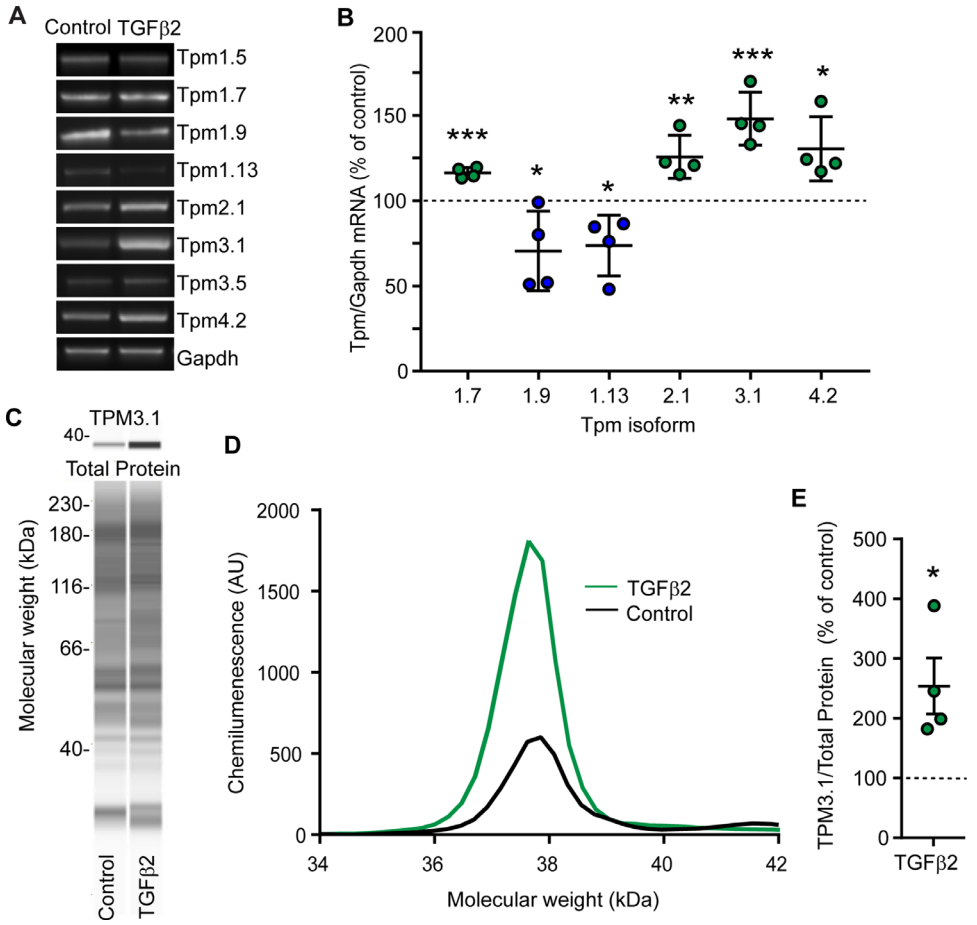

Figure 2. Lens epithelial cells express eight Tpm isoforms including Tpm 3.1, a stress fiber associated Tpm, that is upregulated by TGFβ2 treatment. (**A**) Semi-quantitative RT-PCR for Tpms in imLECs treated with TGFβ2 for 1 day, compared to untreated (control) imLECs. The bands in gels were excised followed by extraction of cDNA to confirm transcript identity by gene sequencing. (**B**) TGFβ2 modulates specific Tpm mRNA levels by downregulating Tpm1.9 and 1.13. TGFβ2 also upregulates known stress fiber associated Tpms: Tpm1.7, 2.1, 3.1, and 4.2, with the highest upregulation of Tpm3.1. (**C–E**) WES capillary-based immunoassay demonstrates increased Tpm3.1 protein level. Panel **C** shows a capillary western blot (WES) immunoassay virtual gel visualizing the band of Tpm3.1 (~36–38 kDa) and total protein (12–230 kDa). **D** Electropherogram of Tpm3.1 and **E** dot-plot of cumulative data points for Tpm3.1 protein level following TGFβ2 treatment. *P < 0.05; **P < 0.01; ***P < 0.001 as compared to control.

findings, we chose to use 1 µM of TR100 for subsequent experiments.

To determine if Tpm3.1 inhibition prevents stress fiber assembly, we exposed imLECs to TGFβ2 supplemented with TR100. As expected, the treatment of imLECs with TGFβ2 results in prominent F-actin assembly into stress fibers that are associated with Tpm3.1 (Fig. 3C). By contrast, TR100 treatment reduces the formation of stress fibers in TGFβ2-treated imLECs along with a substantially reduced association of Tpm3.1 along F-actin stress fibers. To confirm our immunocytochemistry findings, we used WES immunoassay to examine the proportion of Tpm3.1 protein co-sedimenting with the F-actin cytoskeleton after Triton X-100 extraction. We found that Tpm3.1 is present in both Triton-soluble and insoluble fractions of untreated cells (Figs. 3D, 3E) and that treatment with TGFβ2 results in a higher proportion of Tpm3.1 in the Triton-insoluble fraction, consistent with enhanced Tpm3.1 assembly with F-actin into stress fibers. Similar to the immunofluorescence staining results (Fig. 3C), treatment with TR100 reduces the proportion of Triton-insoluble Tpm3.1 in TGFβ2-treated cells (Figs. 3D, 3E). We conclude that selective inhibition of Tpm3.1 function impairs TGFβ2-induced stress fiber formation in imLECs.

