Thymosin β4 is essential for thrombus formation by controlling the G-actin/F-actin equilibrium in platelets

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SUPPLEMENTAL METHODS

Blood parameters

For assessment of platelet count, size and basic blood parameters, mice were bled into EDTA-coated tubes and undiluted blood was immediately measured at an automated cell counter (ScilVet, scil animal care company GmbH, Germany) (1).

Platelet preparation

Mice were bled into heparin (20 U mL^{-1}, Ratiopharm) under isoflurane anesthesia and blood was washed twice using Tyrode-HEPES buffer as described previously (1). Platelet-rich plasma (PRP) was supplemented with 2 µL mL^{-1} apyrase (0.02 U mL^{-1}; A6410, Sigma-Aldrich) and 5 µL mL^{-1} PGI_2 (0.1 µg mL^{-1}; P6188, Sigma-Aldrich) and platelets were pelleted by centrifugation for 5 min at 2800 g, washed twice with Tyrode-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 5 mM HEPES, 5 mM glucose, 0.35% BSA, pH 7.4) containing 2 µL mL^{-1} apyrase and allowed to rest for 30 min prior to experiments.

Flow cytometry

For assessment of platelet activation, 50 µL blood were withdrawn under isoflurane anesthesia, washed twice with Tyrode-HEPES buffer and finally diluted (1:20) in Tyrode-HEPES buffer containing 2 mM Ca^{2+}. Samples were activated with the indicated platelet agonists and concentrations. Activation of αIIbβ3 integrin (JON/A-PE, Emfret, Germany) (2) and P-selectin exposure (WUG 1.9-FITC) were determined using fluorophore-conjugated antibodies (15 min at 37°C). α-granule release and integrin activation were measured after 15 min. Analyses were performed at a FACSCalibur (BD Biosciences) (3).

Platelet lifespan

Platelet lifespan was assessed by *i.v.* injection of a non-cytotoxic Dylight-488-labeled antibody derivative directed against GPIX and measurement of labeled platelets over 5 consecutive days using a FACSCalibur (BD Biosciences).

Aggregometry

Washed platelets or PRP (5 x 10^5 platelets per µL) were either supplemented with 100 µg mL^{-1} fibrinogen (Sigma-Aldrich) or left untreated and light transmission upon activation with the indicated agonists was monitored over time using a four-channel aggregometer (APACT, Laborgeräte und Analysensysteme, Hamburg).

Determination of ATP release

Washed platelets were resuspended to a concentration of 5 x 10^5 per µL in Tyrode-HEPES without Ca^{2+}. 80 µL of this platelet suspension were diluted into 160 µL Tyrode's buffer with 2 mM Ca^{2+}. After addition of 25 µL Chrono-lume luciferase reagent, agonists were added to the continuously stirred (1,000 rpm) platelet suspension. Light transmission and luminescence were recorded on a 700 Whole Blood/Optical Lumi-Aggregometer (Chrono-log) over 10 min. Results were shown in arbitrary units with buffer representing 100% transmission and washed platelet suspension 0% transmission. ATP release was calculated using an ATP standard and the AggroLink 8 software.

Platelet clot retraction

Mice were bled up to 700 µL in 70 µL sodium citrate (0.129 mM) and PRP was isolated by centrifugation at 1,800 rpm for 5 min. Plasma was collected and platelets were resuspended in 1 mL Ca^{2+}-free Tyrode-HEPES supplemented with 2 µL of apyrase (0.02 U mL^{-1}) and 5 µL PGI_2 (0.1 µg mL^{-1}). Platelet count was determined and 7.5 x 10^7 platelets were resuspended in 250 µL plasma. PRP (3 x 10^5 platelets per µL) was recalcified by adding 20 mM $CaCl_2$ and supplemented with 1 µL of red blood cells to visualize the clot. Clot formation was initiated by the addition of 4 U mL^{-1} human thrombin (Sigma). Clot formation and retraction was recorded up to 4 h and the residual volume was determined.

Transmission electron microscopy of platelets

Mice were bled into 300 µl heparin (20 U mL^{-1} in TBS). Platelets were washed and fixed using 2.5% glutaraldehyde (16210; Electron Microscopy Sciences) in 50 mM cacodylate buffer (pH 7.2; 1220 AppliChem) containing 2.5 mM $MgCl_2$ and 50 mM KCl. Samples were embedded in Epon 812 (14900, Electron Microscopy Sciences), ultrathin sections were generated and stained with 2% uranyl acetate (22400, Electron Microscopy Sciences) and lead citrate (17800, Electron Microscopy Sciences). Images were acquired on a Zeiss EM900 electron microscope.

Platinum replica electron microscopy.

Washed platelets were allowed to spread on 100 µg/mL fibrinogen-coated coverslips, permeabilized and fixed at the respective timepoint and finally sequentially incubated with 1% glutaraldehyde, 0.1% tannic acid and 0.2% uranyl acetate. Dehydration was performed by transferring samples through graded acetone. Critical point drying was done in a Leica EM CPD300. Samples were finally coated with 1.2 nm of platinum with rotation at 45°C and 3 nm of carbon at 90°C without rotation under high vacuum in a Leica EM ACE600. Replicas were floated, picked up on formvar-carbon-coated grids and examined on a JEOL JEM-2100.

F-Actin assembly

Assessment of F-actin polymerization was performed as described previously (4). Washed platelets were incubated with a DyLight-649-labeled anti-GPIX antibody derivative (20 µg mL^{-1}). Subsequently, platelets either remained resting or were stimulated with the indicated agonists for 2 min. Platelets were fixed with 0.55 volume of 10% paraformaldehyde in PHEM buffer and permeabilized with 0.1 volume 1% Triton™ X100. Subsequently, platelets were stained with 10 µM phalloidin-FITC (P5282, Sigma-Aldrich) for 30 min and immediately analyzed on a FACSCalibur.

F-actin sedimentation

Washed platelets (3 x 10^5 per µL) in Tyrode-HEPES without Ca^{2+} were lysed by addition of 20 µL 10x PHEM buffer containing 1% Triton X-100, 60 µM taxol, 20 µM phalloidin and protease inhibitors. One set (105 µL) served as whole cell lysate. The other set (105 µL) was separated into a polymerized and a soluble fraction by centrifugation at 56,000 rpm (microtubules) or 75,000 rpm (actin) for 30 min at 37°C in a TLA-100 rotor (Beckman Coulter) (5). Total platelet lysates (T), soluble *supernatant* (S) and insoluble *pellets* (P) were supplemented with 2x Loading Dye and subsequently subjected to SDS-PAGE and immunoblotting.

Platelet spreading on fibrinogen

Rectangular coverslips (24 x 50/60 mm) were coated with 100 µg mL^{-1} human fibrinogen o/N at 4°C in a humid chamber. Slides were blocked with 1% BSA for 1 h at RT. 30-50 µL washed platelets (3 x 10^5 per µL) were mixed with 50-70 µL Tyrode-HEPES with Ca^{2+}, activated with 0.01 U mL^{-1} thrombin (10602400001, Roche) and immediately allowed to spread on the fibrinogen-coated coverslips. After different time points, the adherent platelets were fixed with 300 µL 4% PFA in PBS for 5 min and *differential interference contrast* (DIC) microscopy pictures were taken using an inverted microscope Zeiss HBO 100 (Axiovert 200M, Zeiss). For analysis, the phase abundance of the different spreading stages (1, resting; 2, formation of filopodia; 3, formation of filopodia and lamellipodia; 4, fully spread) was determined.

