Actin/microtubule crosstalk during platelet biogenesis in mice is critically regulated by Twinfilin1 and Cofilin1

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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 300 µL heparin (20 U mL^{-1}, Ratiopharm) under isoflurane anesthesia and blood was washed twice using Tyrode-HEPES buffer as described previously.[1] Briefly, 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 minutes 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 minutes prior to experiments.

Flow cytometry

For assessment of platelet activation, 50 µL of blood were withdrawn under isoflurane anesthesia, washed twice with Tyrode-HEPES buffer and finally diluted (1:20) in Tyrode-HEPES buffer containing 2 mM Ca^{2+}.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 minutes at 37°C). Analyses were performed at a FACSCalibur (BD Biosciences).[3]

Platelet lifespan and recovery

Platelet lifespan in vivo was assessed by i.v. injection of a non-cytotoxic Dylight-488-labeled antibody derivative directed against GPIX and measurement of the proportion of labeled platelets over 5 consecutive days using a FACSCalibur (BD Biosciences). In order to determine the recovery of platelet counts following depletion in vivo, 50 µg of a platelet-depleting antibody (R300, Emfret, Germany) were injected i.p. into mice and platelet size and count were measured over 7 days in the same cohort of mice at a FACSCalibur (BD Biosciences). Analysis was done by normalizing platelet counts to the respective basal values at day 0.

Aggregometry

Washed platelets (1.5 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).

Transmission electron microscopy of platelets

Mice were bled into 300 µl heparin (20 U mL^{-1} in TBS). Platelets were washed as described above and subsequently 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. Granules and microtubule coils in platelets were counted manually using ImageJ Software (NIH).

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 at 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 FACS Calibur (BD Biosciences).

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), anti-α-tubulin Alexa F488 (3.33 µg mL^{-1}, 322588 (B-5-1-2), Invitrogen), anti-acetylated tubulin (4 µg mL^{-1}, sc-23950, Santa Cruz Biotechnology) and anti-detyrosinated tubulin (2 µg mL^{-1}, AB3201, Merck Millipore). For toxin studies, platelets were pretreated with 10 µM colchicine and afterwards allowed to spread on fibrinogen. Samples were mounted with Fluoroshield (F6182, Sigma-Aldrich) and images acquired using a Leica TCS SP8 confocal microscope (Leica Microsystems).

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 an anti-GPIX antibody (56F8, 1.33 mg mL^{-1}), to specifically label platelets and MKs, an anti-CD105 antibody (3.33 mg mL^{-1}, 120402 (MJ7/18), Biolegend) to stain the endothelium as well as Atto647N-conjugated phalloidin (170 nM, 65906, Fluka). 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 and spreading

Bone marrow from femora was flushed, 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) or fibrillary collagen type I (50 µg mL^{-1} Collagen Horm® suspension, Takeda) for 3h at 37°C. For colchicine treatment, day 5 MKs were divided into 2 wells and the respective wells were either treated with 500 µM colchicine or a vehicle control as described previously[5]. Enriched MKs were then resuspended in StemPro medium containing 50 ng mL^{-1} TPO. MKs were then seeded onto coated coverslips and incubated for 3h. Cells were washed with PBS, fixed and permeabilized for 30 min with 4% PFA in PBS containing 0.1% TritonX-100. MKs were stained overnight using anti-α-tubulin Alexa F488 (3.33 mg mL^{-1}, 322588 (B-5-1-2), Invitrogen), phalloidin-Atto647N (170 nM, 65906, Fluka), anti-acetylated tubulin (4 µg mL^{-1}, sc-23950, Santa Cruz Biotechnology) and anti-detyrosinated tubulin (2 µg mL^{-1}, AB3201, Merck Millipore), anti-APC (,1 µg mL^{-1}, ab15270, abcam) and anti-Arp2 (2 µg mL^{-1}, ab49674, abcam). Slides were washed with PBS the following day and mounted using DAPI-containing Fluoroshield (1 µg mL^{-1}, D1306, Invitrogen). Samples were analyzed at a Leica TCS SP8.

qPCR

MKs at day 6 of culture were washed once with PBS once and lysed in 500 µl Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA extraction was performed with the RNeasy Mini Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer's recommendations. Reverse transcription was applied by the iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following the manufacturers' protocol using random primers and a starting amount of 500 ng RNA. Quantitative real-time PCR was performed with the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Relative expression was calculated by the ΔΔCt method in correlation to the housekeeping genes *Sdha* and *Actb*. Primer sequences are as follows:

Apc (ACCATGTACCCAGGCATTG; TACTCCTGCTTGCTGATCC)

Diaph1 (TTAACAGCGCGGCACGAG; GGTGCAACAACCACAGCAG)

Sdha (GACATCAAGACTGGCAAGGTTAC; AGTAGGAGCGGATAGCAGGAG)

Actb (ACCATGTACCCAGGCATTG; TACTCCTGCTTGCTGATCC).

Immunoblotting

For preparation of platelet protein lysates, washed platelets (1 x 10^6 mL^{-1}) were either left untreated or activated with 50 ng mL^{-1} TPO, 10 µM ionomycin or 0.1 U mL^{-1} thrombin with or without 20 µg mL^{-1} CRP and subsequently lysed for 30 min on ice at the indicated time points. Samples were centrifuged and the supernatant was stored at -80°C until analysis. For preparation of MK protein lysates, MKs at day 6 of culture were washed once with PBS/EDTA and lysed using 1x RIPA buffer.