TR100 Represses the Transition of imLECs Into Myofibroblasts

To determine if inhibition of Tpm3.1 could prevent EMT progression in imLECs, we examined the expression of αSMA, the hallmark of myofibroblasts.[28,29] Determination of αSMA mRNA levels using relative, real-time RT-PCR demonstrates that TGFβ2 treatment enhances αSMA mRNA levels 2.3-fold over untreated cells (Fig. 4A). These increases in αSMA mRNA levels can be repressed completely by the inclusion of TR100 during the TGFβ2 treatment. WES immunoassay (Figs. 4B–4D) also indicates that TGFβ2 treatment enhances αSMA protein levels 5.7-fold, an increase which is nearly entirely repressed by TR100 treatment. Immunostaining for αSMA reveals heterogeneity of imLECS in terms of αSMA assembly into F-actin within cells; treatment of TGFβ2 results in 49% of cells that incorporate αSMA into F-actin stress fibers (Figs. 4E, 4F). This incorporation of

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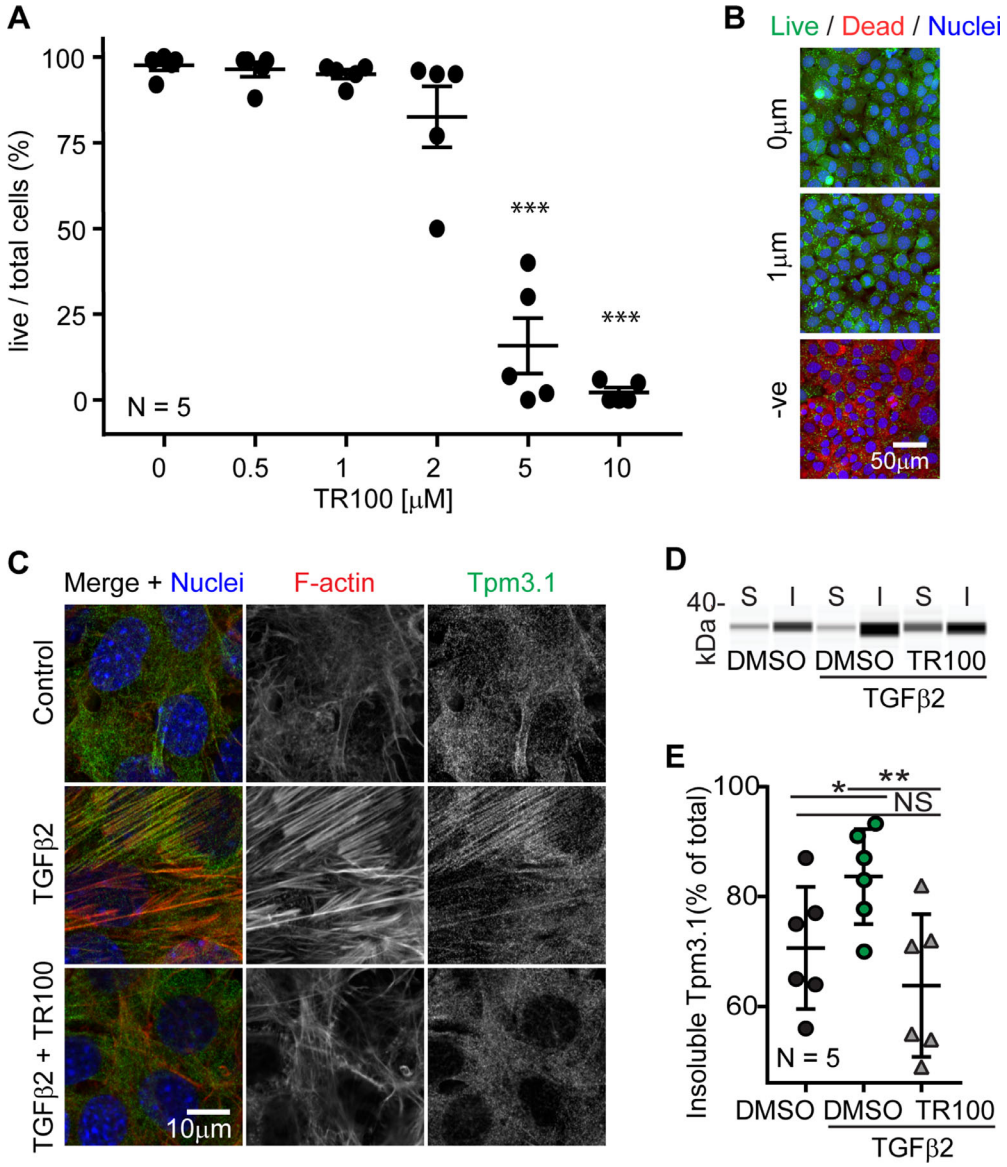

Figure 3. Using 1 μM of TR100 treatment reduces the induction of actin stress fibers by TGFβ2. (**A**) Dot plot showing the percentage of live cells following treatment with different concentrations of TR100. (**B**) The imLECs are viable following treatment with 1 μM of TR100 for 1 day as seen by positive staining for Calcein AM staining (live cells; green) and negative staining for TO-PRO-3 (dead cells; red). Cells fixed in 4% paraformaldehyde are used as a negative, dead cell control. Images were acquired using a 20× objective lens at 1× zoom and are single optical sections. (**C**) Immunostaining of imLECs for F-actin (red) and Tpm3.1 (green). TGFβ2 treatment leads to association of Tpm3.1 with F-actin and actin stress fiber formation. Inclusion of TR100 with TGFβ2 reduces Tpm3.1 association with F-actin and represses stress fiber formation. Images were acquired using a 63× objective lens at 1× zoom and are single optical sections. (**D**) WES capillary immunoassay virtual blot and corresponding (**E**) dot-plot showing an increase in Tpm3.1 in the cytoskeletal (Triton insoluble) fractions of imLECs. *P < 0.05; **P < 0.01; ***P < 0.001 as compared to DMSO (0 μM TR100) control.