Immunostaining of spread platelets

Coverslips were either coated with Poly-L-Lysine (P8920, Sigma-Aldrich) or fibrinogen (100 µg mL^{-1}; F4883, Sigma-Aldrich) overnight at 4°C. Resting platelets were seeded onto poly-L-lysine-coated slides while the remaining platelets were allowed to spread on fibrinogen in the presence of 0.01 U mL^{-1} thrombin (10602400001, Roche). At the indicated time points, platelets were fixed and permeabilized in PHEM buffer (60 mM piperazine-N,N-bis-2-ethanesulfonic acid (PIPES), 25 mM N-2-hydroxyethyl-piperazine-N′2-ethanesulfonic acid (HEPES), 10 mM ethylene glycol tetraacetic acid (EGTA), 2 mM $MgCl_2$, pH 6.9) supplemented

with 4% para-formaldehyde (PFA) and 1% IGEPAL® CA-630 and either analyzed by DIC microscopy at a Zeiss Axiovert 200 inverted microscope or further stained with phalloidin-Atto647N (170 nM, 65906, Fluka) and anti-α-tubulin Alexa F488 (3.33 µg mL^{-1}, 322588 (B-5-1-2), Invitrogen) or DNaseI-488 (0.3 µM, D12371, Thermo Fisher) (6) Samples were mounted with Fluoroshield (F6182, Sigma-Aldrich) and images acquired using a Leica TCS SP5 confocal microscope (Leica Microsystems).

Fibrin formation

Washed platelets (156.000/µl f.c.) were labeled with an anti-GPIX Alexa 647 derivative and added to a mixture of unlabeled fibrinogen (2 mg/ml) and Alexa Fluor A488-labeled fibrinogen (50 µg/ml f.c.). Platelets were stimulated with 0.1 U/ml thrombin and clotting was initiated by addition of 5 mM $CaCl_2$. The mixture was immediately transferred to an uncoated 8 Well Chamber Slide (Ibidi GmbH, Gräfelfing, Germany), and placed in a dark humidity chamber for 2 h at room temperature to allow clots to form. Images were obtained using a Leica SP8 inverted microscope with a 63x oil immersion lens. Optical z-stacks (15 µm, 0.1 step size, Nyquist conform) were deconvolved (Huygens Essential Software) and are shown as maximum projection (Image J software). Adapted from Campbell *et al*., Blood 2009 (7).

Immunoblotting

For analysis of platelet signaling, washed platelets (1 x 10^6 mL^{-1}) were immediately lysed after centrifugation in the respective volume of IP buffer and lysed for 30 min on ice at the indicated time-points. Samples were centrifuged and the supernatant was kept at -80°C until analysis.

Denatured proteins were separated by SDS-PAGE and blotted onto PVDF membranes. Membranes were probed for α-tubulin (1 µg mL^{-1}, T6074, Sigma-Aldrich), P-Pfn1 (1 µg mL^{-1}, PK6930, ECM Biosciences), Pfn1 (1 µg mL^{-1}, PK6930, ECM Biosciences), NMIIa (1 µg mL^{-1}, #3403, Cell Signaling Technology (CST)), phosphotyrosine (clone 4G10, 1 µg mL^{-1}, 05-321, Merck Millipore), GAPDH (1 µg mL^{-1}, G5262, Sigma-Aldrich) and β-actin (1 µg mL^{-1}, #4970, CST) and bound antibodies were detected using horseradish-peroxidase-conjugated secondary antibodies (0.33 µg mL^{-1}) and enhanced chemiluminescence solution (JM-K820-500, MoBiTec). Images were acquired with an Amersham Image 680 (GE Healthcare).

Protein levels of thymosin β4, myosin light chain 2 (MLC2), integrin β1, Syk and phosphorylation state of Syk were assessed with an automated capillary-based immunoassay platform (Jess, ProteinSimple) according to the manufacturers' recommendations. Separation matrix loading time was set to 200 s stacking matrix loading time to 15 s, sample loading time to 9 s, separation time to 30 min, separation voltage to 375 V, antibody diluent time to 5 min, primary antibody incubation time to 90 min and secondary antibody incubation time to 30 min. For recording of the chemiluminescent signal a High Dynamic Range (HDR) profile was used.

The optimized antibody dilutions were as follows: anti-thymosin β4 (19850-1-AP Proteintech) 1:20, anti-integrin β1 (#34971, CST) 1:10, Syk (#12358, CST) 1:40, Syk p-Tyr525/6 (#2711 CST) 1:10, and MLC2 (#3672, CST) 1:10.

Platelet adhesion on collagen under flow

Mice were bled into heparin and platelets and red blood cells (RBCs) were washed using Tyrode-HEPES or RBC wash buffer (140 mM NaCl, 10 mM HEPES, glucose), respectively. Washed platelets and RBCs were mixed and diluted in Tyrode-HEPES containing 2 mM Ca^{2+} and 250 µg mL^{-1} fibrinogen and platelets were fluorescently labeled for 5 min at 37 °C using a Dylight488-labeled anti-GPIX antibody derivative (0.1 µg mL^{-1}). Reconstituted blood was perfused over collagen I-coated coverslips at a shear rate of 1000 s^{-1} and aggregate formation was monitored using a Leica DMI600 (63x objective). Analysis was done using ImageJ software (National Institute of Health, USA).

For experiments on immobilized vWF, heparinized whole blood was perfused over coverslips, which were first coated o/N at 4°C with 200 µL 1:500 diluted polyclonal rabbit anti-human vWF antibody (A0082; Dako), blocked for 1 h with 1% BSA and incubated with 200 µL heparinized wild-type mouse plasma for 2 h at 37°C at a shear rate of 1700 s^{-1}. Platelet adhesion was monitored using a Leica DMI600 (63x objective). Analysis was done using ImageJ software (National Institute of Health, USA).

Histology

3 µm sections of paraformaldehyde-fixed and paraffin-embedded femora or spleens were stained with hematoxylin (MHS32, Sigma-Aldrich) and eosin (318906, Sigma-Aldrich). MK numbers were counted at a Leica DMI 4000 B microscope.

Immunofluorescence staining of whole femora cryosections

Femora and spleen of mice were isolated, fixed with 4% PFA (A3813, AppliChem) and 5 mM sucrose (S0389, Sigma-Aldrich), transferred into 10% sucrose in PBS and dehydrated using a graded sucrose series. Subsequently, the samples were embedded in Cryo-Gel (39475237, Leica Biosystems) and shock frozen in liquid nitrogen. Frozen samples were stored at -80°C. Cryosections were prepared using the CryoJane tape transfer system (Leica Biosystems) and probed with Alexa488-conjugated anti-GPIb antibodies (13G12, 7A9, 1.33 mg mL^{-1}), to specifically label platelets and MKs, and Alexa647-conjugated anti-CD105 antibodies (3.33 mg mL^{-1}, 120402 (MJ7/18), Biolegend) to stain the endothelium. Nuclei were stained using DAPI (40,6-diamidino-2-phenylindole; 1 mg mL^{-1}, D1306, Invitrogen). Femora were visualized using a Leica TCS SP8 confocal microscope (Leica Microsystems).

Megakaryocyte differentiation for spreading assays and staining

Bone marrow (BM) from femora was flushed (8), passed through a cell strainer and cultured for 2 days in StemPro Medium (Gibco) containing 50 ng mL^{-1} stem cell factor (SCF) (R&D Systems). On day 2, medium was changed to StemPro containing 50 ng mL^{-1} SCF and 50 ng mL^{-1} thrombopoietin (TPO). On day 4, medium was again changed to StemPro containing only TPO. On day 5, the megakaryocytic fraction was isolated by a BSA density gradient. Cells were cultured for another day in StemPro containing 50 ng mL^{-1} TPO.

