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Supplementary Information for

Thrombomodulin is essential for maintaining quiescence in vascular endothelial cells

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Title: TM regulates inflammation

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This PDF file includes:

Other supplementary materials for this manuscript include the following:

Supplementary Information Text

Materials & methods

Cell culture and reagents

Transformed HUVECs (EA.hy926 cells) were obtained from ATCC (Manassas, VA, USA) and were maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, 100 µg/mL penicillin, 100 µg/mL streptomycin, 1X HAT supplement (#25-046 from Mediatech Inc. Manassas, VA, USA), 2 mM L-glutamine. Protease-phosphatase inhibitor cocktail was obtained from Thermo Fisher (Rockford, IL, USA). Anti-PAR1 non-blocking antibody (Clone S19) #sc-8204, and GAPDH antibody (#sc-47724) was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Human TNF-α, Goat anti-mouse TM (#AF3894) and Rat anti-mouse TM (MAB3894) was from R&D (Minneapolis, MN, USA). Fluorescein (FITC)-conjugated ICAM-1 antibody (#353108), Phycoerythrin (PE)-conjugated VCAM-1 antibody (#305806) and PE-conjugated anti-E-selectin antibody were purchased from Biolegend (San Diego, CA, USA). PE-conjugated TM antibody (#130-113-318) and PE-conjugated mouse isotype control antibody (#130-113-762) were purchased from Miltenyi Biotec, Germany. VE-cadherin antibody (#2500), ZO1 (#8193) and β-actin (#4967) was from Cell Signaling Technology (Danvers, MA, USA). Alexa Fluor 488-conjugated goat anti-rabbit IgG (A-11008), Alexa Fluor 568-conjugated goat anti-rabbit IgG (A-11011), Alexa Fluor 568-conjugated goat anti-mouse IgG (A-11004) and Alexa Fluor 488-conjugated donkey anti-goat IgG (A-11057) and Alexa Fluor 568-conjugated Phalloidin was from Thermo Fisher Scientific (Rockford, IL, USA). Anti-PAR-1 blocking antibody, clone ATAP2. Catalog No: MABF244 (Millipore, Burlington, MA, USA). Tamoxifen and Evans blue dye was from Sigma (St. Louis, MO, USA).

Generation of TM-deficient endothelial cells and overexpression of TM by lentivirus-based methods

Heterozygous and homozygous TM-deficient EA,hy926 endothelial cells were generated by CRISPR/Cas9 technology (schematic representation in Supplementary Figure S1). For re-expression of TM in TM-deficient and WT type endothelial cells, recombinant lentivirus particles containing full-length human TM (vector: pLV-EGFP-EF1A-hTM) and pLV-EGFP-:T2A:Puro vector control were from Vector Builder Inc. Chicago, IL). Sub-confluent cells (70-80%) were transduced with lentivirus particles at an MOI (Multiplicity of infection) of 4 with polybrene (5µg/ml). After 5 days, cells positive for TM were selected and sorted by cell sorter. All selected cells were maintained the same as normal EA.hy926 cells.

Knockdown of VWF in TM-deficient EA.hy926 cells

VWF shRNA vectors (#VB191114 and VB191114) were purchased from Vector builder (Vector Builder Inc. Chicago, IL). Both vectors were transfected into $TM^{-/-}$ EA.hy926 cells using Lipofectamine 2000 and stable cells were selected based on puromycin resistance. Cells were maintained as normal EA.hy926 cells.

TM knockout mouse model

Thrombomodulin flox/flox ($TM^{f/f}$) and estrogen receptor-Cre-recombinase (ERcre) fusion protein under the transcriptional control of the ROSA26 locus mice were generated as described previously (1). $TM^{f/f}$ mice were crossed with ERCre mice to generate ERcre-$TM^{f/f}$ mice. To obtain TM deficient (TM-KO) mice, 8-16 weeks old ERcre-$TM^{f/f}$ mice were administered with intraperitoneal injection of tamoxifen (2mg per mouse) for 5 consecutive days to induce Cre-mediated recombination. Tamoxifen administered $TM^{f/f}$ mice were used as wild type control. Mice were harvested and blood samples were collected at day 12 after first tamoxifen injection. Blood coagulation assays- plasma clotting time (PCT) and activated partial thromboplastin time (APTT) were measure as described previously (2, 3). Tissue samples were analyzed by Western-blotting, enzyme linked immunosorbent assay (ELISA), immunohistochemistry as described in sections below. All mice protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Oklahoma Medical Research Foundation (OMRF).

ELISA

WT and $TM^{-/-}$ EA.hy926 cells were cultured in a 6 well plate and medium supernatant was collected from confluent cells. In some cases, cells were treated with 100nM of soluble TM (sTM) or thrombin (10nM) or TNFα (100ng/ml) for 16 hours. VWF and Ang2 release were measured using a sandwich ELISA. VWF

ELISA was done as described previously (4). Ang2 was quantified by the Human Angiopoitein-2 DuoSet ELISA kit #DY623 (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Mouse plasma VWF, lung tissue antigen levels of VWF and TM were measured by mouse VWF matched antibody pair kit from Abcam, Cambridge, UK (#ab213466) and mouse TM Duo set kit from R&D Systems, Minneapolis, MN (#DY3894) according to the manufacturer's instructions. For this, equal amount of protein (0.67µg) was taken along with the standards provided in the kit. Final values were plotted as amount of antigen in ng per µg of protein in the lung lysate. Plasma Ang2 levels were measured by mouse Ang2 matched antibody pair kit from Abcam, Cambridge, UK (#ab219520).

VWF-platelet string formation assay

WT and *TM*$^{-/-}$ EA.hy926 were cultured in 35mm tissue culture dishes and platelet string formation was monitored as described earlier (4). In these experiments, platelets were fluorescently labeled with 5-chloromethylfluorescein diacetate (2.5 µmol/L) before perfusion and a shear rate of 8 dynes/cm^2 was applied. Images were captured with a monochromatic charge-coupled device camera (Hamamatsu C11440) in an Axiovert 200 fluorescence microscope (Zeiss) using a 20X objective, and videos were recorded using NIS-Elements software (Nikon) between 8 and 12 minutes after flow was initiated. Platelet strings were counted at 5 to 10 random fields and string formation was defined as ≥3 platelets aligning in the direction of the flow.