αSMA into stress fibers by TGFβ2 is reduced by TR100 treatment. Thus, TR100 represses both αSMA mRNA and protein levels, as well as αSMA assembly into stress fibers, in TGFβ-treated imLECS and impairs EMT.

Native Mouse Lens Epithelial Cells Express Eight Tpm Isoforms Including Tpm3.1

To characterize the role of Tpm3.1 in a physiological model, we examined Tpm3.1 in the lens epithelia of native lenses. First, we determined Tpm isoform expression in lens epithelial cells and compared this to Tpm isoform expression in fiber masses, after separating lens epithelia from fiber masses before RNA extraction. Because there is approximately 6.3-fold less RNA in lens epithelium as compared to fiber masses from an individual lens (data not shown), we reverse-transcribed an equivalent amount of RNA from lens epithelium and fibers and then performed semiquantitative PCR followed by Sanger sequencing of cDNA products. Native mouse lens epithelial cells express eight Tpm isoforms (Fig. 5A and Fig. 1B). Five of the isoforms (Tpm1.5, 1.7, 1.9, 2.3, and 3.1) are detected solely in the lens epithelium but not in fiber cells, whereas three of the isoforms (Tpm1.13, 3.5, and 4.2) are expressed in both the lens epithelium and fiber cells. Tpm isoform expression of native

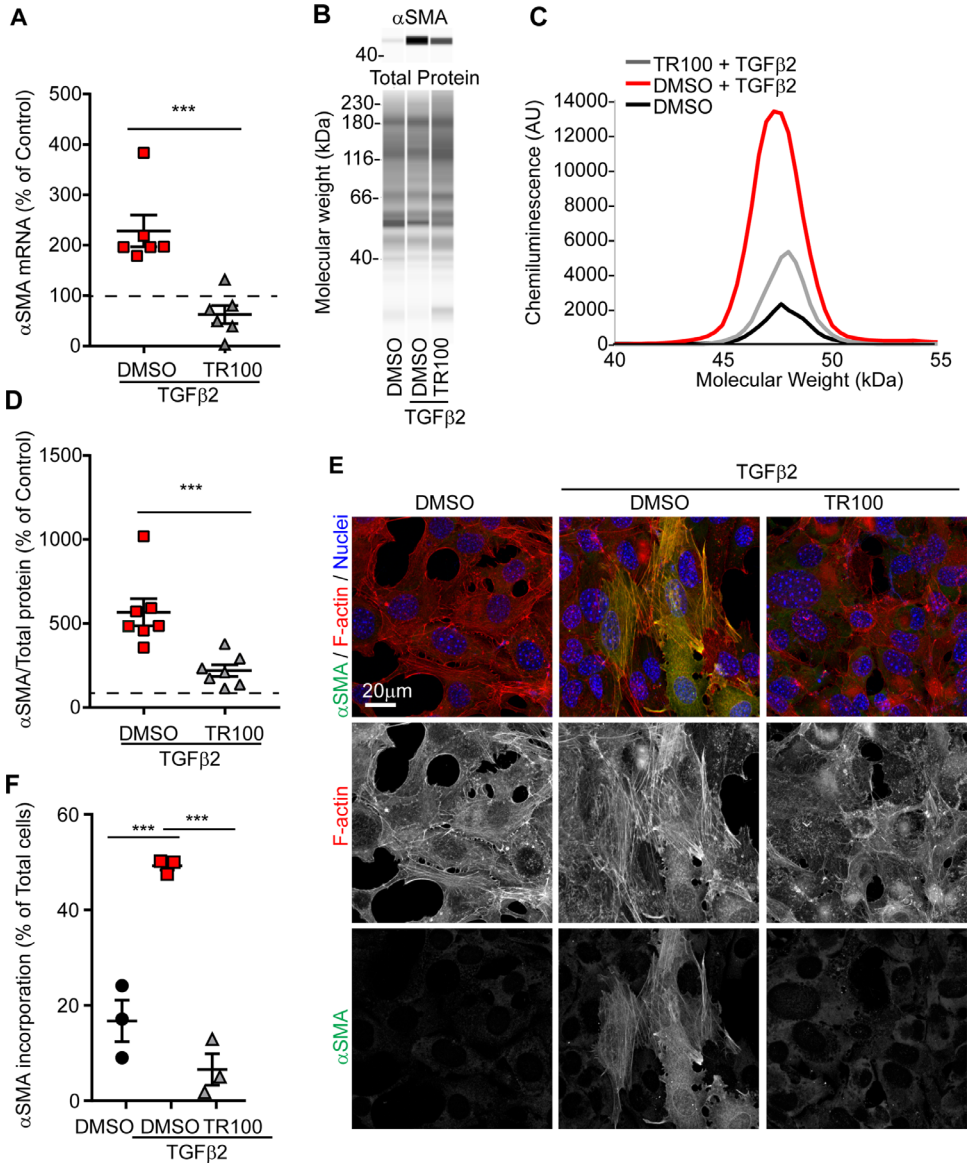

Figure 4. TR100 reduces αSMA expression in imLECs. (**A**) Relative real-time RT-PCR demonstrates that TR100 treatment represses the induction of αSMA mRNA levels by TGFβ2 after 1 day treatment. (**B–D**) WES capillary-based immunoassay demonstrates repression of αSMA Tpm 3.1 protein level by TR100. **B** Capillary western blot (WES) immunoassay virtual gel visualizing the band for αSMA (~47 kDa) as well as for total proteins (12–230 kDa). The corresponding electropherogram for αSMA is shown in **C**. Peak areas for αSMA and total protein were calculated. **D** Corresponding dot-plot showing that TR100 treatment represses the induction of αSMA protein levels after 2 days. (**E**) Immunostaining of imLECs for F-actin (red), αSMA (green), and nuclei (blue). Images were acquired using a 63× objective lens at 0.6× zoom and are single optical sections. (**F**) TR100 treatment reduces the proportion of cells that have αSMA incorporated within F-actin stress fibers as determined by counting of cells that have stress fibers containing αSMA. A total of 1642 cells were counted from three separate experiments that were performed on different occasions; $N = 3$. ***$P < 0.001$ as compared to DMSO + TGFβ2 treated.