For adhesion experiments, coverslips were coated with fibrinogen (100 µg mL^{-1}, F4883, Sigma-Aldrich) for 3 h at 37°C. After 30 min of incubation at 37°C, cells were centrifuged and resuspended in StemPro medium containing 50 ng mL^{-1} TPO. MKs were then seeded onto coated coverslips and adhesion was allowed for 3 h. Cells were washed with PBS, fixed and permeabilized for 30 min in 4% PFA in PBS containing 1% NP-40. MKs were stained overnight using anti-α-tubulin Alexa F488 (3.33 mg mL^{-1}, 322588 (B-5-1-2), Invitrogen) and phalloidin-Atto647N (170 nM, 65906, Fluka) antibodies. MKs were washed with PBS the following day and slides were mounted with DAPI-containing Fluoroshield (1 µg mL^{-1}, D1306, Invitrogen). Samples were observed at a Leica TCS SP8.

PPF of BM MKs

Male and female mice were anaesthetized and a 1 cm incision was made along the midline to expose the frontoparietal skull, while carefully avoiding damage to the bone tissue. The mouse was placed on a customized metal stage equipped with a stereotactic holder to immobilize its head (9). BM vasculature was visualized by injection of BSA-Alexa Fluor 546 and anti-CD105-Alexa Fluor 546 (6 µg and 0.6 µg per gram bodyweight, respectively). Platelets and MKs were antibody stained (0.6 µg per gram body weight anti-GPIX-Alexa Fluor 488). Images were acquired with a fluorescence microscope equipped with a 20x water objective with a numerical aperture of 0.95 and a TriM Scope II multiphoton system (LaVision BioTec), controlled by ImSpector Pro-V380 software (LaVision BioTec). Emission was detected with HQ535/50-nm and ET605/70-nm filters. A tunable broad-band Ti:Sa laser (Chameleon, Coherent) was used at 760 nm to capture Alexa Fluor 488 and Alexa Fluor 546 fluorescence. ImageJ software (NIH) was used to generate movies.

Transmission electron microscopy of BM MKs

Bones were isolated, cut into 3 mm long pieces and fixed in Karnovsky fixative (2% PFA, 2.5% glutaraldehyde in 0.1 M cacodylate buffer) overnight at 4°C. Subsequently, bones were decalcified using 10% EDTA/ PBS over 5 consecutive days. Afterwards, fatty components of the samples were fixed with 2% osmium tetroxide in 50 mM sodium cacodylate (pH 7.2), stained with 0.5% aqueous uranyl acetate, dehydrated with a graded ethanol series and

embedded in Epon 812. Ultra-thin sections were stained with 2% uranyl acetate (in 100% ethanol) followed by lead citrate. Images were acquired on a Zeiss EM900 TEM.

Determination of MK ploidy

To determine BM MK ploidy, both femora were isolated, and the BM was flushed and homogenized. Unspecific binding sites were blocked by incubation of the cell suspension with 0.02 mg mL^{-1} anti-FcgR antibody (553,142 (2.4G2), BD Pharmingen). Afterwards, MKs were stained using a fluorescein isothiocyanate-conjugated anti-GPIIb antibody (10 mg mL^{-1}, clone MWReg30). Finally, the cells were fixed, permeabilized and the DNA was stained using 50 mg mL^{-1} propidium iodide (P3561, Invitrogen) staining solution with 100 mg mL^{-1} RNaseA (EN0202, Fermentas) in PBS. Analysis was performed by flow cytometry and FlowJo software (Tree Star Inc., Ashland, USA).

Two-photon intravital microscopy of the BM

Male and female mice were anaesthetized and a 1 cm incision was made along the midline to expose the frontoparietal skull, while carefully avoiding damage to the bone tissue. The mouse was placed on a customized metal stage equipped with a stereotactic holder to immobilize its head (9). BM vasculature was visualized by injection of BSA-FITC and anti-CD105-Alexa Fluor 488 (100 µg and 20 µg, respectively). Platelets and MKs were antibody stained (0.6 mg per gram body weight anti-GPIX-Alexa Fluor 546). Images were acquired with a fluorescence microscope equipped with a 20x water objective with a numerical aperture of 0.95 and a TriM Scope II multiphoton system (LaVision BioTec), controlled by ImSpector Pro-V380 software (LaVision BioTec). Emission was detected with HQ535/50-nm and ET605/70-nm filters. A tunable broad-band Ti:Sa laser (Chameleon, Coherent) was used at 760 nm to capture FITC/Alexa Fluor 488 and Alexa Fluor 546 fluorescence. ImageJ software (NIH) was used to generate movies.

Tail bleeding time

Mice were anaesthetized and a 2 mm segment of the tail tip was removed using a scalpel. Tail bleeding was monitored by gently absorbing blood on a filter paper at 20 s intervals without touching the wound site. Bleeding was determined to have ceased when no blood was observed on the paper. Experiments were stopped after 20 min by cauterization. Differences between the mean bleeding times were statistically assessed using unpaired Student's t-test and differences between occluded and non-occluded vessels were determined by Fisher's exact test.

Aorta injury model

The abdominal cavity of anaesthetized mice was opened to expose the abdominal aorta. An ultrasonic flow probe (0.5PSB699; Transonic Systems, USA) was placed around the abdominal aorta, and thrombus formation was induced by a single firm compression with a forceps upstream of the flow probe. Blood flow was monitored for 30 minutes or until vessel occlusion occurred (blood flow stopped for > 5 minutes). Differences between occluded and non-occluded vessels were statistically assessed using the Fisher's t-test.

$FeCl_3$-induced injury of mesenteric arterioles

The mesentery of 3- to 4-week old anaesthetized mice was exteriorized by a midline abdominal incision and endothelial damage in mesenteric arterioles was induced by topical application of a filter paper soaked with 20% $FeCl_3$. Arterioles were visualized using a Zeiss Axiovert 200 inverted microscope equipped with a 100-W HBO fluorescent lamp source and a CoolSNAP-EZ camera (Visitron). Digital images were recorded and analyzed using the Metavue software. Adhesion and aggregation of fluorescently labeled platelets (Dylight488-conjugated anti-GPIX derivative) was monitored until complete occlusion occurred (blood flow stopped for > 1 min).

SUPPLEMENTAL REFERENCES

1. Aurbach K, Spindler M, Haining EJ, Bender M, Pleines I. Blood collection, platelet isolation and measurement of platelet count and size in mice-a practical guide. Platelets. 2019;30(6):698-707.
2. Bergmeier W, Schulte V, Brockhoff G, Bier U, Zirngibl H, Nieswandt B. Flow cytometric detection of activated mouse integrin alphaIIbbeta3 with a novel monoclonal antibody. Cytometry. 2002 Jun 1;48(2):80-6.
3. Stritt S, Wolf K, Lorenz V, Vogtle T, Gupta S, Bosl MR, et al. Rap1-GTP-interacting adaptor molecule (RIAM) is dispensable for platelet integrin activation and function in mice. Blood. 2015 Jan 8;125(2):219-22.
4. Stritt S, Beck S, Becker IC, Vogtle T, Hakala M, Heinze KG, et al. Twinfilin 2a regulates platelet reactivity and turnover in mice. Blood. 2017 Oct 12;130(15):1746-56.
5. Bender M, Stritt S, Nurden P, van Eeuwijk JM, Zieger B, Kentouche K, et al. Megakaryocyte-specific Profilin1-deficiency alters microtubule stability and causes a Wiskott-Aldrich syndrome-like platelet defect. Nat Commun. 2014 Sep 4;5:4746.
6. Cramer LP, Briggs LJ, Dawe HR. Use of fluorescently labelled deoxyribonuclease I to spatially measure G-actin levels in migrating and non-migrating cells. Cell Motil Cytoskeleton. 2002 Jan;51(1):27-38.
7. Campbell RA, Overmyer KA, Selzman CH, Sheridan BC, Wolberg AS. Contributions of extravascular and intravascular cells to fibrin network formation, structure, and stability. Blood. 2009 Nov 26;114(23):4886-96.
8. Heib T, Gross C, Müller ML, Stegner D, Pleines I. Isolation of murine bone marrow by centrifugation or flushing for the analysis of hematopoietic cells - a comparative study. Platelets. 2020 Jul 29:1-7.
9. Zhang L, Orban M, Lorenz M, Barocke V, Braun D, Urtz N, et al. A novel role of sphingosine 1-phosphate receptor S1pr1 in mouse thrombopoiesis. The Journal of experimental medicine. 2012;209(12):2165-81.
10. May F, Hagedorn I, Pleines I, Bender M, Vögtle T, Eble J, et al. CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis. Blood. 2009;114(16):3464-72.