Endothelial cell permeability assay

Cell permeability was monitored by spectrophotometric analysis of the leakage of Evans blue-bound albumin across endothelial cell monolayer in a modified two-compartment chamber model as described previously (5). WT, *TM*$^{-/-}$, TM^{match} and TM^{high} EA.hy926 cells (2×10^5 cells/well) were seeded on trans-well inserts (3.0 µm pore size) in complete growth medium and allowed to become confluent. Thereafter, cells were serum starved with serum free DMEM media containing 0.5% BSA for 3 hours followed by stimulation of cells with thrombin (1nM) for 20 minutes or TNFα for 4 hours. At the end of treatment, Evans blue dye (0.67 mg/mL) and BSA (4%) was added to the upper chamber. Permeability was measured by collecting media from the lower chamber and measuring the absorbance of leaked Evans blue dye at 650 nm. Final values were plotted as fold change to untreated controls.

Cell index measurement

XCELLigence, RTCA DP instrument (ACEA Biosciences, San Diego, CA) was used to monitor the thrombin induced changes in transcellular resistance across the cell monolayer. 16-well plates were coated with gelatin (0.5%) in HEPES for 20min followed by addition of 100ul of growth medium. EAhy.926 cells (30,000 cells in 100µl) were seeded in each well in the plate. Cells were allowed to grow for two days on the electrodes with continuous measurement of cell index. Once the cells became confluent as observed by the cell index curve, they were serum starved for 3 hours and stimulated with thrombin as in the transwell assay. To analyze results, cell index values were normalized (divided by their values at the time of thrombin addition) as described previously (6). All obtained normalized values were subtracted from normalized values of untreated wells by the software (RTCA software Ver 2.0.0.1301) called baseline correction. Final values were plotted as baseline normalized cell index at various time points.

Immunofluorescence microscopy

WT and *TM*$^{-/-}$ EA.hy926 cells were cultured on glass coverslips coated with 0.5% gelatin. Sub-confluent cultures washed with PBS and fixed with 2% paraformaldehyde in PBS for 15min followed by permeabilization with 0.1% Triton X-100/PBS for 5min at room temperature. Cells were then washed with PBS and blocked for 1 hour with 2% bovine serum albumin in PBS. For analysis of VE-cadherin, VWF, Ang2 and TM, rabbit monoclonal anti-human VE-cadherin, rabbit polyclonal anti-human VWF, goat polyclonal anti-human Ang2 and mouse monoclonal anti-human TM antibodies were incubated overnight at 4^{o}C. After washing with PBS, cells were incubated with the respective secondary antibodies; goat anti-rabbit Alexa Fluor 488-conjugated antibody, goat anti-rabbit Alexa Fluor 568-conjugated antibody, goat anti-mouse Alexa Fluor 568-conjugated antibody, donkey anti- goat Alexa Fluor 568-conjugated antibody and counterstained with Hoechst 33342 or directly by mounting with Vecta Shield mounting medium containing DAPI (Vector Laboratories Inc., Burlingame, CA). Images were captured with a Nikon C2

Confocal Microscope (Melville, NY, USA). In some cases, a 3D surface plot was generated from a single cell by using Image J software (Version 1.52a, National Institute of Health, USA) with plugins; Interactive 3D Surface Plot (Version 2.4, National Institute of Health, USA).

Whole mount immunostaining of the trachea

The cylindrical tracheas were dissected from *TM*$^{f/f}$ and TM-KO mice and fixed with 2% PFA for 1 hour at RT. After washing with 1XPBS, tracheas were permeabilized with PBS containing 0.3% Triton X-100 (PBS-T). Tracheas were then incubated with PBS-T containing 5% normal goat or donkey serum, 0.2% BSA for 1 hour at room temperature to block nonspecific antibody binding. Tracheas were stained with antibodies for TM (1:50) and VWF (1:100) overnight at +4°C for 2 days. Tracheas were washed with PBS-T for 5-6 times before addition of secondary antibody. Secondary antibodies were incubated overnight at +4°C for one day and then tracheas were washed with PBS-T for 8-10 times before mounting on glass slides. Three-dimensional projections were digitally reconstructed from confocal z-stacks using Nikon C2 Confocal Microscope (Melville, NY, USA).

SDS-PAGE and Western-blotting

Cell lysates were prepared from confluent cells before or after treatment with TNFα for various time points. The composition of the lysis buffer used was: 50mM Tris-pH-7.4, 150mM NaCl, 0.5% Triton X-100, 0.5% Nonidet P-40, 5 mM EDTA and protease inhibitor cocktail along with 1mM sodium orthovanadate. For preparation of the lung lysate, all above components were mixed in 1X PBS. After clearing the lysate at high speed centrifugation at 12000rpm, protein estimation was done by the BCA protein assay kit from Thermo Fisher, Rockford, IL, USA. All lysate samples were boiled in loading buffer with 5% β-mercaptoethanol and resolved on 7.5-10% SDS-polyacrylamide gels. The protein samples were transferred to nitrocellulose membrane and incubated with respective antibodies followed by horseradish peroxidase-linked anti-rabbit IgG, anti-mouse IgG and anti-goat IgG as appropriate. Protein bands were detected using an ECL substrate from Thermo Fisher, Rockford, IL, USA and band intensity was quantified using Image J software (Version 1.52a, National Institute of Health, USA).

RNA isolation and qRT-PCR analysis

Total RNA was isolated using the kit from Qiagen, Hilden, Germany. RNA was reverse-transcribed to cDNA according to the manufacturer's instructions. qRT-PCR reactions were carried out using the iQ SYBR Green Supermix (Bio-Rad) and a BIO-RAD C1000 Thermal cycler according to standardized protocols. The relative fold change in transcription levels of TM, Ang2 and VWF were determined using the comparative CT (threshold cycle) method and GAPDH was used as an internal control. Data from at least three independent experiments were combined and presented. All qPCR primers used in this study were derived from previously published studies (7,8) with the exception of the primer for the TM-forward 5'-GGGCTTGCTCATAGGCATCT-3' and the primer for the TM-reverse 5'-GCAGCACTACCTCCTTGGAA-3'.