Denatured proteins were separated by SDS-PAGE and blotted onto PVDF membranes. Membranes were probed for Twf1 (1 µg mL^{-1}, #8461, Cell Signaling Technology), Cof1 (1 µg mL^{-1}, #3318, Cell Signaling Technology), α-tubulin (1 µg mL^{-1}, T6074, Sigma-Aldrich), P-Jak2 (1 µg mL^{-1}, #3771, Cell Signaling Technologies), Jak2 (1 µg mL^{-1}, #3230, Cell Signaling Technologies), P-Pfn1 (1 µg mL^{-1}, PK6930, ECM Biosciences), Pfn1 (1 µg mL^{-1}, PK6930, ECM Biosciences), P-Lim-Kinase (1 µg mL^{-1}, #3841, Cell Signaling Technologies), Lim-Kinase (1 µg mL^{-1}, #3842, Cell Signaling Technologies), APC (2 µg mL^{-1}, ab15270, abcam), mDia1 (2 µg mL^{-1}, ab96784, abcam), RhoA (2 µg mL^{-1}, ARH04, Cytoskeleton), anti-acetylated tubulin (4 µg mL^{-1}, sc-23950, Santa Cruz Biotechnology), anti-detyrosinated tubulin (2 µg mL^{-1}, AB3201, Merck Millipore), GAPDH (1 µg mL^{-1}, G5262, Sigma-Aldrich) and β3-integrin (1 µg mL^{-1})[6] 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 at an Amersham Image 680 (GE Healthcare).

TPO Signaling in MKs

350.000 MKs were seeded into a 24-well plate and starved for 4 h in DMEM containing 0.5 % FCS. Afterwards, they were stimulated using 50 ng mL^{-1} TPO for 10 min, washed once and immediately lysed on ice using 1x RIPA containing 1x Halt protease and phosphatase inhibitor (78440, ThermoFisher Scientific). JAK2 and STAT3 total protein and/or phosphorylation levels were analyzed using an automated capillary-based immunoassay platform; Jess (ProteinSimple). Briefly, lysates were prepared by the addition of 5 x master mix containing 200 mM dithiothreitol (DTT), 5 x sample buffer and fluorescent standards (Standard Pack 1, PS-ST01-8) and boiled for 5 minutes at 95°C according to the manufacturer's instructions. All primary antibodies were diluted in Antibody Diluent 2 (042-203). Samples, Antibody Diluent 2, primary and secondary antibodies, Luminol-S (043-311) and Peroxide (043-379) mix and wash

buffer were displaced into 12-230 kDa prefilled microplates, (pre-filled with separation matrix 2, stacking matrix 2, split running buffer 2 and matrix removal buffer, SM-W004). To operate Jess and analyze results Compass Software for Simple Western was used (version 4.1.0, ProteinSimple). Separation matrix loading time was set to 200 seconds, stacking matrix loading time to 15 seconds, sample loading time to 9 seconds, separation time to 31 minutes, separation voltage to 375 volts, antibody diluent time to 5 minutes, primary antibody incubation time to 90 minutes and secondary antibody incubation time to 30 minutes. To record the chemiluminescent signal, the High Dynamic Range (HDR) profile was used. The optimized dilution for the JAK2 antibody (#3230, CST) was 1:20 on 0.5 mg/ml lysate concentration, for the JAK2 p-Tyr1007/1008 antibody (#3771, CST) was 1:10 on 0.5 mg/ml lysate concentration, for the STAT3 antibody (#9139, CST) was 1:40 on 0.2 mg/ml lysate concentration and for the STAT3 p-Tyr705 antibody (#9138, CST) was 1:10 on 0.2 mg/ml lysate concentration.

PPF of BM MKs

BM from femora and tibias was flushed and lineage depletion was performed by magnetic separation using an antibody mixture (Lineage depletion panel, 133307, Biolegend) as well as magnetic beads (CD4 untouched, Miltenyi Biotec, Germany). Cells were incubated for 72 h in the presence of 50 ng mL^{-1} TPO as well as 100 U mL^{-1} hirudin (rHirudin, Hyphen Biomed, RE120A) in Dulbecco's Modified Eagle Medium. On day 3, a BSA density gradient was performed and cells were incubated overnight in medium containing TPO and rHirudin. Proplatelet formation was analyzed using a Zeiss PrimoVert. Approximately 600 cells were counted per mouse. For immunofluorescent stainings, round glass slides were coated with 0.01% Poly-L-Lysine (P8920, Sigma-Aldrich). MK culture was diluted in platelet buffer and spun down onto the prepared glass slides. Cells were fixed, permeabilized and stained with anti-α-tubulin Alexa F488 (3.33 mg mL^{-1}, 322588 (B-5-1-2), Invitrogen), phalloidin-Atto647N (170 nM, 65906, Fluka), anti-vWF, anti-acetylated tubulin (4 µg mL^{-1}, sc-23950, Santa Cruz Biotechnology) and anti-detyrosinated tubulin (2 µg mL^{-1}, AB3201, Merck Millipore). Proplatelet morphology was assessed using a Leica TCS SP8. For the analysis of F-actin distribution in round MKs, the cells were enriched by BSA density gradient, washed in PBS/EDTA and afterwards stained in suspension using phalloidin-Atto647N and an Alexa488-conjugated anti-GPIX antibody (56F8, 1.33 mg mL^{-1}). MKs were imaged using a Leica TCS SP8 inverted confocal microscope. Maximum intensity projections were analyzed using ImageJ software (NIH).

Live Imaging of PPF

Following enrichment by BSA density gradient, TPO- and rHirudin-conditioned MKs were

diluted in CO_2-independent medium (5.33 mM KCl, 0.441 KH_2PO_4, 0.338 mM Na_2HPO_4, 5.56 mM glucose, 138 mM sodium chloride, 185 mg L^{-1}, 4.17 mM $NaHCO_3$, 97.6 mg L^{-1} $MgSO_4$, 1 % L-glutamine, 1 % penicillin/streptomycin, 2 % essential amino-acids, 1% non-essential amino acids, 2.5 % HEPES pH 7, 10 % FCS) and imaged for 24h at 37°C using a confocal microscope (Leica TCS SP8). Analysis was done using ImageJ Software (NIH).

Plasma TPO levels

Plasma TPO levels were determined using a Mouse Thrombopoietin Quantikine ELISA Kit (DY488, R&D Systems). Briefly, plasma was collected, diluted (1:5 in Reagent Diluent) and immediately applied as duplicates onto the anti-TPO-IgG coated 96-well plate and incubated for 2 h at RT. The plate was washed five times and incubated for 2 h with 100 µL of horseradish peroxidase-conjugated anti-mouse TPO antibodies. After another five washing steps, tetramethylbenzidine solution was added and incubated for 30 min at RT. The reaction was stopped by adding 100 µL of diluted hydrochloric acid. Optical densities of the samples were determined using a Multiskan Ascent (96/384) plate reader (MTX Lab Systems) at 450 nm. Wavelength correction was performed at 570 nm.