lens epithelial cells differs from the imLECs by only one isoform (Fig. 1B); native lens epithelial cells express Tpm2.3 (Fig. 5A) whereas imLECs express Tpm2.1 (Fig. 2A). Of note, Tpm3.1 is expressed in native lens epithelial cells but not in lens fiber cells. The faint, slightly higher, cDNA band that appears in gels of the Tpm3.1 RT-PCR reaction for fiber cells is not Tpm3.1 based on Sanger sequencing.

To determine the presence and localization of Tpm3.1 protein within native lens epithelial cells, we developed a methodology to permeabilize the capsule of fixed lenses to allow fluorescent immunostaining of proteins in lens epithelial cells in whole lenses. Using this methodology, we imaged Tpm3.1 in epithelial cells of native lenses (Figs. 5B, 5C). The intensity of Tpm3.1 staining is highest at the basal regions of lens epithelial cells with minimal staining in the middle and apical regions. At the basal regions, there are no apparent stress fibers, and Tpm3.1 is localized in a punctate pattern partially colocalized with F-actin (Fig. 5B). The XZ plane reconstruction of confocal images shows that while F-actin staining is most intense in the apical region of epithelial cells, Tpm3.1 staining is most intense in the basal region of cells (Fig. 5C). This distribution of Tpm3.1 within cells was confirmed by a basal-to-apical line scan analysis of fluorescent intensity (Fig. 5D).

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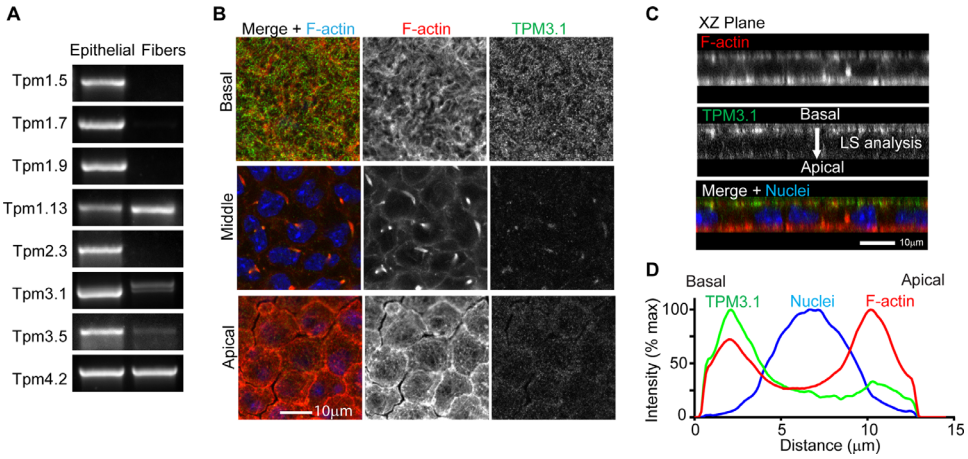

Figure 5. Tpm3.1 is expressed in native lens epithelial cells and associates with basal F-actin. (**A**) Semi-quantitative RT-PCR from equivalent amounts of RNA isolated from lens epithelial peels or fiber cells reveals the expression of eight Tpms in native lens epithelium, including the expression of Tpm3.1. (**B**) Whole mount images of single (*x,y*-plane view) optical sections of basal, middle, and apical regions of the native lens epithelium, showing F-actin (phalloidin; red), Tpm3.1 (green) and nuclei (Hoecsht; blue). Z-stack images were acquired using a 100× objective lens at 1× zoom. (**C**) Sagittal (*x,z*-plane view) optical sections from 3D reconstructions of confocal z-stacks showing Tpm3.1 (green) associates with F-actin (phalloidin; red) at the basal regions of cells. This was confirmed by (**D**) line scan analysis of images.

Tpm3.1 Knockout or TR100 Treatment Represses Stress Fiber Formation in Lens Epithelial Cells of Native Lenses

Next, we used an ex vivo culture model for EMT using whole lenses.[26,30] Whole organ culture of lenses is a useful tool to study lens EMT as the overall native three-dimensional architecture of the lens is preserved.[26,31] In this model, to stimulate EMT, we cultured mouse lenses in medium 199 in the absence and presence of TGFβ2 and compared to noncultured (control) lenses. After two days of culture, we determined the cellular region where F-actin stress fibers form during lens epithelial cell EMT using whole-mount confocal imaging of lenses stained with phalloidin. Lens culture in the absence of exogenous TGFβ2 led to the formation of some stress fibers at the basal regions of lens epithelial cells, which are absent in freshly isolated, uncultured lenses (Supplementary Fig. S1). However, lenses cultured in medium 199 supplemented with TGFβ2 led to considerably more stress fiber formation than lenses cultured in medium 199 alone. Thus, to stimulate EMT in subsequent studies, we cultured lenses in medium 199 supplemented with TGFβ2.