Flow cytometry

Confluent cultures of WT and *TM*$^{-/-}$ EA.hy926 cells were treated with and without TNFα (1 or 10ng/ml) for 4 hours. Cells were detached using HBSS containing 10mM EDTA, washed and resuspended in HBSS containing 2mM EDTA and 0.1% human serum albumin (HSA). Cells were stained using FITC conjugated anti-ICAM-1 antibody, PE-conjugated anti-VCAM-1 antibody, PE-conjugated anti-E-selectin antibody or PE-conjugated anti-TM antibody. Cell surface levels of ICAM-1, VCAM-1, E-selectin and TM were detected using FACS Celesta and data was analyzed by FlowJo software (BD Biosciences) as described with minor modification (9). For detecting PAR1 and EPCR, anti-PAR1 or anti-EPCR antibodies were incubated with cells followed by detection using Alexa Fluor 488 conjugated anti-mouse or anti-rabbit IgG antibodies.

Cell adhesion assays under flow conditions

Cells were treated with or without TNFα (1ng/ml) for 4 hours and washed with 1X HBSS. HL-60 cells were fluorescently labeled with 5-chloromethylfluorescein diacetate (2.5 μM/L) and suspended in

HBSS with HSA at 1×10^6 cells/ml. HL-60 cells were perfused over the EA.hy926 monolayer via a syringe pump at a shear stress of 0.25 dyne/cm^2. The interaction between HL-60 cells and endothelial cell monolayer was visualized and recorded by monochromatic charge-coupled device camera (Hamamatsu C11440) in an Axiovert 200 fluorescence microscope (Zeiss) using a 20X objective, and videos were captured using NIS-Elements software (Nikon). Video recordings were made between 10 and 12 minutes after flow was initiated.. Images were evaluated afterward, and the total number of adhering cells (cells not moving for more than 10 seconds) in 10 random fields of view was calculated.

Histological analysis

Mouse lung tissues were freshly harvested, fixed with 10% neutral buffered formalin (Thermo Fisher; Rockford, IL, USA), embedded in paraffin and sectioned. All 5µm sections were stained with hematoxylin and eosin (H&E). The pictures were obtained with an Eclipse E1000 microscope (Nikon). Histologic severity of inflammation was assessed in a blinded fashion.

Protein C activation

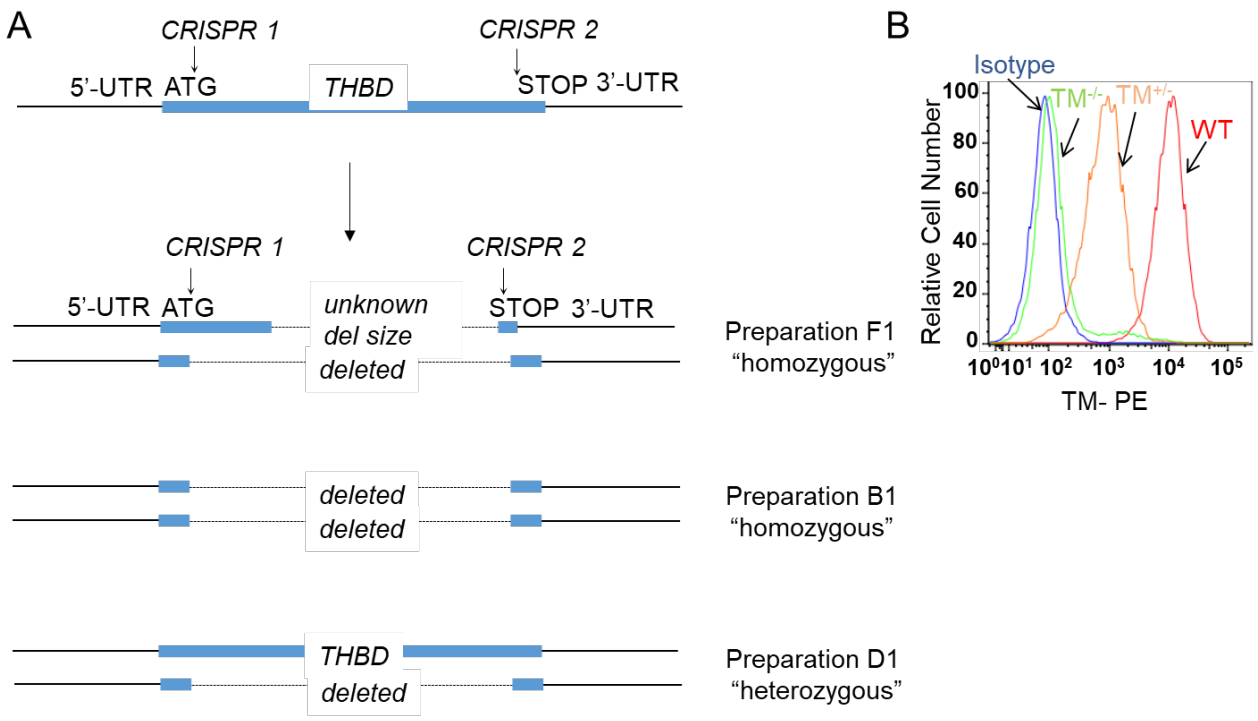
Activation of protein C by thrombin was monitored on confluent monolayer cultures of WT, *TM*$^{-/-}$, TM^{match} and TM^{high} endothelial cells as described previously (10).