SUPPLEMENTAL TABLE

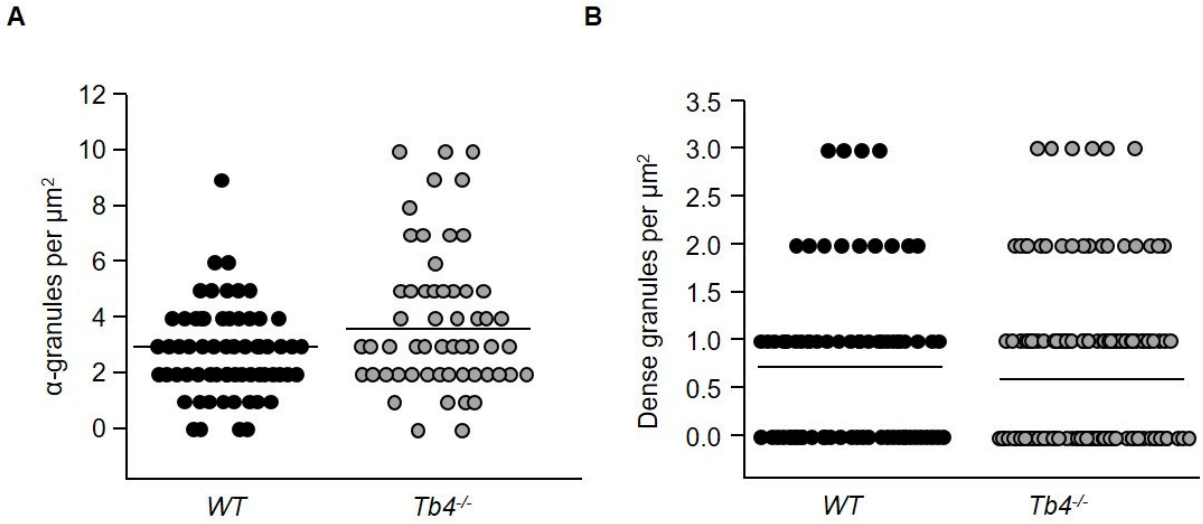
Supplemental Table 1. Basic blood parameters of WT and *Tmsb4x* KO platelets. For assessment of platelet count, size and basic blood parameters, mice were bled into EDTA-coated tubes and undiluted blood was immediately measured at an automated cell counter. Results are given as mean ± SD of at least 4 mice per group (3 independent experiments).

	WT	***Tb4*$^{-/-}$**	
Platelets [nl-1]	920±125	595±44	***
Mean platelet volume [fl]	5.3±0.1	5.8±0.2	*
Red blood cells [pl-1]	9±0	9±1	ns
Hemoglobin [g dl-1]	15±1	15±1	ns
Hematocrit [%]	44±3	43±4	ns
Mean red blood cell volume [fl]	50±2	50±1	ns
WBC	5±3	3±1	ns

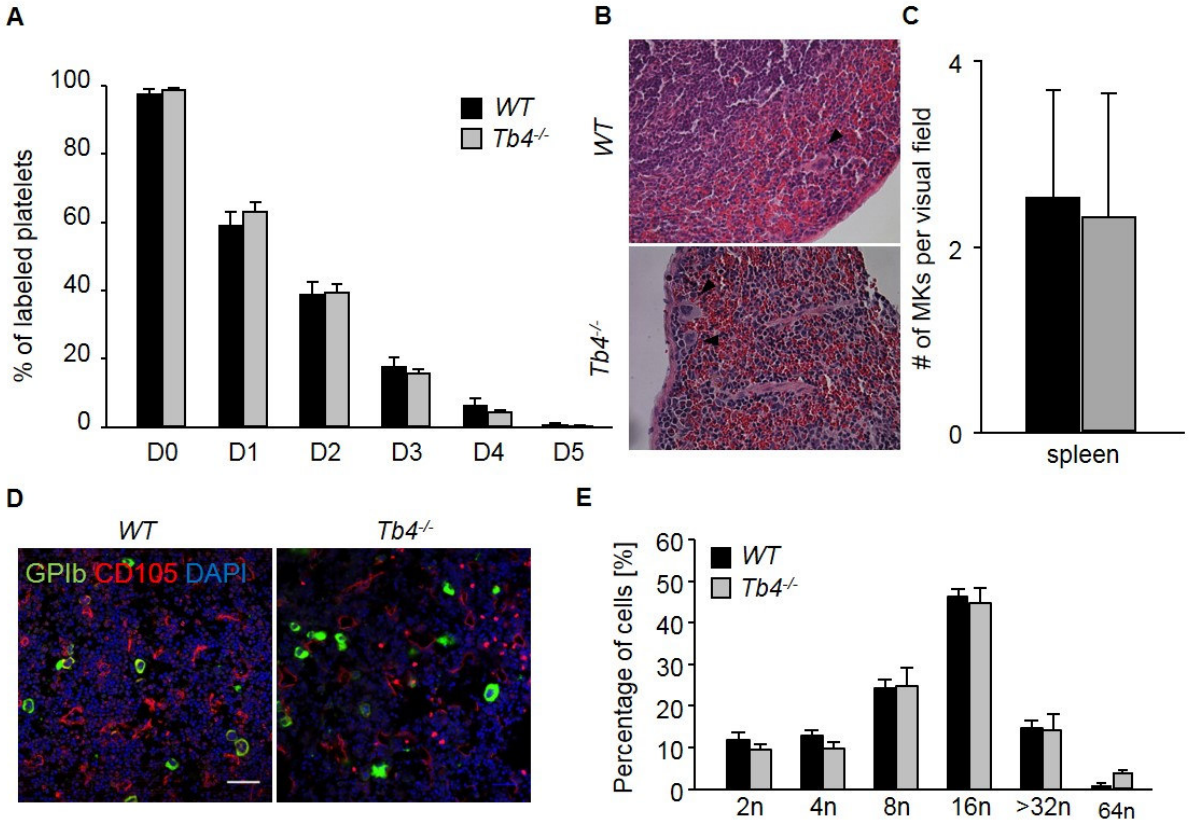
Supplemental Table 2. Platelet surface glycoprotein expression in WT and *Tmsb4x* KO platelets. For assessment of platelet glycoprotein expression, diluted whole blood was stained with fluorophore-labeled antibodies and analyzed on a FACSCalibur (Becton Dickinson, Heidelberg). Platelets were gated by FSC/SSC characteristics. Results are given as MFI ± SD of at least 4 mice per group (3 independent experiments).