MK progenitor FACS

Flushed BM was poured through a 70 µm cell strainer, centrifuged and resuspended in 1 mL MACS buffer (1x PBS, 5% FCS, 0.1% sodium acidic). 100 µL of the total BM were used for the subsequent FACS measurements. Antibody mixture (1:200 in MACS buffer, CD34-PE, c-Kit-AF700, Sca-1-AF647, CD150-Bv650, CD48-PacB, Flk2/Flt3-Cy5.5, FITC-labeled lineage mixture, CD16/32-Cy7, GPIX-546) was added and cells were stained for 30 min at 4°C, followed by a dead cell marker staining (1:300 in PBS) for 30 min at 4°C. The pellet was resuspended in 250 µL MACS buffer and cells were analyzed using a FACS Celesta Cell Analyzer (BD Biosciences).

Determination of MK ploidy

Femora of mice 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 5D7). Finally, the cells were fixed, permeabilized and the DNA was stained using 50 mg mL^{-1} propidium iodide (P3561, Invitrogen) staining solution containing 100 mg mL^{-1} RNaseA (EN0202, Fermentas) in PBS.

Analysis was performed by flow cytometry and FlowJo software (Tree Star Inc., Ashland, USA).

Tail bleeding assay

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 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. Mice were sacrificed after 20 min. Differences between the mean bleeding times were statistically assessed using unpaired Student's t-test and differences between definite and infinite bleeding were determined by Fisher's exact test.

Imaging of proplatelet formation in vivo by 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.[7] 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.

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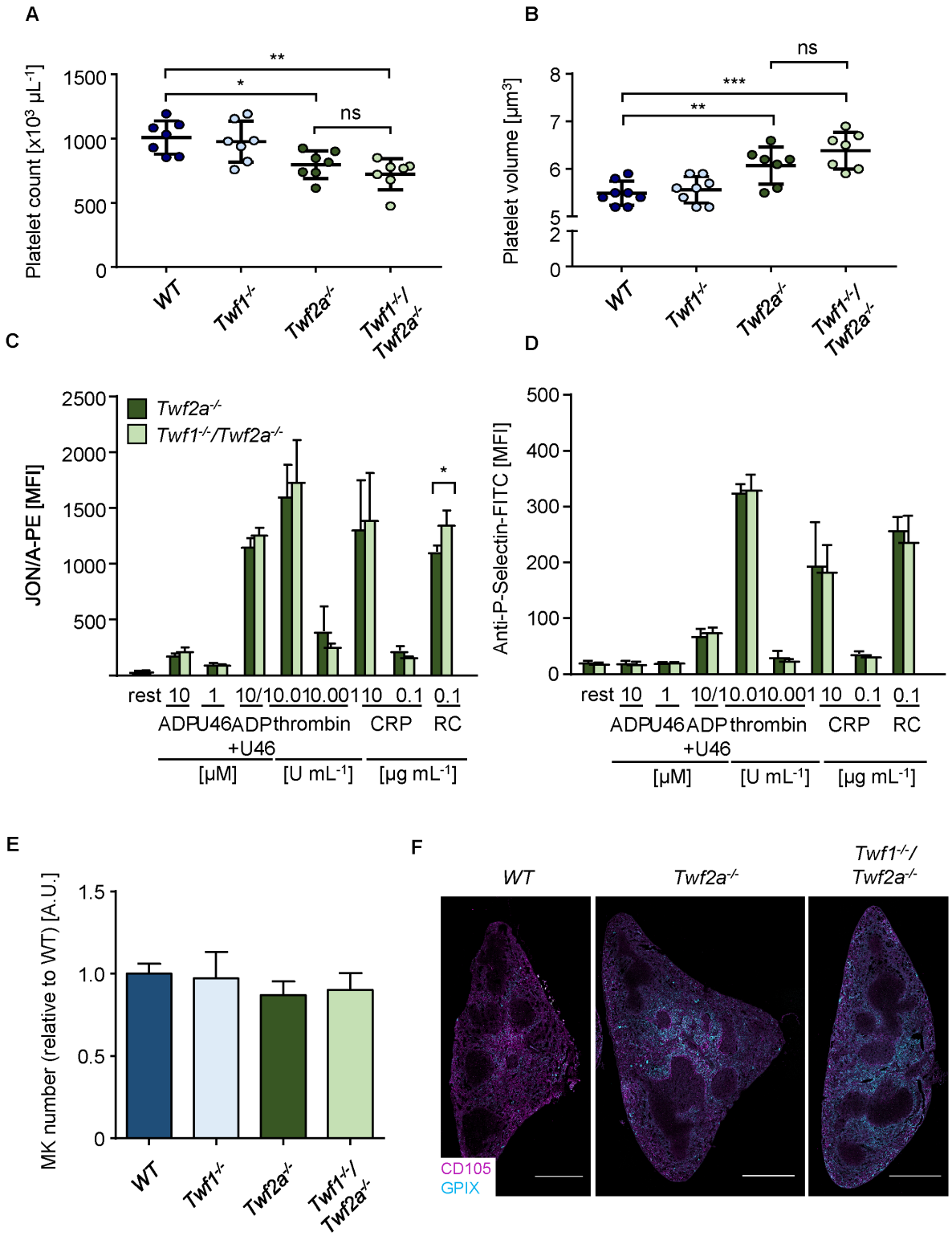
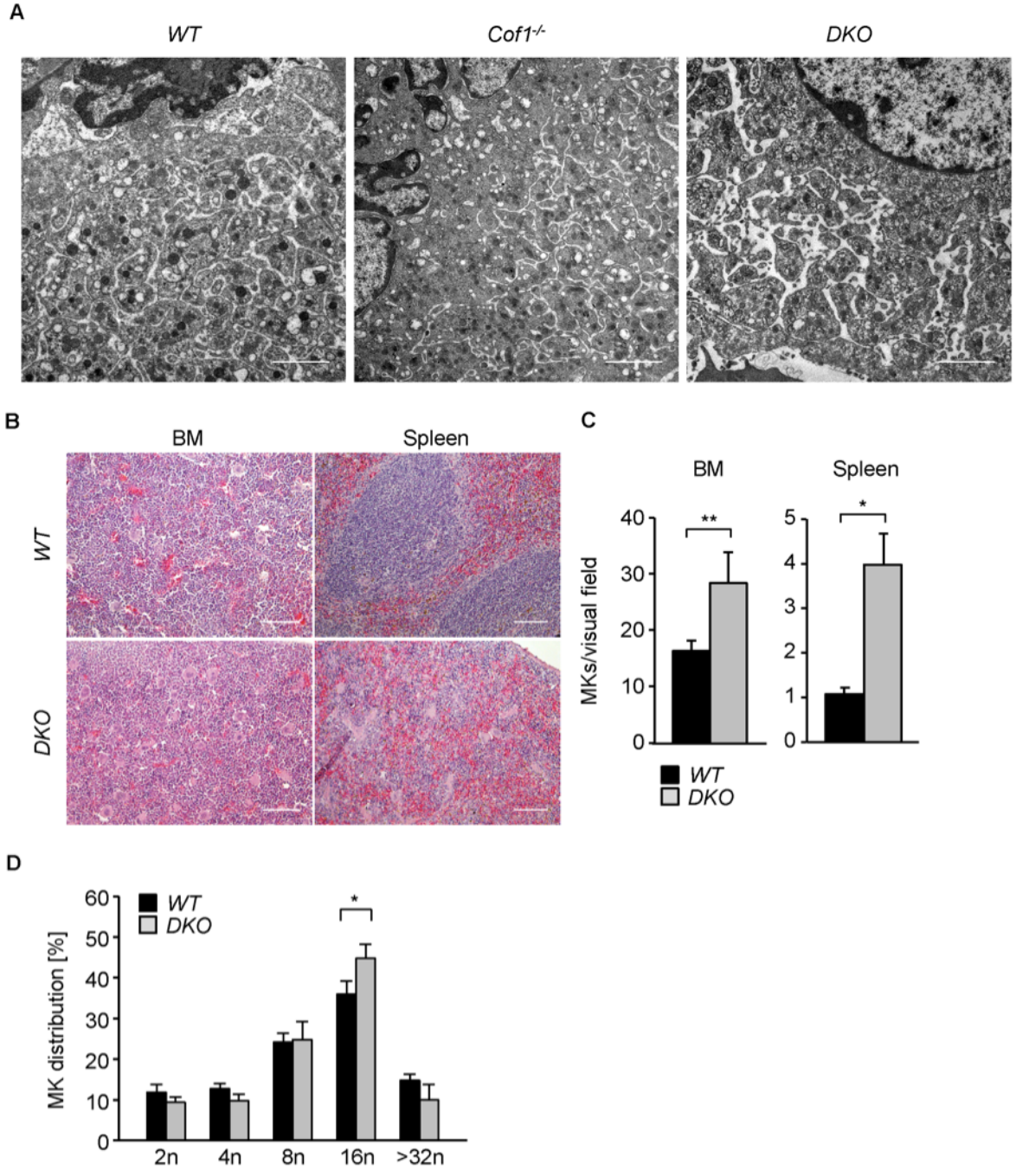
SUPPLEMENTAL FIGURES & LEGENDS