VWF multimer analysis

Cell supernatant and mouse plasma samples were diluted in (1:4) and (1:20) ratio respectively in multimer sample buffer: 0.5 M Tris-Hcl (pH 6.8), 0.8 mM EDTA, 20% wt/vol SDS, 90 mM urea, 0.02% bromophenol blue. Samples were heated at 60^{o}C for 20 min and 5µl was loaded into 1.3% agarose HGT gel (50050, Seakem, Lonza, Basel, Switzerland) prepared in 100 mM Tris base (pH 8.8), 100 mM glycine, and 0.4% SDS. After running, the gel was dried overnight on the hydrophilic side of GelBond ® Film from Lonza, Basel, Switzerland. For immunoblotting, the gel was blocked with 5% milk in 0.05% TBS-T for 2 h at room temperature and probed with rabbit anti-human VWF IgG at 1:2000 dilution with gentle rocking overnight at 4 °C. After washing, HRP labeled goat anti-rabbit IgG (1:5000 dilution) was added and incubated for 1 h at room temperature. Protein bands were detected using an ECL substrate from Thermo Fisher, Rockford, IL, USA.

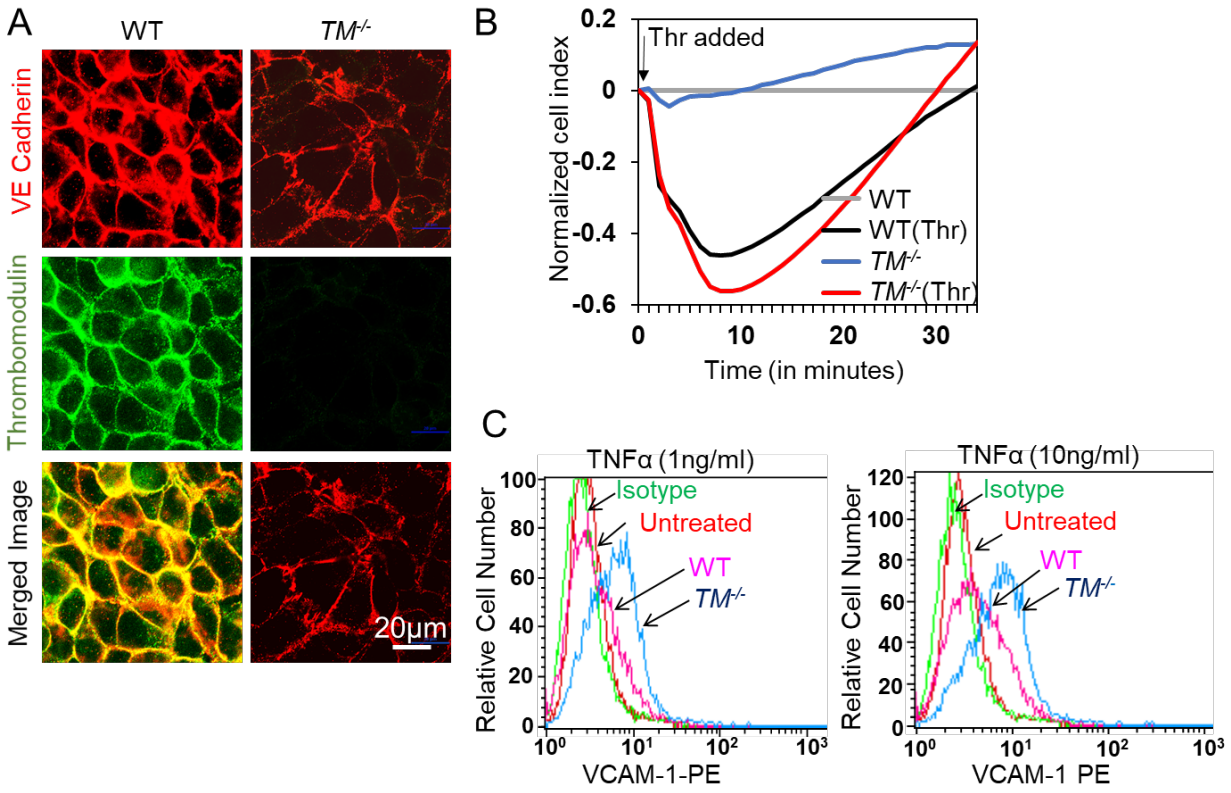
Statistical analysis

Data are presented as mean ± standard error of mean (SEM) from ≥3 independent experiments. Data between two groups were analyzed by the Student t-test, and multiple groups were analyzed by Analysis of Variance (ANOVA) using Graph Pad Prism 8.3.0 (Graph Pad Prism, San Diego, CA). p values are indicated by asterisks: *P<0.05, **P<0.01, ***P<0.001, and ****P≤0.0001.