To study the role of Tpm3.1, we examined the actin stress fibers at the basal regions of lens epithelial cells in Tpm3.1 knockout (*Tpm3/*Δ*exon9d*$^{-/-}$) mice. Freshly isolated and fixed lenses from *Tpm3/*Δ*exon9d*$^{-/-}$ mice had similar actin organization to lenses from wildtype, *Tpm3/*Δ*exon9*$^{+/+}$ mice (Fig. 6; Supplementary Fig. S2). Notably, actin stress fibers are absent in the basal regions of the epithelial cells from either genotype (Fig. 6). Exposure of wildtype, *Tpm3/*Δ*exon9*$^{+/+}$ lenses to TGFβ2 led to the formation of actin stress fibers, as shown above (Supplementary Fig. S1). However, stress fiber formation at the basal region of epithelial cells in TGFβ2-treated *Tpm3/*Δ*exon9d*$^{-/-}$ lenses is repressed as compared to *Tpm3/*Δ*exon9*$^{+/+}$ lenses. By contrast, F-actin organization in the middle and apical region of *Tpm3/*Δ*exon9d*$^{+/+}$ and *Tpm3/*Δ*exon9d*$^{-/-}$ lens epithelial cells was similar following TGFβ2 treatment (Supplementary Fig. S2). Thus, knockout of Tpm3.1 represses TGFβ2 induced F-actin stress fiber formation at the basal region of epithelial cells.

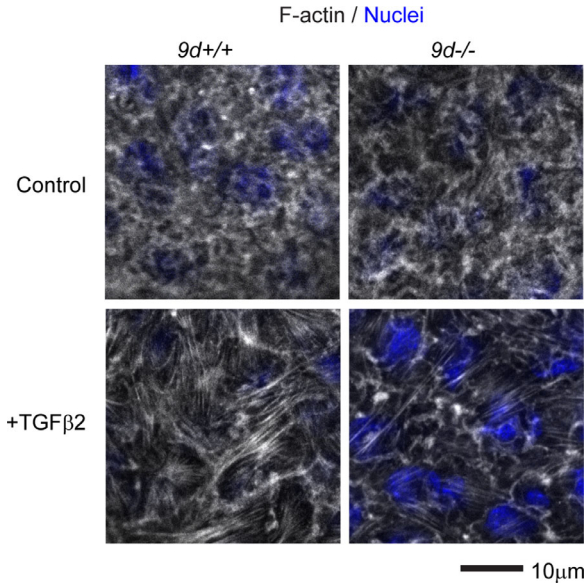

Figure 6. Stress fiber formation is repressed in TGFβ2-treated *Tpm3/*Δ*exon9d*$^{-/-}$ lenses. Whole mount images showing single (*x,y*-plane view) optical sections of basal regions of the lens epithelium in noncultured and lenses cultured with TGFβ2 for 2 days. Z-stack images were acquired using a 63× objective lens at 2× zoom. In noncultured lenses, F-actin staining (phalloidin; grayscale) is similar in *Tpm3/*Δ*exon9d*$^{-/-}$ (*9d-/-*) lenses as compared to *Tpm3/*Δ*exon9*$^{+/+}$ (*9d+/+*) lenses. However, stress fiber formation at the basal epithelial regions is repressed in *Tpm3/*Δ*exon9d*$^{-/-}$ (9d-/-) lenses as compared with *Tpm3/*Δ*exon9d*$^{+/+}$ (9D+/+) lenses following TGFβ2 treatment.

To determine if the Tpm3.1 inhibitor TR100 also prevents stress fiber assembly in native lens epithelial cells, we exposed whole wild-type lenses to TGFβ2 in the presence

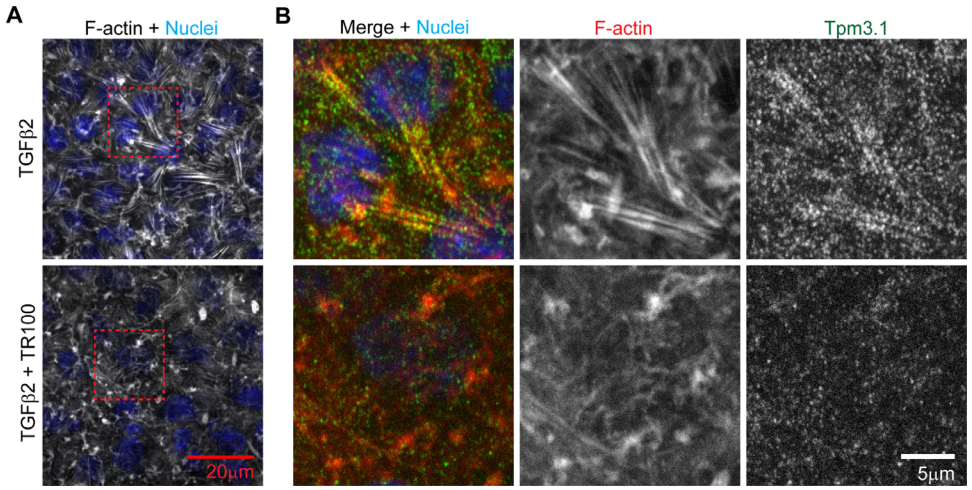

Figure 7. TGFβ2-induced stress fiber formation at the basal regions of native lens epithelial cells is repressed by TR100. Whole mount images of (**A**) single (*x,y*-plane view) optical section of the basal region of lens epithelial cells revealing that stress fiber formation is reduced in TGFβ2-treated lenses in the presence of TR100 for 2 days. Images were acquired using a 100× objective lens at **A** 1× or (**B**) 2× zoom. Boxes outlined with dashed red lines in **A** are shown at higher magnification (2× zoom) in **B**, where Tpm3.1 (green) immunostaining demonstrates association with F-actin (red) in stress fibers in TGFβ2-treated control (top panels) but not in TGFβ2 plus TR100-treated lenses (bottom panels).