	WT	***Tb4*$^{-/-}$**	
GPIb	276±11	260±21	ns
GPV	155±11	171±9	ns
GPIX	263±29	270±24	ns
CD9	599±42	544±56	ns
GPVI	27±2	25±1	ns
CLEC-2	97±12	103±8	ns
α2	41±1	43±2	ns
αIIbβ3	270±23	283±6	ns

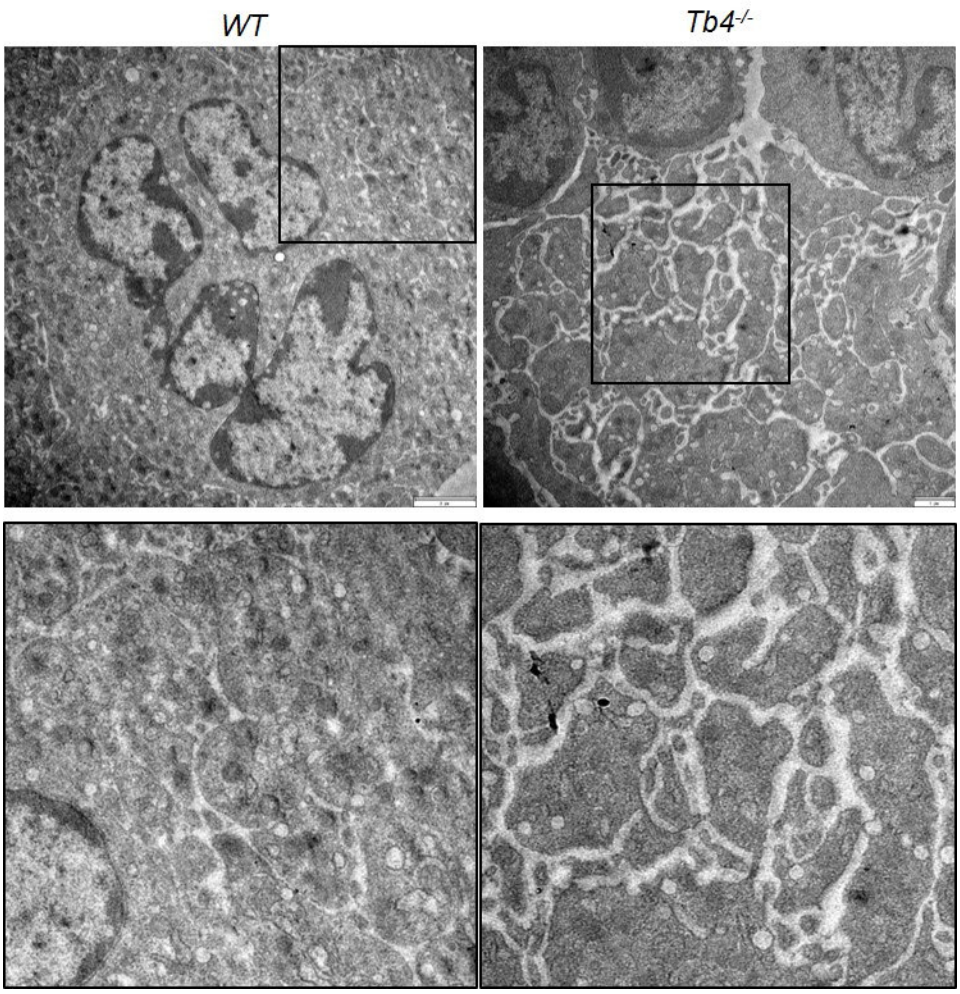
SUPPLEMENTAL FIGURES & LEGENDS



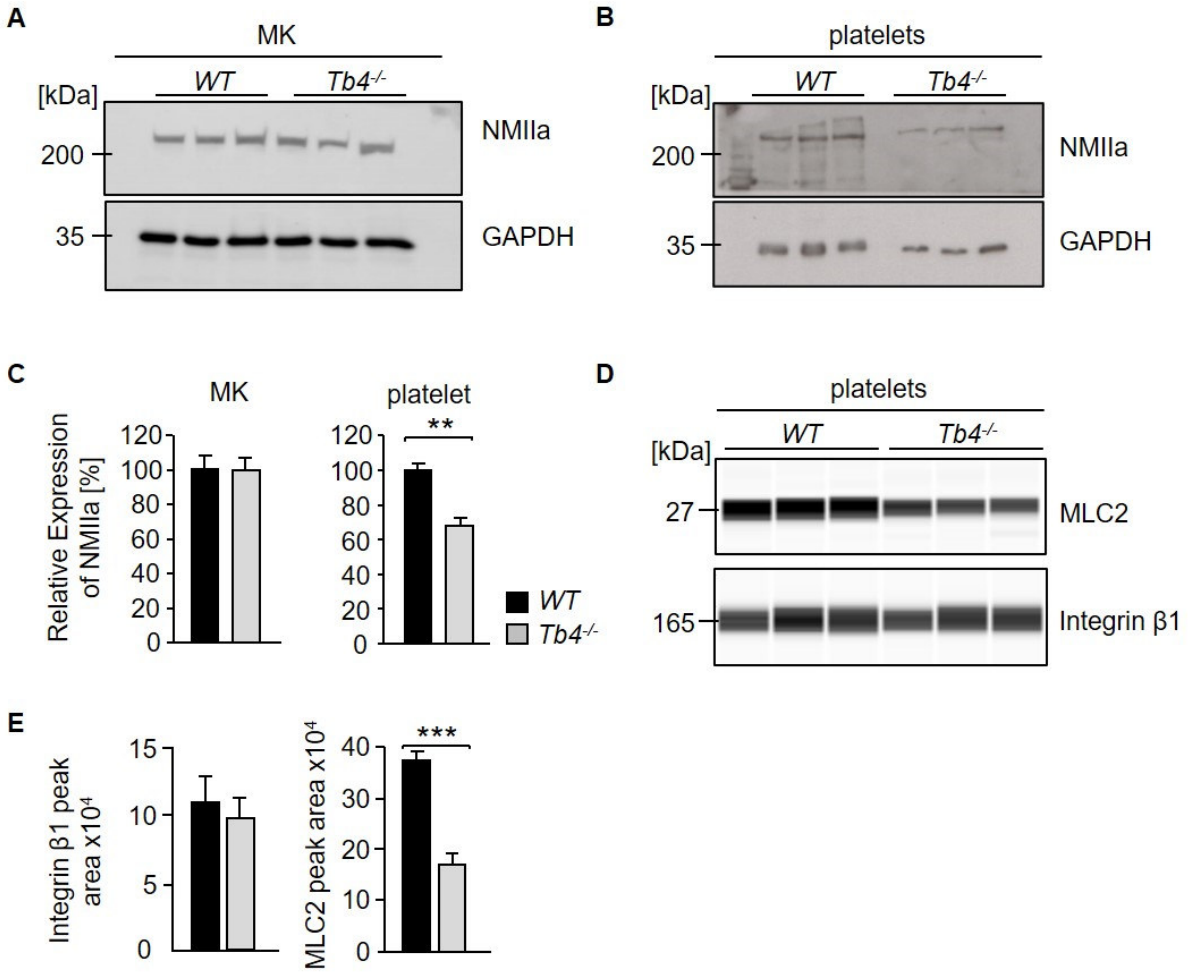
Supplemental Figure 1. Unaltered granule content in *Tmsb4x* KO mice. Analysis of α-granule (**A**) and dense granule (**B**) numbers in WT and *Tmsb4x* KO platelets from transmission electron microscopic images. Values are mean ± SD of at least 80 platelets per genotype.