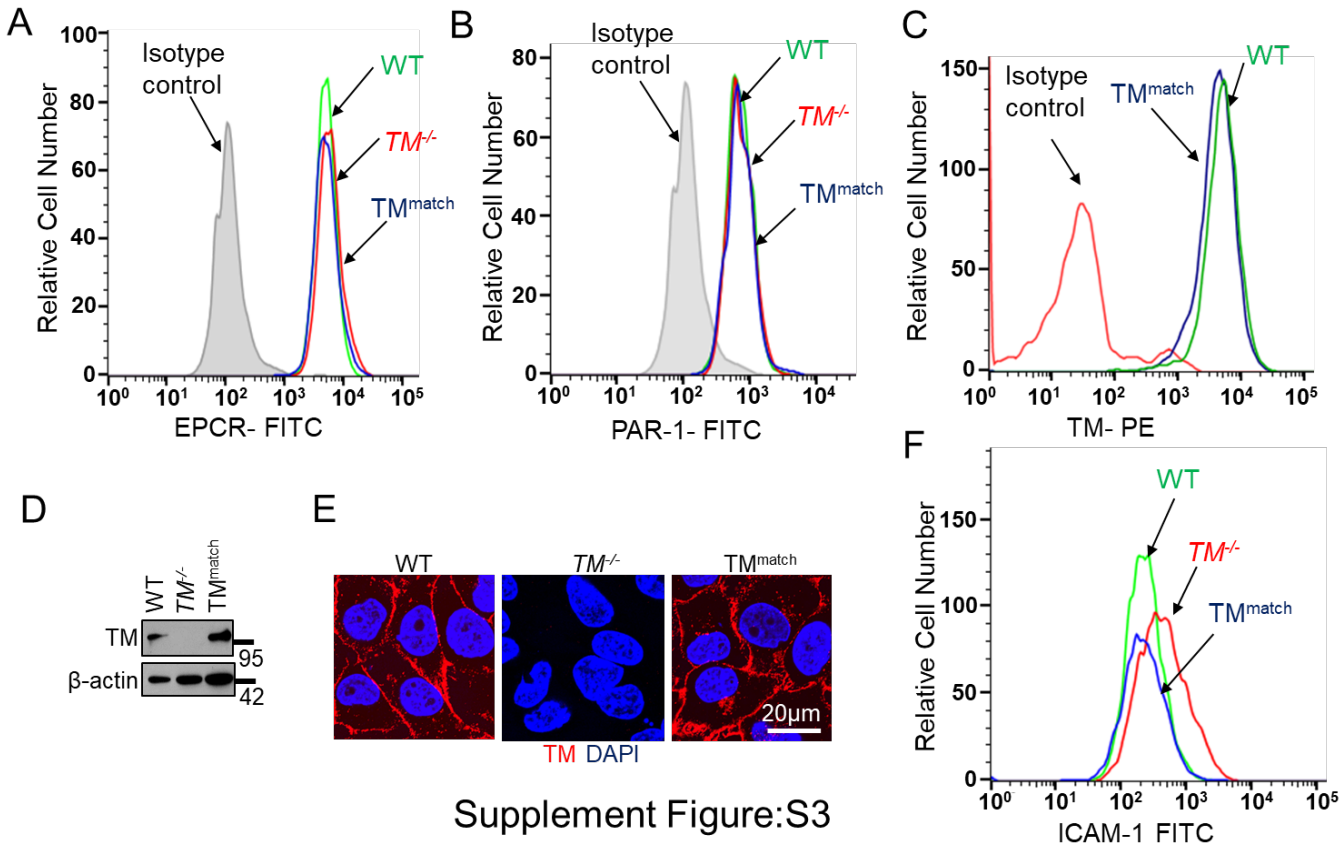
Supplement Figure:S1

Supplemental Figure S1. Generation of TM knockout EA.hy926 cells. A) Schematic representation of the generation of homozygous and heterozygous TM knockout EA.hy926 cells by CRISPR/Cas9 technology. B) Cell surface expression levels of TM on WT, *TM*$^{-/-}$ and *TM*$^{+/-}$ cells.