of 1 μM TR100. Similar to our results with imLECs (Fig. 3C), TR100 reduces F-actin stress fiber formation in the basal region of native lens epithelial cells (Fig. 7A) and reduces the association of Tpm3.1 with F-actin at the basal region of the cell (Fig. 7B). Reduction of TGFβ2-induced stress fibers in the absence of Tpm3.1 in *Tpm3/*Δ*exon9*$^{-/-}$ lenses or in TR100-treated wild-type lenses provides evidence that normal levels and function of Tpm3.1 are essential for stress fiber formation at the basal region of lens epithelial cells.

TGFβ2 Induction of αSMA is Repressed in Tpm3/Δexon9$^{-/-}$ Lenses or by TR100 Treatment of Wild-Type Lenses

To determine if knockout of Tpm3.1 or inhibition with TR100 reduces EMT progression into myofibroblasts, we examined αSMA protein levels. For this analysis, we extracted protein from capsular-epithelial peels. By performing WES immunoassay, a sensitive method to detect protein in a low number of cells,[32] we can determine αSMA protein expression from individual capsule-epithelial peels (Fig. 8). These experiments show that TGFβ2 treatment leads to a 2.8-fold increase in αSMA protein levels in the lens epithelium as compared to control. Knockout of Tpm3.1 (*Tpm3/*Δ*exon9*$^{-/-}$ lenses) represses the increase in αSMA levels as compared to wild-type. In addition, exposure to TR100 also reduces αSMA levels in TGFβ2 treated wildtype lenses (Fig. 8). Therefore, knockout or inhibition of Tpm3.1 represses EMT progression in native lens epithelial cells.

Discussion

This study provides new insights into targeting stress fiber formation via inhibition of specific tropomyosin isoforms during lens EMT. We discovered that eight Tpm isoforms are expressed in lens epithelial cells. We found that four of these Tpm isoforms are upregulated in imLECS following TGFβ2 treatment, including Tpm3.1, which is the most highly upregulated. Tpm3.1 is expressed in mouse imLECs and native mouse lens epithelial cells but not fiber cells. We determined that Tpm3.1 is required for lens EMT. During TGFβ2 induced EMT in imLECs or whole lenses, Tpm3.1 assembles with F-actin in stress fibers (Fig. 9A). Knockout of Tpm3.1 or inhibition with the Tpm3.1-binding molecule TR100 prevents TGFβ2-induced stress fiber formation and EMT progression in lens epithelial cells (Fig. 9B).

TGFβ2 modulates the expression of diverse Tpm isoforms, suggesting it may be imperative to target specific isoforms to prevent EMT. Previously, Kubo et al. have suggested a role for Tpm1 and 2 in EMT and cataracts.[14,15] They showed that TGFβ1 or TGFβ2 treatment results in an increase in Tpm1 and Tpm2 gene expression. Furthermore, they showed that knockdown of Tpm1 and 2 with a mixture of siRNAs represses the TGFβ-induced elevation of αSMA expression. However, these previous studies do not elucidate which specific Tpm isoforms were either upregulated or targeted by the siRNAS, which may be problematic as we determined that lens epithelial cells express four isoforms from the Tpm1 gene (Tpm1.5, 1.7, 1.9, and 1.13). Although Tpm1.7 is a known stress fiber associated Tpm,[12,13] and, therefore, may be another target to prevent EMT, the other isoforms are not known to be stress fiber-associated. These other isoforms likely stabilize different networks of actin in the lens and may be crucial for the maintenance of the epithelial phenotype. For instance, Tpm1.9 is associated with F-actin at adherens junctions in other (LLC-PK1 kidney) epithelial cells,[33] and we found, in the present study, that Tpm1.9 is downregulated in imLECs by TGFβ2 treatment. To effectively prevent EMT, it is likely that it would be necessary to retain expression of Tpm isoforms that stabilize F-actin at the lens epithelial apical and lateral domains, while reducing expression of Tpms that stabilize F-actin stress fibers.

Alternative splicing mechanisms regulate Tpm isoforms expression in cells and a further understanding of these mechanisms is required for the lens EMT. TGFβ stimulation alters RNA binding protein activity, which contributes to differential splicing. For instance, TGFβ treatment in

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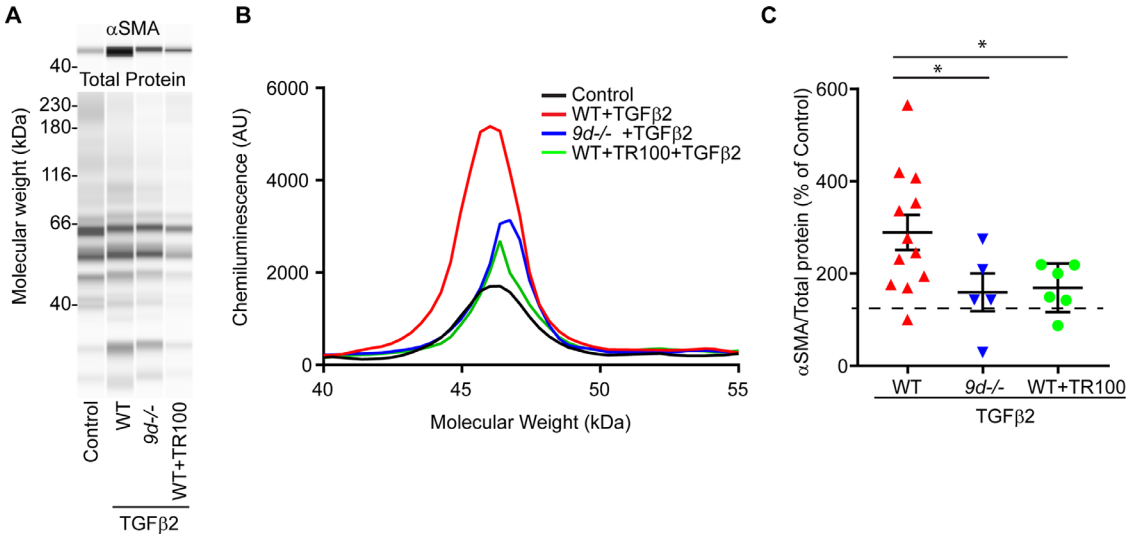