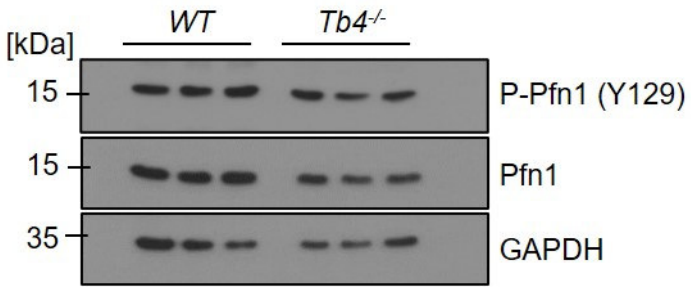
Supplemental Figure 2. Unaltered platelet lifespan, MK number and ploidy in *Tmsb4x* KO mice. (**A**) Platelet lifespan was assessed by flow cytometric measurement of the fluorescence-positive platelet population at the indicated time points after injection of a fluorophore-conjugated anti-GPIX antibody derivative (n = 6). (**B**) Hematoxylin-Eosin stainings of spleen paraffin sections of WT and *Tmsb4x* KO mice and (**C**) quantification of MK numbers. Arrow heads indicate the MKs. Values are mean ± SD (n = 3). (**D**) Confocal fluorescence microscopic images of femora cryo-sections of WT and *Tmsb4x* KO mice (Leica TCS SP5). Scale bars: 50 µm. MKs, proplatelets and platelets are shown by GPIb staining in green. CD105 staining (red) labels vessels. Nuclei were counterstained using DAPI (blue). (**E**) Flushed BM MKs were stained with a megakaryocyte-specific antibody (anti-αIIbβ3 integrin) and DNA was labeled using propidium iodide. DNA distribution was determined by flow cytometric analysis at a FACSCalibur (BD Biosciences) (n = 6). Values are mean ± SD.



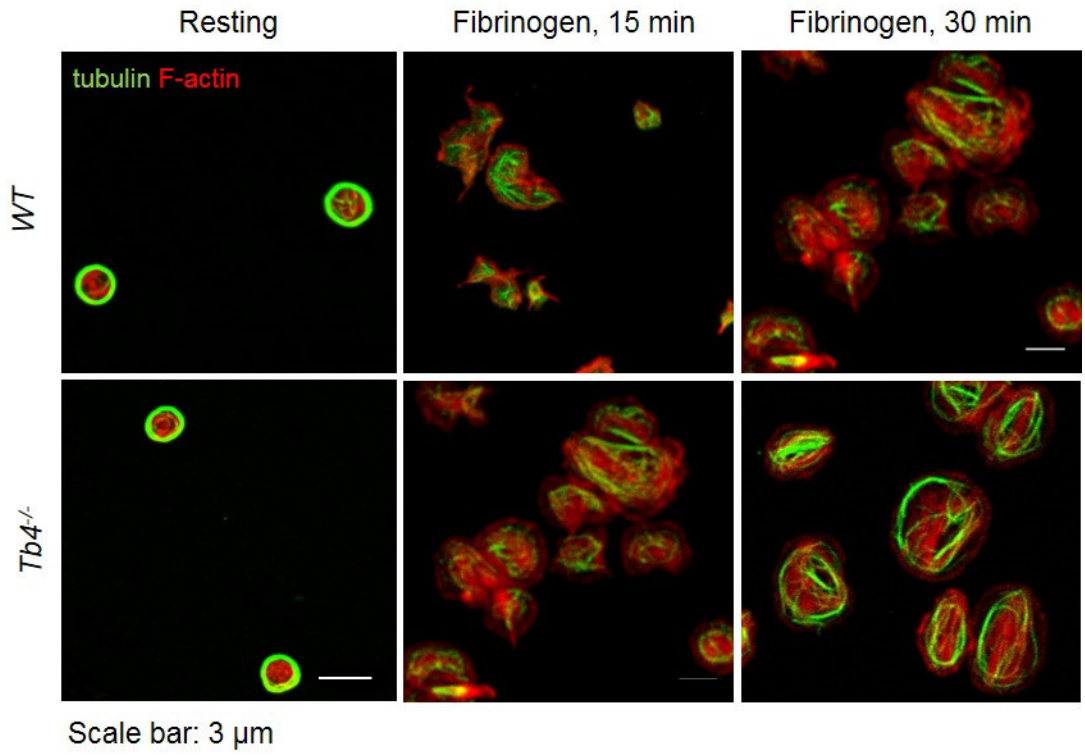
Supplemental Figure 3. Altered MK morphology in *Tmsb4x* KO mice. BM MKs from WT and *Tmsb4x* KO mice were analyzed by transmission electron microscopy. Scale bars: 1 µm. Representative images of at least 4 animals per group.



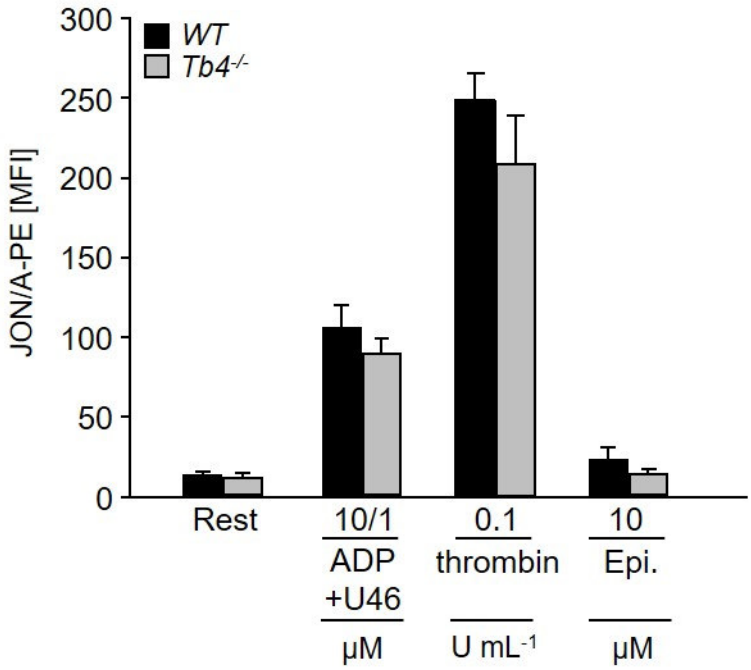
Supplemental Figure 4. Reduced non-muscle myosin IIA (NMIIa) and MLC2 levels in thymosin β4-deficient platelets. MKs (**A**) and platelets (**B**) were left untreated, lysed, and processed for immunoblotting. Total NMIIa was probed with the respective antibody and analyzed by densitometry (**C**). GAPDH served as loading control. Values are mean ± SD (n = 3) and given as relative expression compared to WT levels. Unpaired, two-tailed Student's t-test. **P< 0.005. (**D, E**) Protein levels of MLC2 in WT and *Tmsb4x* KO platelets were analyzed (**D**) and quantified (**E**) by an automated quantitative capillary-based immunoassay platform. Integrin β1 served as loading control. Values are mean ± SD (n = 3). Unpaired, two-tailed Student's t-test. ***P< 0.001.