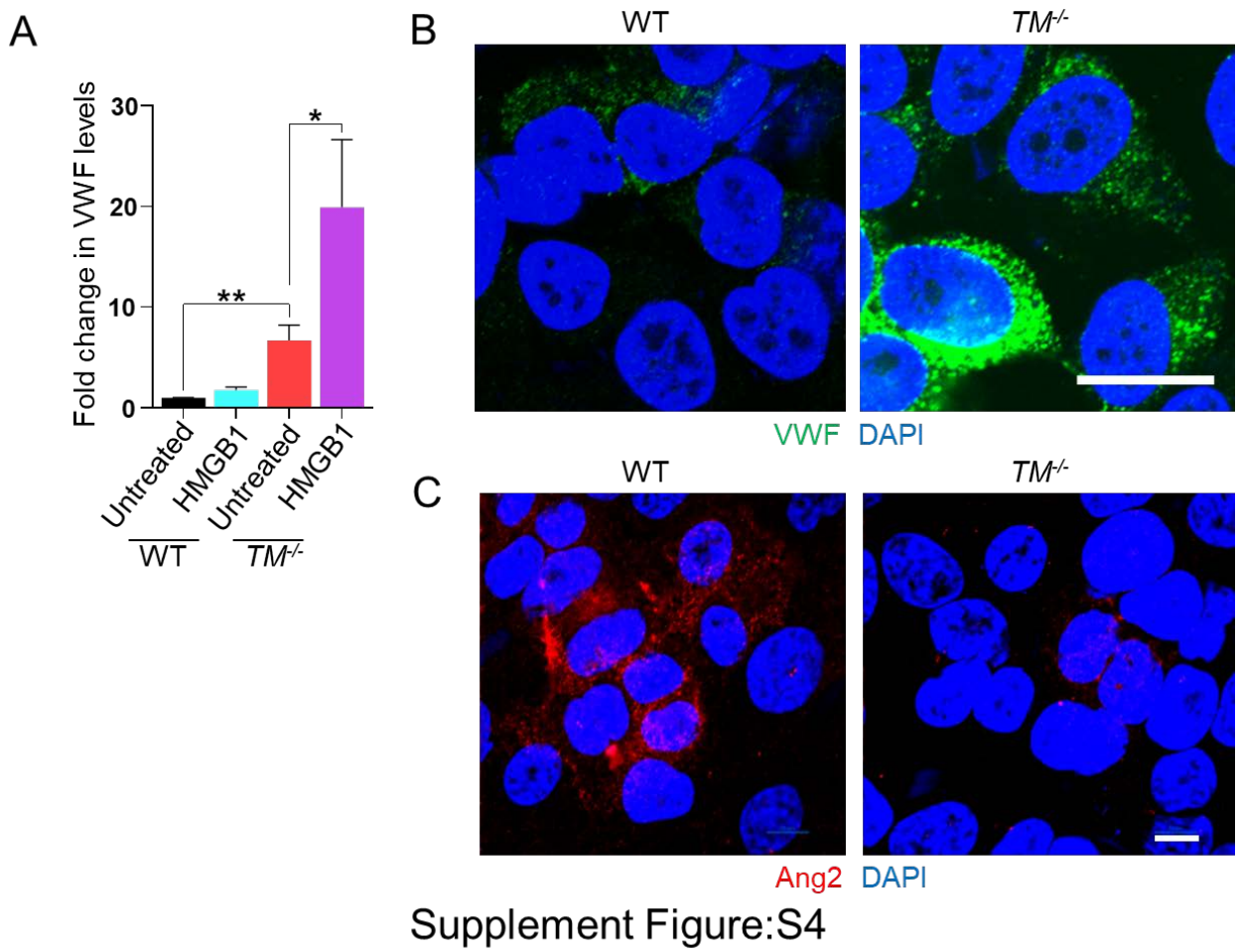


Supplement Figure:S2

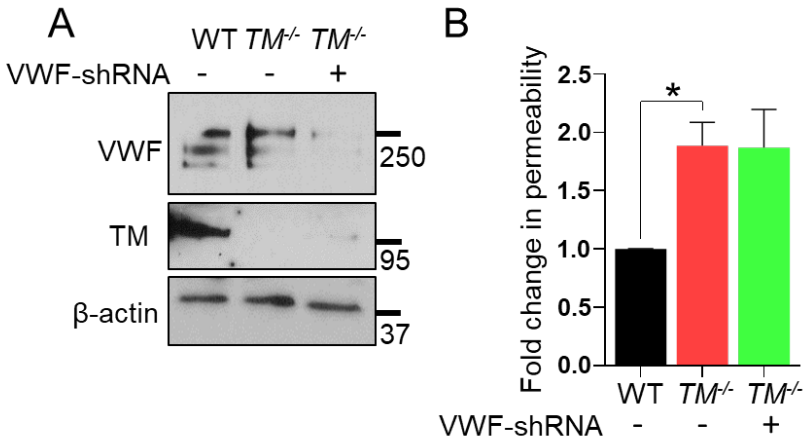
Supplemental Figure S2. TM deficiency leads to loss of VE-cadherin at junctions and decreased cell index by thrombin. A) Confluent WT and *TM*$^{-/-}$ cells were fixed, permeabilized and VE-cadherin stained with rabbit anti-VE-cadherin antibody and Alexa Fluor 568-conjugated goat anti-rabbit IgG, TM stained with mouse anti-TM antibody and Alexa Fluor 488-conjugated donkey anti-mouse IgG. Immunofluorescence images were obtained with confocal microscopy. Scale bar=20μm. B) Representative baseline normalized cell index tracings of WT and *TM*$^{-/-}$ cells with or without thrombin (1nM) treatment. Each line represents the average of two technical replicates. The black arrow indicates the 0 time-point where thrombin was added. C) Cell surface levels of VCAM-1 on WT and *TM*$^{-/-}$ cells, treated with TNFα (1 and 10ng/ml) for 4h, were measured by flow cytometry.



Supplemental Figure S3. Surface expression levels of EPCR, PAR1 and TM. A) Cell surface levels of EPCR and B) PAR1 on WT, *TM*$^{-/-}$ and TM^{match} cells under basal conditions. C) Cell surface levels of TM on WT and TM^{match} cells selected by FACS. D) Confluent WT, *TM*$^{-/-}$ and TM^{match} cells were lysed and protein levels of TM and β-actin were analyzed by Western-blotting. E) Confluent WT, *TM*$^{-/-}$ and TM^{match} cells were fixed, permeabilized and stained with mouse anti-TM antibody and Alexa Fluor 568-conjugated goat anti-mouse IgG. The nucleus was stained with DAPI. Immunofluorescence images were obtained with confocal microscopy. Scale bar=20μm**.** F) Cell surface levels of ICAM-1 on WT, *TM*$^{-/-}$ and TM^{match} cells under basal conditions.

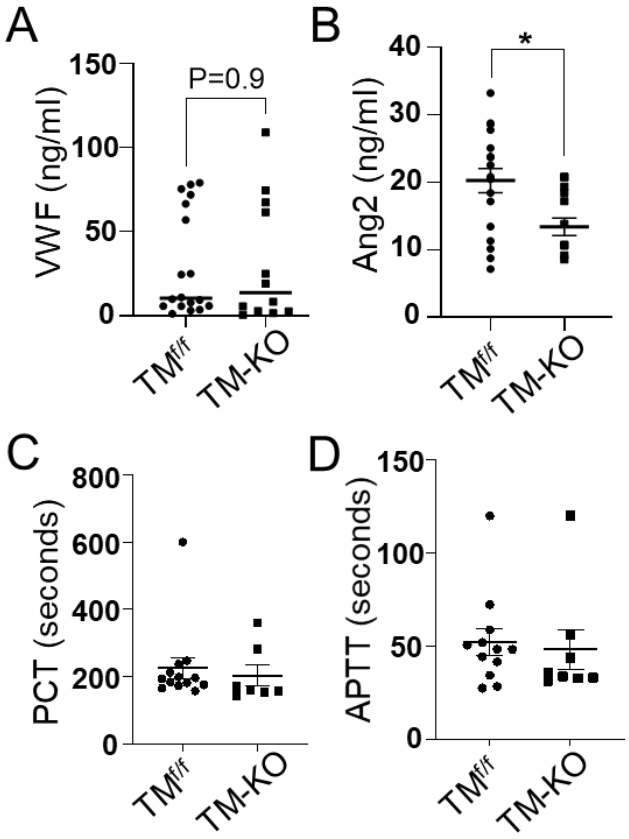


Supplemental Figure S4. HMGB1-induced VWF release and analysis of VWF and Ang2 by immunostaining in WT and TM-deficient cells. A) Levels of VWF in medium supernatant collected from confluent WT and *TM*$^{-/-}$ cells treated with HMGB1 (20nM) for 18h were measured by a sandwich ELISA. B) Confluent WT and *TM*$^{-/-}$ cells were fixed, permeabilized and stained with rabbit anti-VWF antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG. The nucleus was stained with Hoechst 33342. Immunofluorescence images were obtained with confocal microscopy. Scale bar=20μm. C) Similar to panel B except cells were stained with goat anti-Ang2 antibody and Alexa Fluor 568-conjugated donkey anti-goat IgG. Scale bar=10μm. One-way ANOVA: *$p < 0.05$ and**$p < 0.01$.