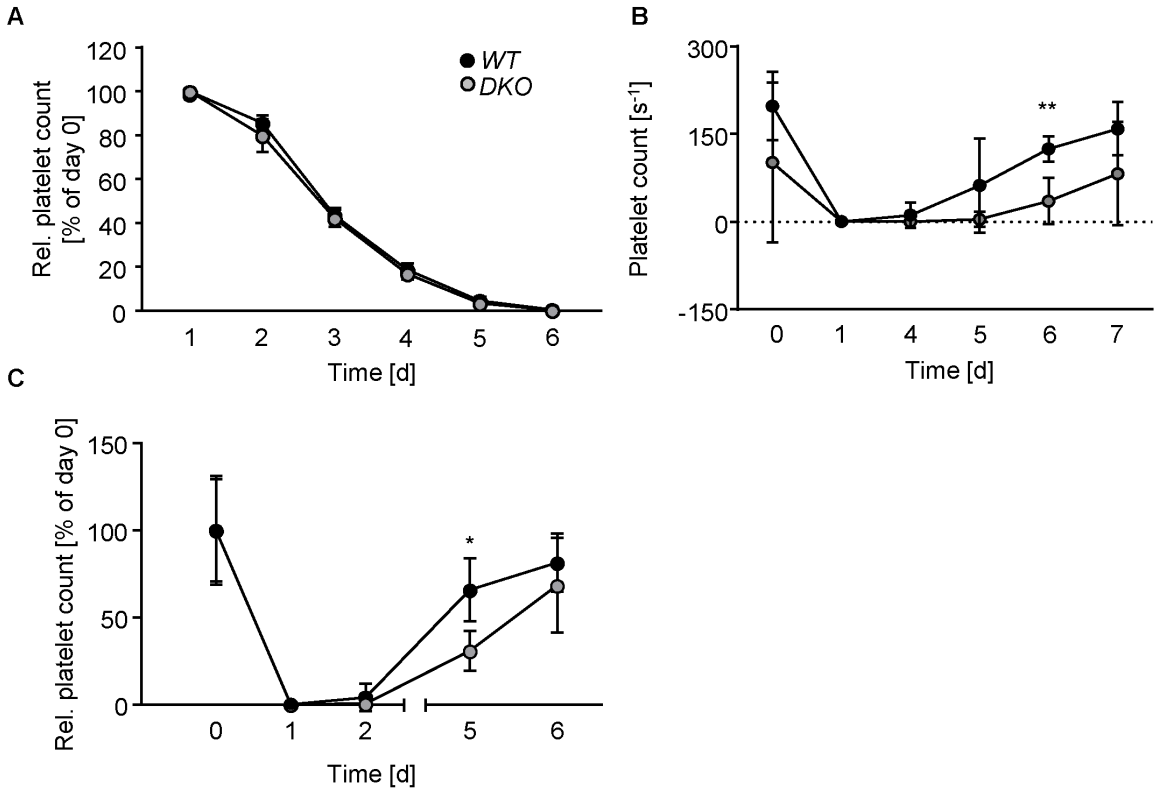


Supplemental Figure 1. Twf1 and Twf2a have non-redundant functions. (**A, B**) Platelet count (**A**) and size (**B**) were determined using an automated blood cell analyzer (ScilVet, Germany). Values are mean ± SD (n = 7). One-way ANOVA with Sidak correction for multiple comparisons. *P < 0.05; **P < 0.01; ***P < 0.001. (**C, D**) Integrin αIIbβ3 activation (**C**) and

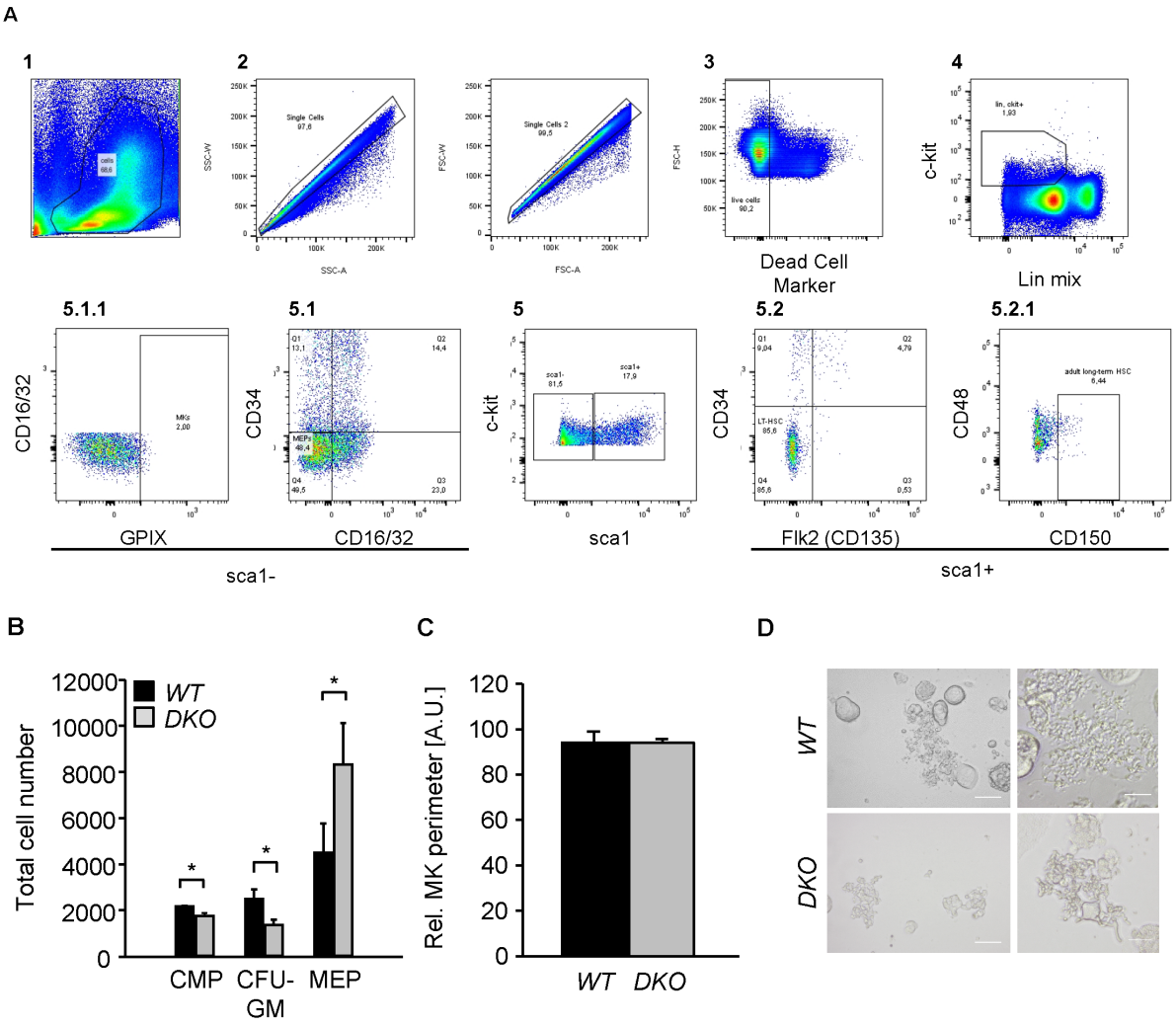
degranulation (**D**) of *Twf2a*$^{-/-}$ and *Twf1/2a*$^{-/-}$ platelets upon stimulation were analyzed by flow cytometry. Values are mean ± SD (n = 4). Unpaired, two-tailed Student's t-test. *P < 0.05. (**E**) MK numbers in whole femora cryosections were counted using ImageJ software. Values were normalized to *WT* levels. Mean ± SD (n = 3). (**F**) Cryosections of spleens were stained for CD105 and GPIX and analyzed by confocal microscopy (Leica TCS SP8; 25x objective). Scale bars: 750 µm.