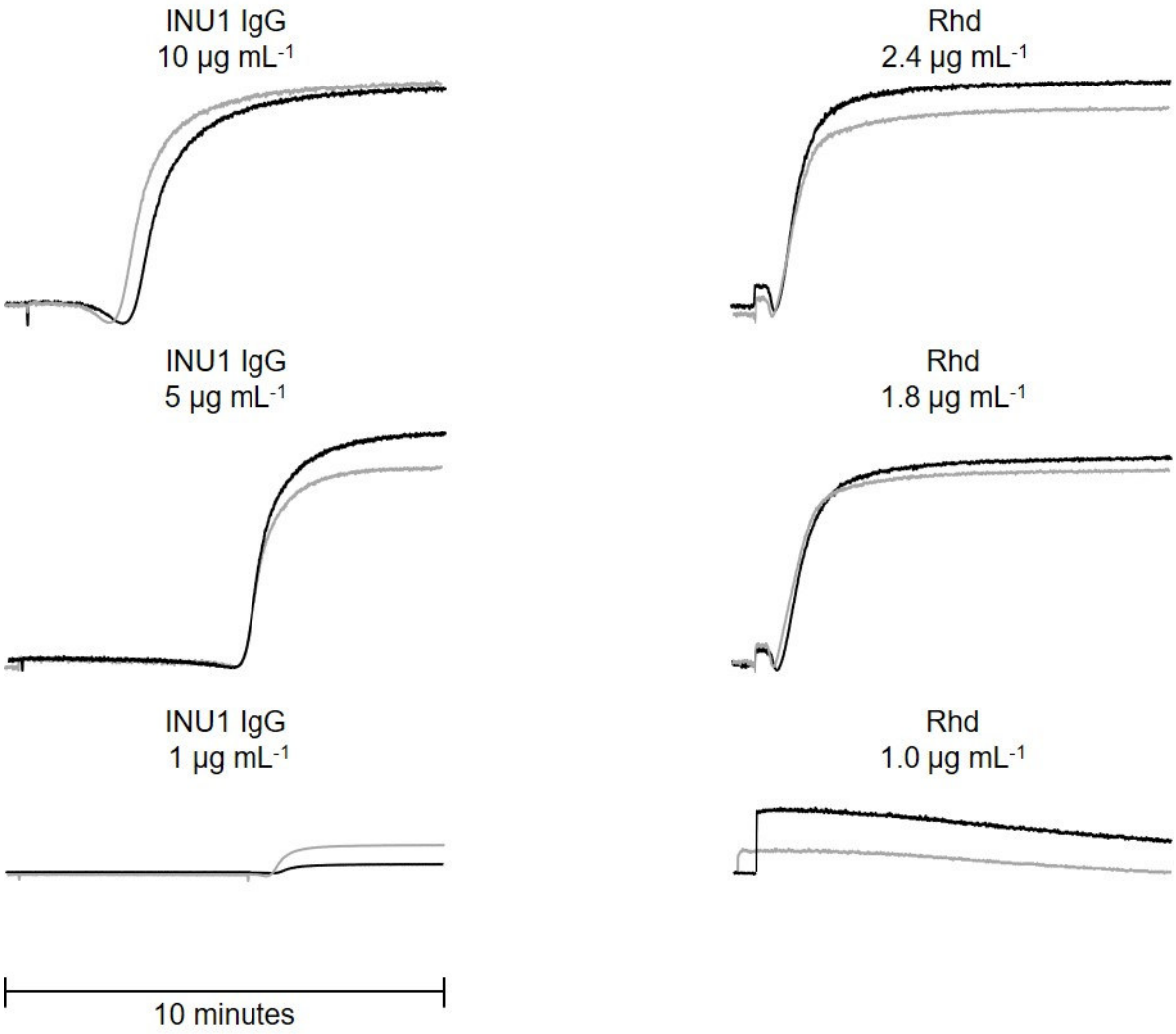
Supplemental Figure 5. Loss of thymosin β4 does not affect the expression levels of Profilin 1. Platelets were left untreated, lysed, and processed for immunoblotting. Phosphorylated Pfn1 and total Pfn1 were probed with the respective antibodies. GAPDH served as loading control.



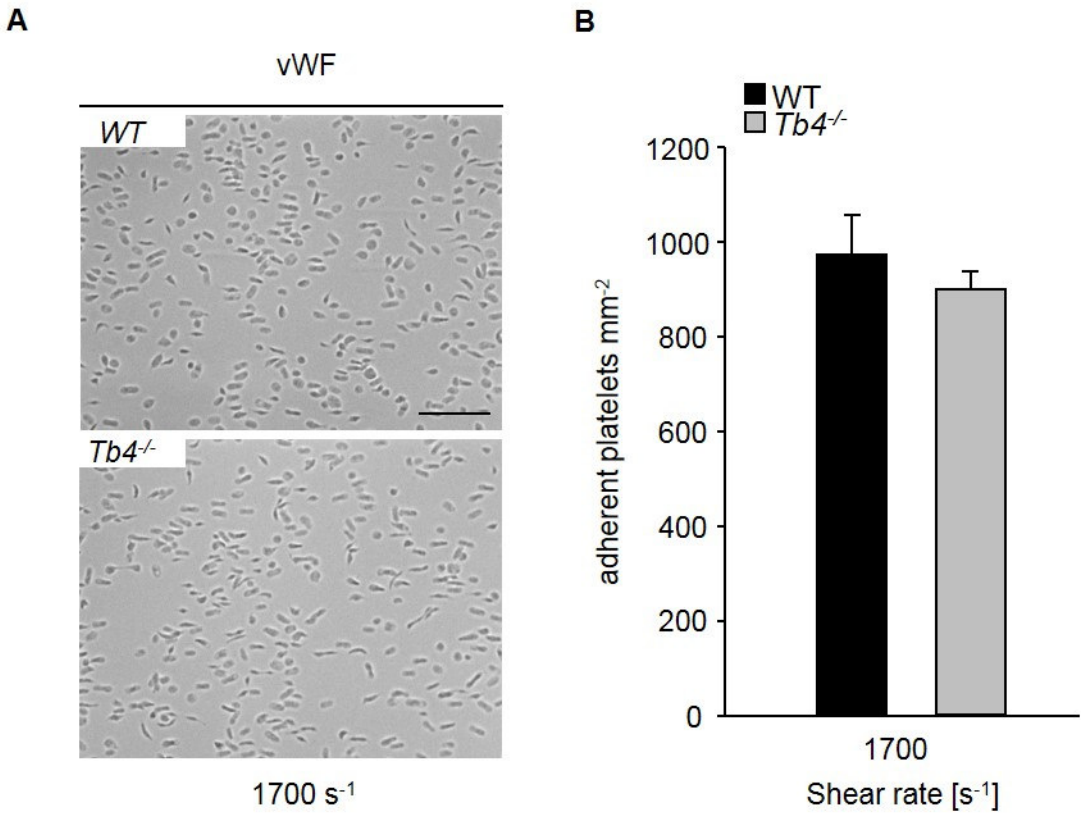
Supplemental Figure 6. No major changes in the F-actin structure in *Tmsb4x* KO platelets. Confocal fluorescence microscopy images of resting (poly-L-lysine) and spread platelets (15 and 30 min) on fibrinogen (100 µg mL^{-1}) immunostained for F-actin (red) and α-tubulin (green). Scale bar: 3 µm. Images were acquired with a TCS SP5 confocal microscope (100x/1.4 oil STED WHITE objective, Leica Microsystems) and are representative of at least 3 mice per group.