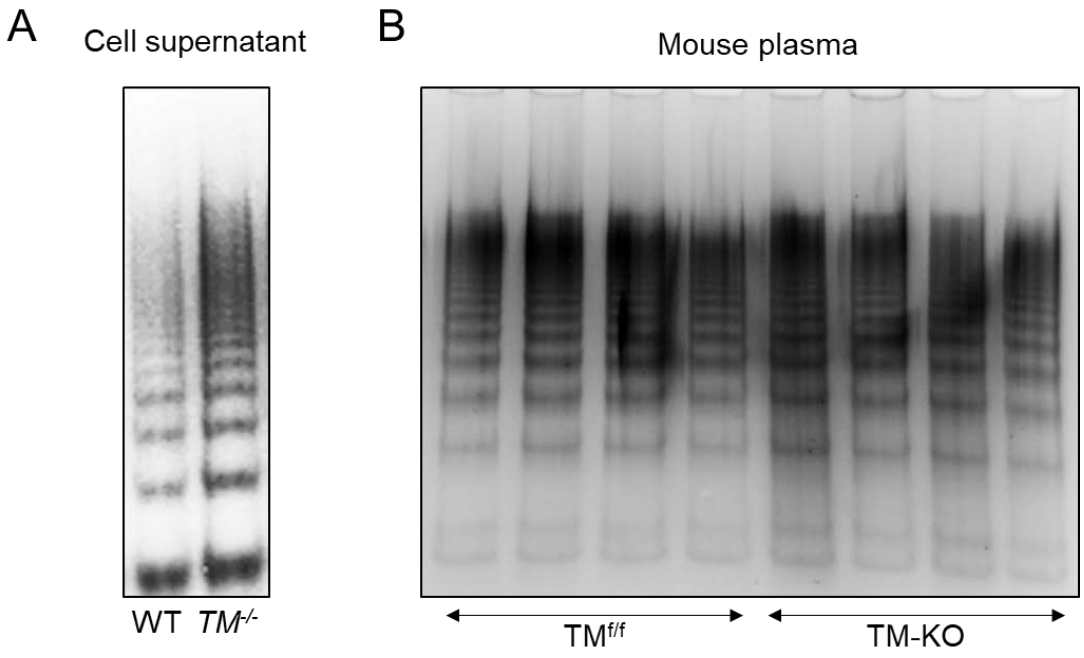


Supplement Figure:S5

Supplemental Figure S5. Knockdown (KD) of VWF in TM-deficient cells failed to protect basal permeability. A) Confluent WT, *TM*$^{-/-}$ and *TM*$^{-/-}$*/VWF*-KD cells were lysed and levels of VWF, TM and β-actin were analyzed by Western-blotting. B) Confluent WT, *TM*$^{-/-}$ and *TM*$^{-/-}$/VWF-KD cells were serum starved with basal medium containing 0.5% BSA for 3h and then amount of Evans blue dye that leaked into the lower chamber in the Trans-well assay plate was measured as described under materials and methods.



Supplemental Figure S6. Plasma levels of VWF, Ang2, and clotting assays (PCT & APTT) in *TM$^{f/f}$* and TM-KO mice A) Plasma levels of VWF and B) Ang2 in *TM$^{f/f}$* (n=18) and TM-KO (n=14) mice were measured by a sandwich ELISA. C) Plasma clotting time (PCT) and D) activated partial thromboplastin time (APTT) were measured in the blood plasma of *TM$^{f/f}$* (n=13) and TM-KO (n=8) mice.



Supplement Figure: S7

Supplemental Figure S7. Multimer analysis of VWF by SDS agarose gel electrophoresis A) VWF multimer analysis of cell supernatants collected from confluent WT and *TM*$^{-/-}$ cells. B) VWF multimer analysis of plasma samples from *TM*$^{f/f}$ (n=4) and TM-KO (n=4) mice.

Movies

Movie S1 (separate file): TNFα-induced adhesion of HL-60 cells on WT cells under flow conditions.

Movie S2 (separate file): TNFα-induced adhesion of HL-60 cells on *TM*$^{-/-}$ cells under flow conditions.

Movie S3 (separate file): TNFα-induced adhesion of HL-60 cells on TM^{match} cells under flow conditions.

Movie S4 (separate file): HMGB1-induced platelet string formation on WT cells under flow conditions.

Movie S5 (separate file): HMGB1-induced platelet string formation on *TM*$^{-/-}$ cells under flow conditions.

Movie S6 (separate file): HMGB1-induced platelet string formation on TM^{match} cells under flow conditions.