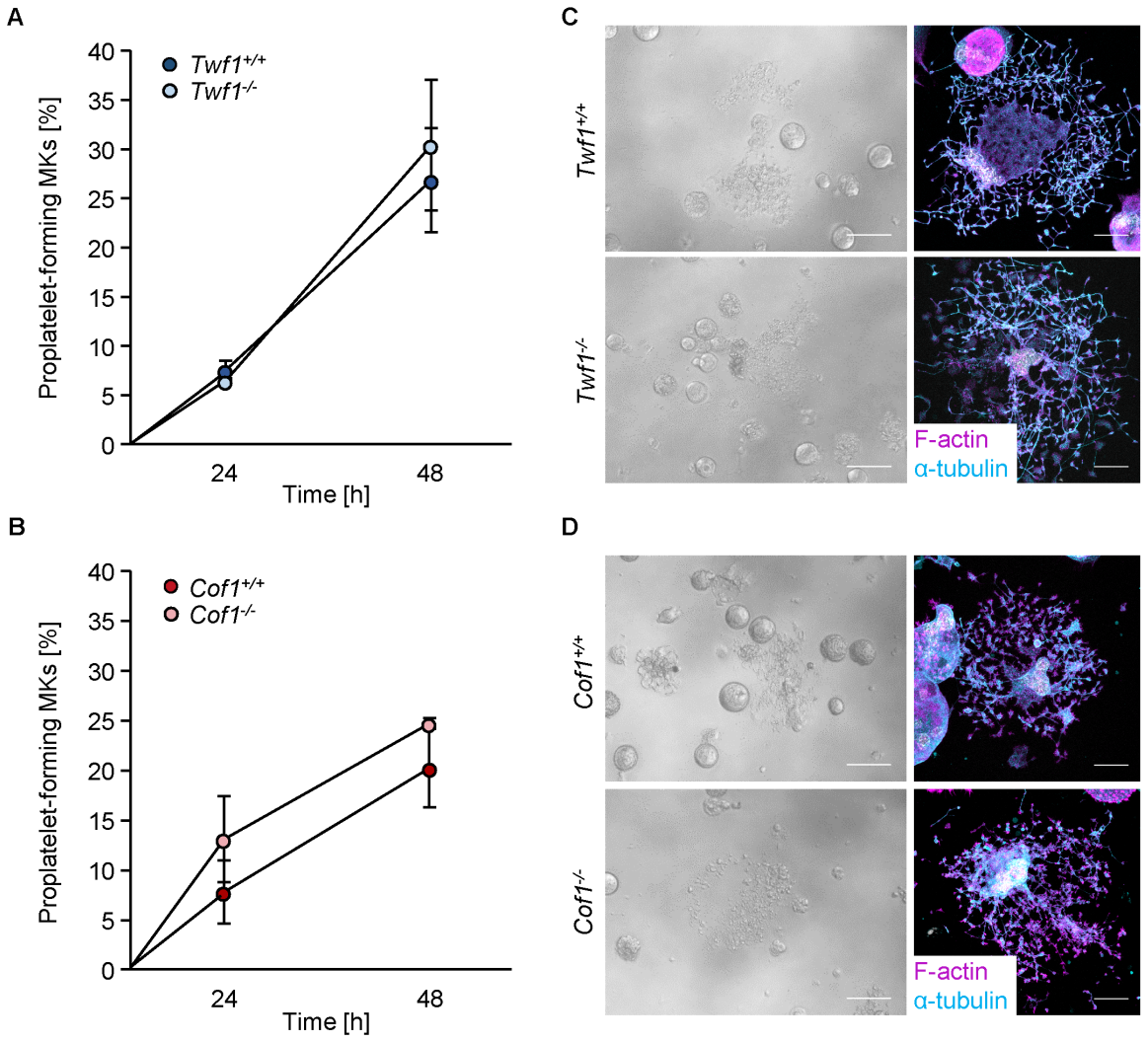
Supplemental Figure 2. Altered MK morphology and increased MK numbers in BM and spleen of Twf1/Cof1-deficient mice. (**A**) Transmission electron micrographs of *WT*, *Cof1*$^{-/-}$ and *DKO* BM MKs. Scale bars: 2 µm. (**B, C**) Hematoxylin-Eosin stainings of femur and spleen paraffin sections of *WT* and *DKO* mice (**B**) and quantification (**C**) of MK numbers. Scale bars: 100 µm. Values are mean ± SD (n = 3). Unpaired, two-tailed Student's t-test. *P < 0.05; **P < 0.01. (**D**) 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 FACS Calibur (BD Biosciences). Values are mean ± SD (n = 3). Unpaired, two-tailed Student's t-test. *P < 0.05.