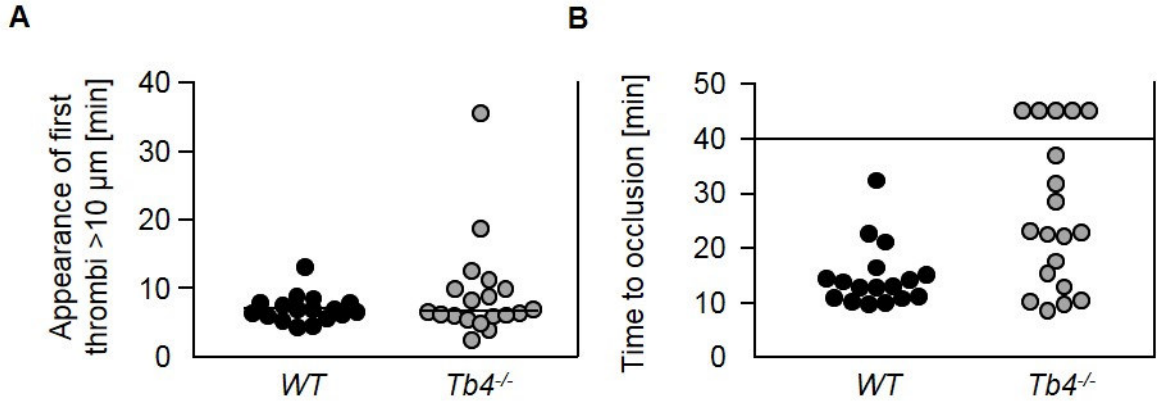
Supplemental Figure 7. *Tmsb4x* KO platelets are not pre-activated. Activation of platelet αIIbβ3 integrin (JON/A-PE) in WT and *Tmsb4x* KO platelets upon stimulation with the indicated agonists was determined by flow cytometry (n = 4). U46, U46619; Epi, epinephrine. Unpaired, two-tailed Student's t-test. *P<0.05, **P<0.005.



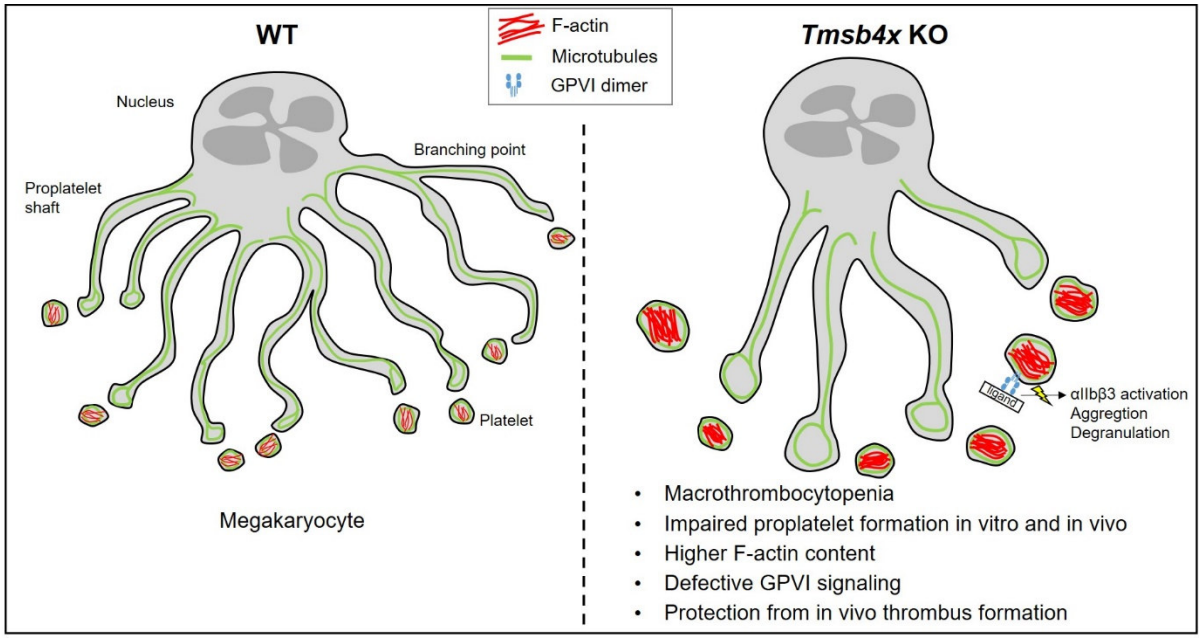
Supplemental Figure 8. Unaltered aggregation of thymosin β4 KO platelets in response to CLEC-2 stimulation. Aggregation responses of washed platelets in turbidometric aggregometry in response to the anti-CLEC-2 antibody INU1 (10), or Rhd (rhodocytin). Displayed are representative traces of 6 mice per group.



Supplemental Figure 9. *Tmsb4x* KO platelets adhere to vWF under flow. (**A, B**) Heparinized whole blood of WT and *Tmsb4x* KO mice was perfused over a vWF-coated cover slip for 4 min at a shear rate of 1700 s^{-1}. (**A**) Representative phase contrast images taken at the end of the perfusion time and (**B**) analysis of the number of adherent platelets per mm^2 ± SD was performed. Images were acquired with a Zeiss Axiovert 200 inverted microscope (40x/0.6 oil objective). Images are representative of at least 12 animals per group. Scale bar: 50 µm. Unpaired, two-tailed Student's *t*-test.



Supplemental Figure 10. $FeCl_3$-induced arterial thrombosis. (**A, B**) Adhesion of platelets (**A**) and occlusion of mesenteric arterioles (**B**) in WT and *Tmsb4x* KO mice upon $FeCl_3$-induced injury of the endothelial barrier. Each symbol represents 1 mesenteric arteriole (n = 9 mice per genotype).



Supplemental Figure 11. Thymosin β4 is essential for platelet formation and function. Loss of thymosin β4 results in defective proplatelet formation in vitro and in vivo leading to a macrothrombocytopenia with only mildly increased platelet volume and an unaltered platelet life span. Thymosin β4 deficient platelets display markedly decreased G-actin levels and concomitantly increased F-actin levels resulting in accelerated spreading on fibrinogen. Moreover, Thymosin β4 deficient platelets show enhanced clot retraction, activation defects and an impaired immunoreceptor tyrosine-based activation motif (ITAM) signaling downstream of the activating collagen receptor glycoprotein (GP) VI. Together, these defects translate into impaired aggregate formation under flow, protection from occlusive arterial thrombus formation in vivo and increased tail bleeding times.

Supplemental Video 1. Rapid proplatelet formation in WT mice. Intravital two-photon microscopy of BM MKs in the skull of a control animal. MKs were labeled using an anti-GPIX antibody derivative coupled to Alexa Fluor 488. Vessels were visualized using BSA-Alexa Fluor 546 as well as an anti-CD105 antibody coupled to Alexa Fluor 546. (20x objective; Frame: 5450.52 ms; 1017x1017 pixel). Scale bar: 25 µm.

Supplemental Video 2. Thickened proplatelet protrusions in *Tmsb4x* KO mice. Altered proplatelet morphology of BM MKs in the skull of *Tmsb4x* KO mice as visualized by two-photon microscopy. MKs and vessels were stained as described above. (20x objective; Frame: 5568.42 ms; 1039x1039 pixel). Scale bar: 25 µm.

Supplemental Video 3. $FeCl_3$-induced arterial thrombus formation in WT mice. Intravital microscopy of arterial occlusive thrombus formation after $FeCl_3$-induced injury of a mesenteric arteriole. Platelets were labeled using an anti-GPIX antibody derivative coupled to Dylight488. (10x objective; Zeiss Axiovert 200 inverted microscope equipped with a 100-W HBO fluorescent lamp source and a CoolSNAP-EZ camera). Images were acquired every second.

Supplemental Video 4. $FeCl_3$-induced arterial thrombus formation in *Tmsb4x* KO mice. Intravital microscopy of arterial thrombus formation and embolization after $FeCl_3$-induced injury of a mesenteric arteriole. Platelets were labeled using an anti-GPIX antibody derivative coupled to Dylight488. (10x objective; Zeiss Axiovert 200 inverted microscope equipped with a 100-W HBO fluorescent lamp source and a CoolSNAP-EZ camera). Images were acquired every second.