References

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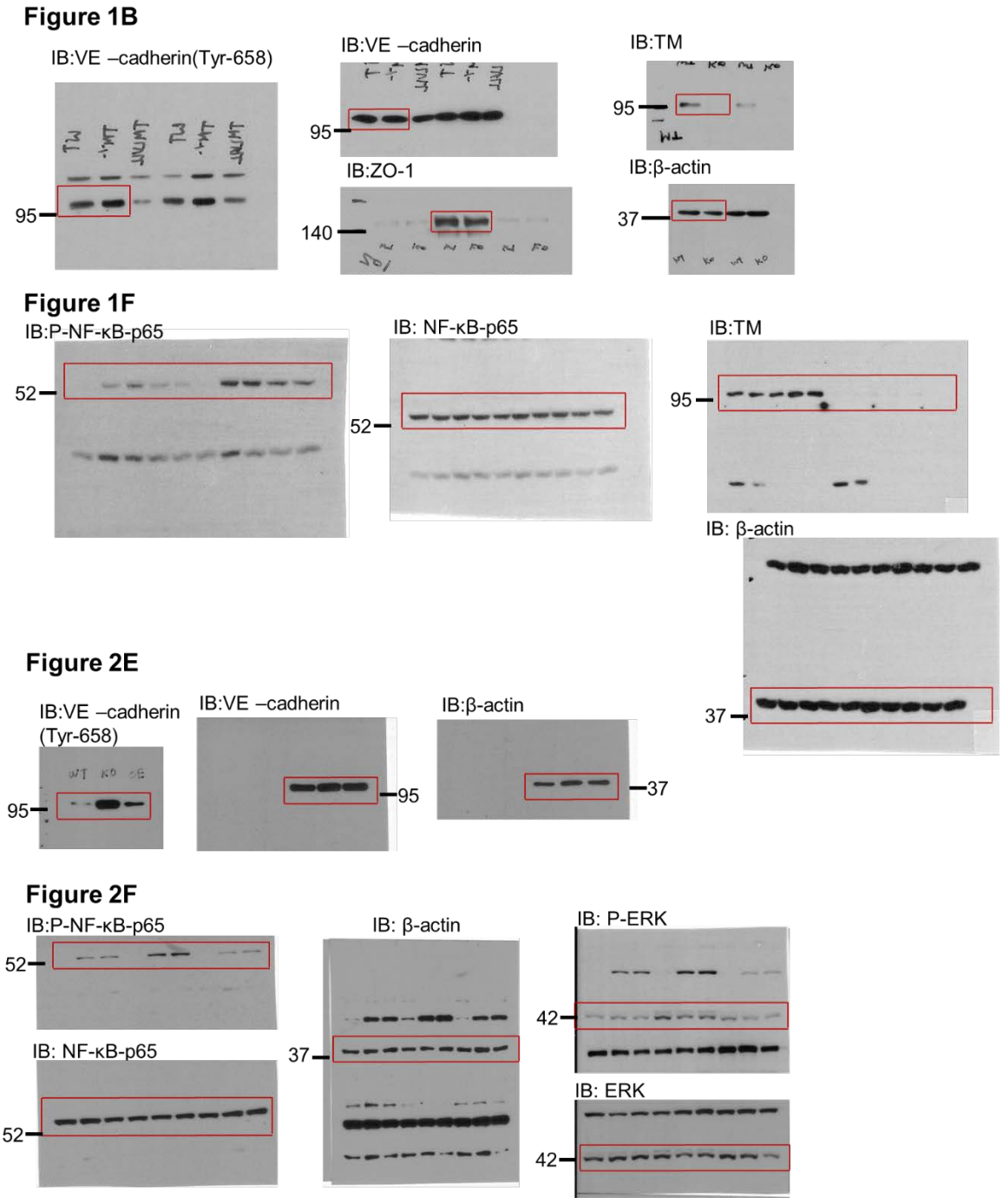
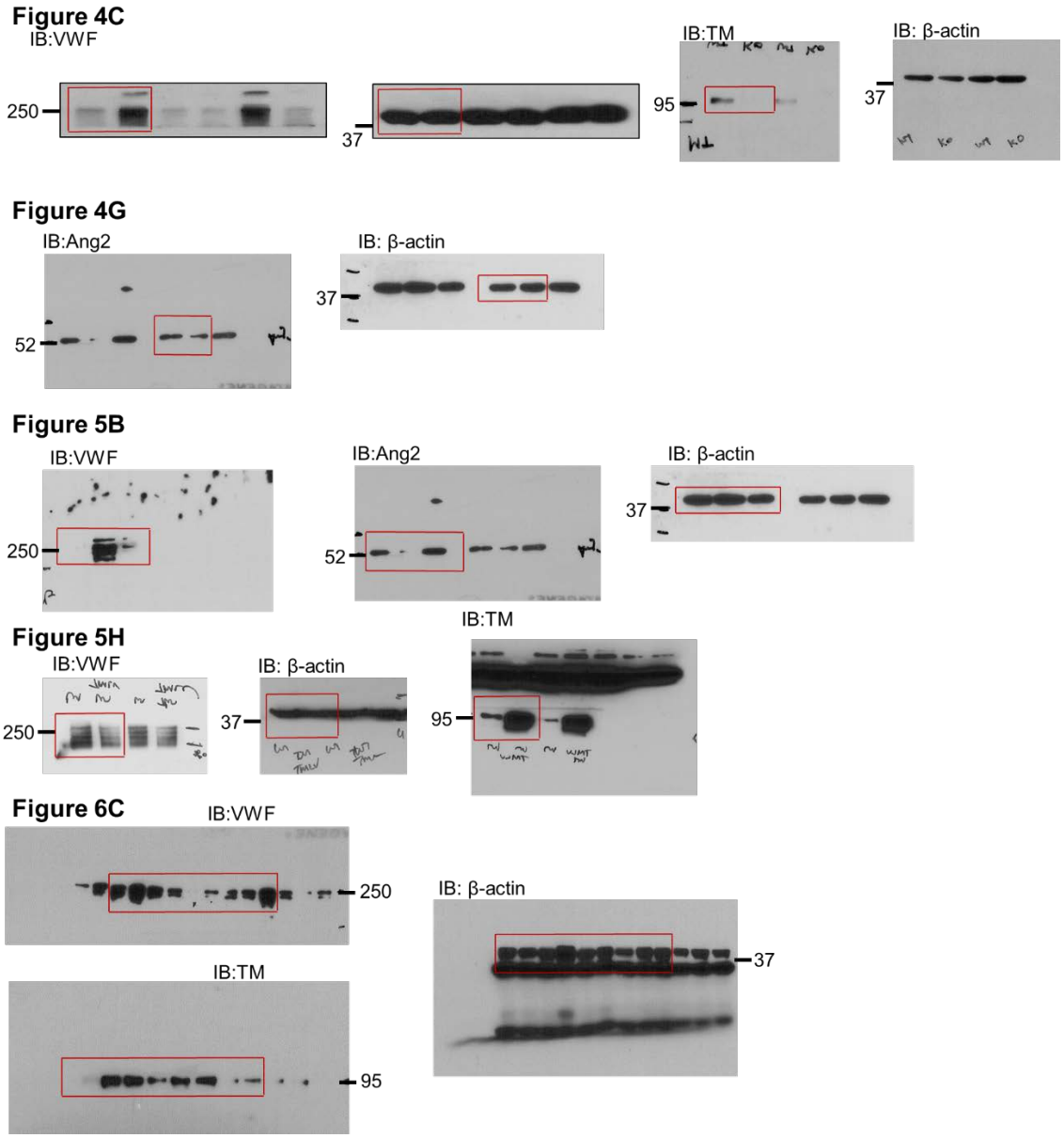
Datasets



Dataset S1: Full-length Western blot membranes for Figs. 1B, 1F, 2E and 2F. Red boxes highlight lanes used in figures.



Dataset S2: Full-length Western blot membranes for Figs. 4C, 4G, 5B, 5H and 6C. Red boxes highlight lanes used in figures.