Figure 8. Knockout or inhibition of Tpm3.1 reduces TGFβ2-induction of αSMA expression in native lens epithelial cells. (**A**) Capillary electrophoresis immunoassay (WES) virtual gel visualizing the αSMA (~45–47 kDa) band as well as bands for total protein (12–230 kDa). The corresponding electropherogram for αSMA is shown in (**B**). (**C**) corresponding dot-plot showing αSMA protein levels in wild-type (WT; *Tpm3/*Δ*exon9d*$^{+/+}$) or knockout (*9d-/-*; *Tpm3/*Δ*exon9d*$^{-/-}$) mouse lens epithelial peels. Lens epithelial peels were from control lenses (harvested fresh), cultured for 4 days with TGFβ2, or cultured for 4 days with TGFβ2 in the presence of TR100. *$P < 0.05$ as compared to wild-type lenses treated with TGFβ2.

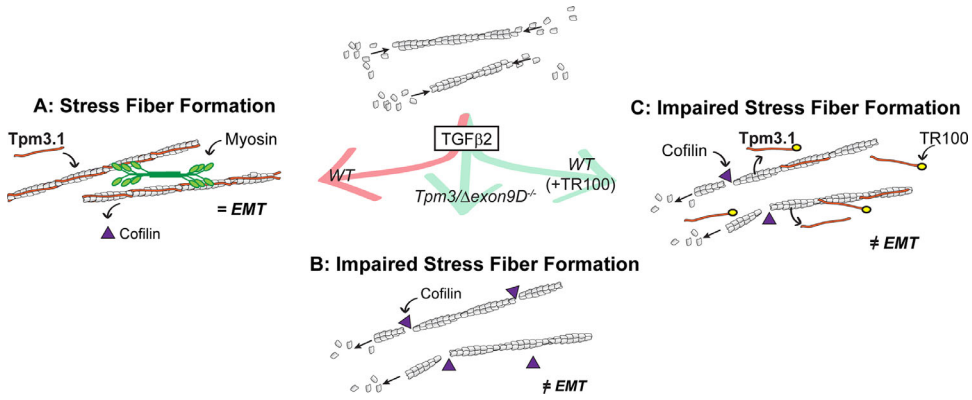

Figure 9. Tpm3.1 contributes to EMT in lens epithelial cells by stabilizing F-actin stress fibers. (**A**) Tpm3.1 is upregulated by TGFβ2 treatment and binds to F-actin. This can promote myosin activity and also prevent actin severing (by molecules such as cofilin) leading to stabilization of stress fibers and EMT progression. Knockout (**B**) or pharmacological inhibition by TR100 (**C**) of Tpm3.1 binding to F-actin can allow actin severing proteins such as cofilin to disassemble F-actin, leading to destabilization of actin stress fibers and EMT repression.

MCF10A breast epithelial cell line leads to CUGBP Elav-Like Family Member 1 (CELF1) induction at a protein level.[34] CELF1 activates splicing of exon 6B in Tpm2, whereas RNA binding protein-polypyrimidine tract binding protein (PTB) represses splicing of exon 6B.[35] PTB also represses splicing of exons 9a region in Tpm1, and this repression can be rescued by activation of RNA binding motif protein 4 (RBM4).[36] Interestingly, CELF, PTB, and RBM family proteins have all been associated with EMT in other cell types.[34,37–39] Thus, it is plausible that EMT induction by TGFβ2 treatment alters RNA binding protein expression or activity, which would lead to differential regulation of Tpm isoforms by alternative splicing. In addition, RNA binding proteins also regulate mRNA stability, transport, localization, and translation,[40] and, therefore, potentially regulate isoform expression in EMT through multiple mechanisms. Further understanding the regulation of Tpms by RNA binding proteins could lead to the development of additional strategies to repress lens EMT.

We focused on the strategy of specifically targeting Tpm3.1 as it is the predominantly upregulated Tpm isoform in imLECS following TGFβ2 treatment and is associated with F-actin in stress fibers. The association of Tpm3.1 with actin stress fibers in lens epithelial cells is in agreement with previous studies on human osteosarcoma (U2OS)[12,41] and human neuroblastoma (SK-N-BE(2))[42] as well as rat neuronal (B35) cells.[9] Tpm3.1 enhances non-muscle myosin IIA phosphorylation and recruitment of non-muscle myosin IIA to stress fibers[9,12] (Fig. 9A). Furthermore, Tpm3.1 can stimulate the activation of myosin II ATPase,[13] and increase the duty ratio of non-muscle myosin IIB on F-actin in vitro,[42] presumably leading to enhanced contractility.