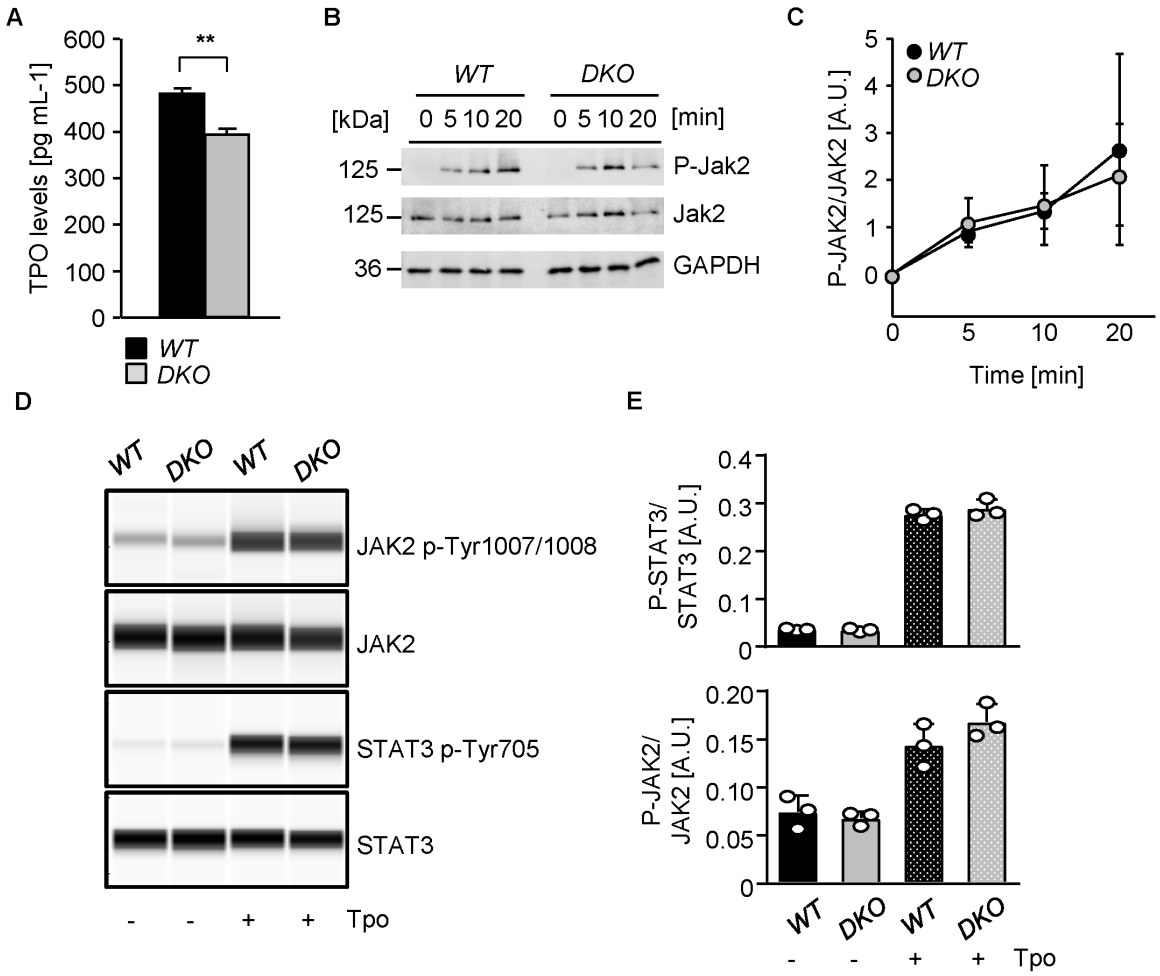
Supplemental Figure 3. Unaltered platelet lifespan, but delayed recovery in Twf1/Cof1-deficient mice. (**A**) Platelet lifespan was assessed by flow cytometric measurement of the fluorescence-positive platelet population at the indicated time points after injection of a fluorophore-conjugated anti-GPIX antibody derivative. (**B, C**) Platelet recovery was assessed upon depletion of platelets in *WT* and *DKO* mice by i.p. injection of a platelet depletion antibody (R300, Emfret). Platelet size and count were measured over 7 days at a FACS Calibur (BD Biosciences). Total platelet count per second (**B**) as well as relative count compared to day 0 (**C**) are shown. Values are mean ± SD (n = 5). Unpaired, two-tailed Student's t-test. *P < 0.05.



Supplemental Figure 4. Altered progenitor populations and MK morphology albeit unaffected maturation of *DKO* MKs in vitro. (**A, B**) Gating strategy and flow-cytometric analysis of MK precursors in flushed BM with adjusted cell count. Values are mean ± SD (n = 6). Unpaired, two-tailed Student's t-test. *P < 0.05. (**C**) TPO-conditioned BM MKs of *WT* and *DKO* mice were imaged and the relative size was analyzed using ImageJ Software. At least 50 MKs per animal were analyzed. Values are mean ± SD (n = 3). (**D**) Proplatelet formation of BM MKs after culturing in rHirudin- and TPO-conditioned medium was analyzed on day 4 using a brightfield microscope (Evos, Thermo Fisher Scientific). Scale bars: left panel: 100 µm, right panel: 50 µm.