Tpm3.1 can also protect against F-actin severing by cofilin/actin-depolymerization factor[9,43] (Figs. 9B and 9C). Thus, selective inhibition or knockout of Tpm3.1 can reduce stress fiber formation and actomyosin contractility in lens epithelial cells through several mechanisms.

In the present study, we found that by inhibiting Tpm3.1, the expression of αSMA as well as its incorporation into stress fibers is repressed, two known factors that lead to increased contractility of myofibroblasts.[28] Previous studies have demonstrated that actin reorganization can directly alter gene expression by regulating nuclear translocation of actin-binding transcription factor MRTF.[44–47] In the nucleus, MRTF acts as a co-activator for serum response factor, which binds to and activates the αSMA promoter. Here, we found that TR100-inhibition of Tpm3.1 reduces TGFβ2-induced nuclear MRTF localization (Supplementary Fig. S3), thus leading to a reduction in transcriptional activation of αSMA, as indicated by reduced αSMA mRNA and protein levels (Figure 4). Therefore, the MRTF-SRF pathway is one mechanism by which Tpm3.1 regulates αSMA expression. The mechanism by which Tpm3.1 modulates the incorporation of αSMA into stress fibers in lens epithelial cells remains elusive. Studies on fibroblasts have demonstrated that specific Tpm isoforms also enhance incorporation of αSMA into F-actin stress fibers.[48] This leads to the postulation that Tpm3.1 protein may directly regulate αSMA assembly into stress fibers in lens epithelial cells by binding and stabilizing the F-actin polymerized from αSMA protein. Although this requires further examination, it is likely that Tpm3.1 regulates lens epithelial αSMA contractility by at least these two mechanisms.

Reduction of stress fiber formation and αSMA incorporation into stress fibers via inhibition of Tpm3.1 may be a direct means to prevent capsular wrinkling, a contributing factor to light scattering in cataracts. Previous studies have shown that parallel bundles of F-actin resembling stress fibers form adjacent to the location of capsular wrinkling.[49] As expected capsular wrinkling can be inhibited by treatment with the actin depolymerizing agent, latrunculin B.[50] Although latrunculin B can inhibit capsular wrinkling, it is a potent G-actin sequestering agent, and nondiscriminately disassembles dynamic F-actin networks. By targeting Tpm3.1, the F-actin stress fibers at the basal regions of epithelial cells adjacent to the lens capsule can be selectively targeted, possibly leading to reduced capsular wrinkling. Selective inhibition of Tpm3.1 could be used in future studies to establish the links between basal stress fiber formation and capsular wrinkling.

Tpm3.1 is a suitable candidate to target cellular structures in lens epithelial cells for two reasons. First, the absence of Tpm3.1 does not affect native lens epithelial actin organization. Freshly isolated Tpm3.1 knockout lenses had similar F-actin organization as wildtype lenses (Supplementary Fig. S2); the F-actin organization in cells adjacent to the capsule (basal regions), cell-cell junctions (middle and apical regions) and in polygonal arrays (apical region) were similar in *Tpm3/Δexon9*$^{+/+}$ and *Tpm3/Δexon9d*$^{-/-}$ lenses. Second, Tpm3.1 knockout represses the acquisition of mesenchymal cell-like features induced by TGFβ2. Not only does the absence of Tpm3.1 selectively abrogate the ability of TGFβ2 to convert the basal F-actin into stress fibers, but Tpm3.1 knockout also results in maintenance of epithelial adherens junction organization in the presence of TGFβ2 (Supplementary Fig. S4). We have determined that Tpm3.1 knockout prevents the reduction of E-cadherin protein levels and loss of E-cadherin organization at adherens junctions caused by TGFβ2 in wild-type lenses. Based on this result, we would speculate that Tpm3.1 knockout leads to a compensatory upregulation of other Tpm isoforms, which stabilize the F-actin networks associated with adherens junctions, leading to increased E-cadherin junctional stability. This will be a subject of future investigations.

In addition to Tpm3.1, we observed that Tpm1.7, 2.1, and 4.2 were upregulated in imLECs by TGFβ2 treatment, which may also be targets against EMT. These Tpms are also reported to associate with actin stress fibers and play diverse roles in promoting stress fibers formation.[12,13,41] Tpm1.7 protects against actin severing by cofilin[13] and can promote the incorporation of αSMA into stress fibers.[48] Furthermore, Tpm2.1 can promote actin filament elongation.[51] Tpm4.2 activates myosin II ATPase and is present mostly in highly bundled F-actin.[13] Stress fiber formation in Tpm3.1 knockout lenses, or in lenses treated with TR100 is impaired, but not completely abolished. Thus, although impairment of Tpm3.1 may compromise stress fiber formation, these other Tpms may also partially compensate to promote stress fiber formation to some extent. Further research into the roles of the other stress fiber-associated Tpms in lens EMT is required.

In conclusion, mouse lens epithelial cells express multiple Tpm isoforms, several of which may be involved in lens EMT. Inhibition of stress fiber-associated Tpms, such as Tpm3.1, may be a means to repress stress fiber formation and EMT during TGFβ2 stimulation of lens epithelial cells. In preliminary studies we have also detected Tpm3.1 expression in human lens epithelial, SRA01/04, cells (data not shown), but future investigation is required to test whether targeting of Tpm3.1 in human lens epithelial cells will prevent TGFβ2-induced stress fiber formation. In addition, studies aimed at elucidating Tpm3.1 expression and assembly using in vivo cataract animal models[24] as well as with in vitro human cataract model systems[52] are required in order to fully ascertain the therapeutic potential of targeting Tpm3.1 in cataracts. Indeed, targeting Tpms could be a promising route to prevent EMT and cataractogenesis.

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