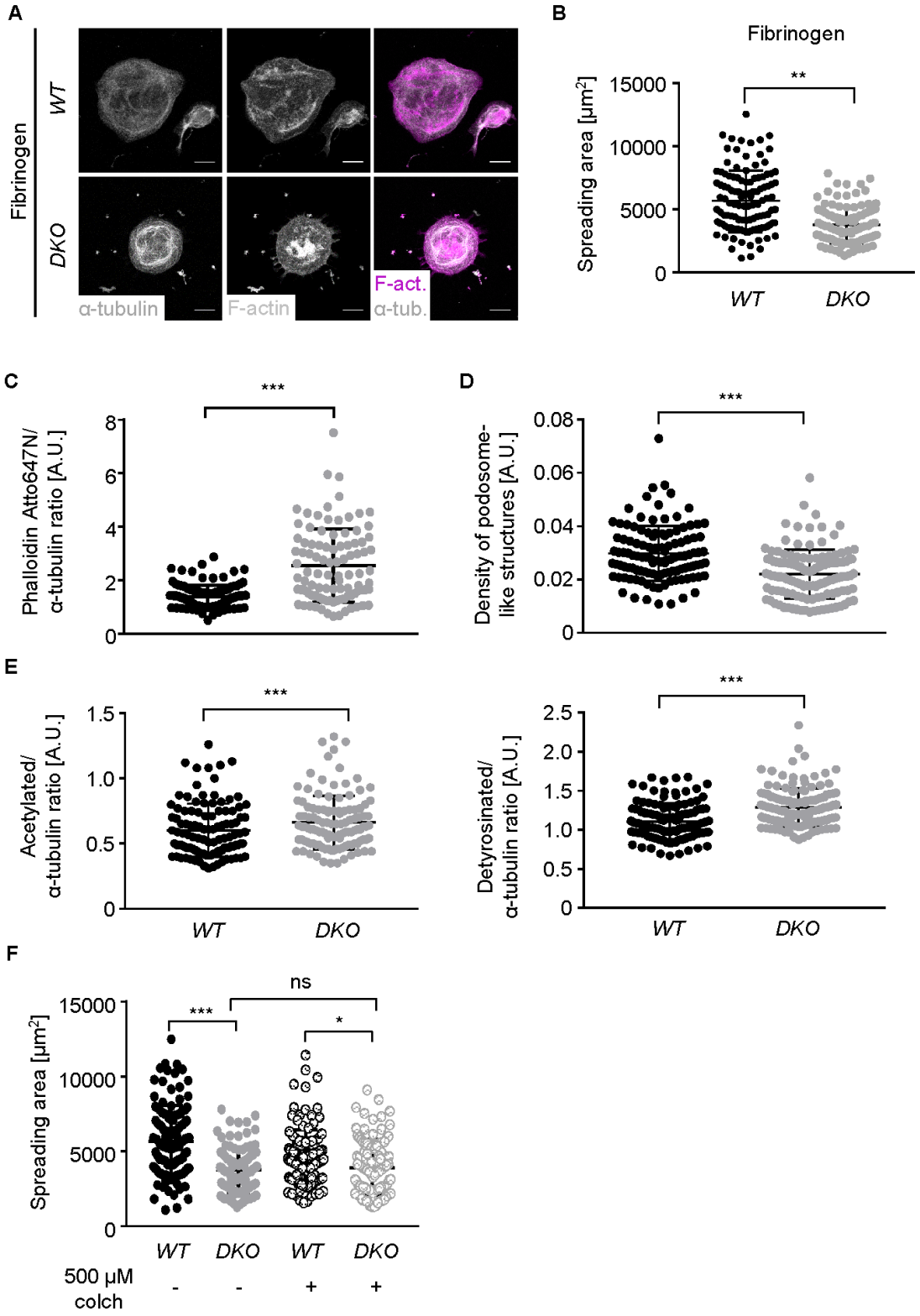


Supplemental Figure 5. Unaltered PPF of *Twf1*$^{-/-}$ and *Cof1*$^{-/-}$ MKs in vitro. PPF of *Twf1*$^{-/-}$ (**A**) or *Cof1*$^{-/-}$ (**B**) BM-derived and rHirudin- and TPO-conditioned MKs was analyzed on day 4 and 5 of culture under a light microscope (Leica). Average of 5 analyzed visual fields per MK culture is shown. Values are mean ± SD (n = 3). (**C, D**) MKs were seeded onto fibrinogen-coated slides and proplatelets were analyzed by brightfield (left panels) or confocal fluorescence microscopy (right panels) (Leica TCS SP8; 40x objective). Scale bars: left panel: 100 µm, right panel: 50 µm.



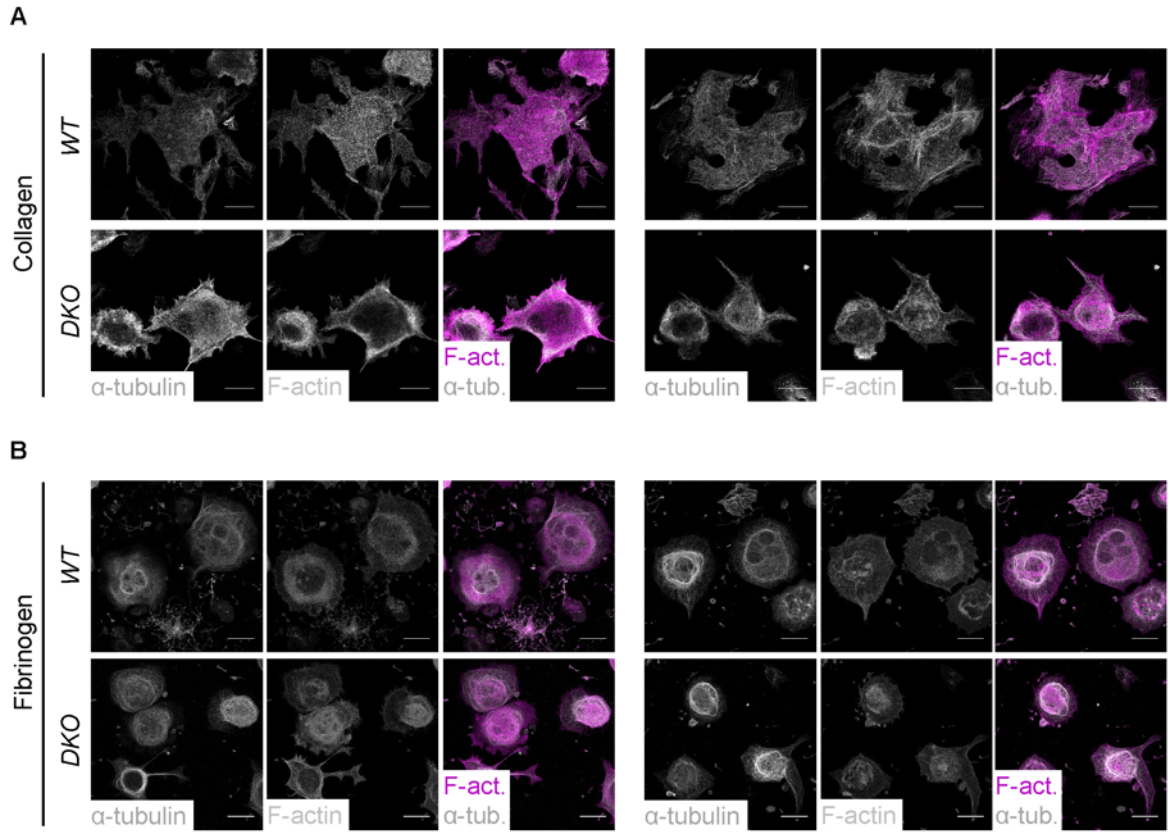
Supplemental Figure 6. Reduced plasma TPO levels but unaltered signaling in *DKO* platelets and MKs. (**A**) TPO-levels in plasma of *WT* and *DKO* mice were assessed in triplicates using a mouse TPO Quantikine ELISA Kit (R&D Systems, United States). Values are mean ± SD (n = 3). Unpaired, two-tailed Student's t-test. **P < 0.01. (**B, C**) *WT* and *DKO* platelets were stimulated with 50 ng mL^{-1} TPO for 20 min and JAK2 phosphorylation (Tyr1007/1008) was assessed by immunoblotting. (**B**) Images were acquired at an Amersham Image 680 (GE Healthcare). (**C**) Densitometric analysis was performed using ImageJ Software. Values are mean ± SD (n = 3). (**D, E**) Automated immunoblot (**D**) and densitometric quantification (**E**) of STAT3 and JAK2 phosphorylation following stimulation of MKs with TPO for 10 min. Images were acquired at an automated Western Blot machine (Jess,

Proteinsimple). Values are mean ± SD (n = 3).

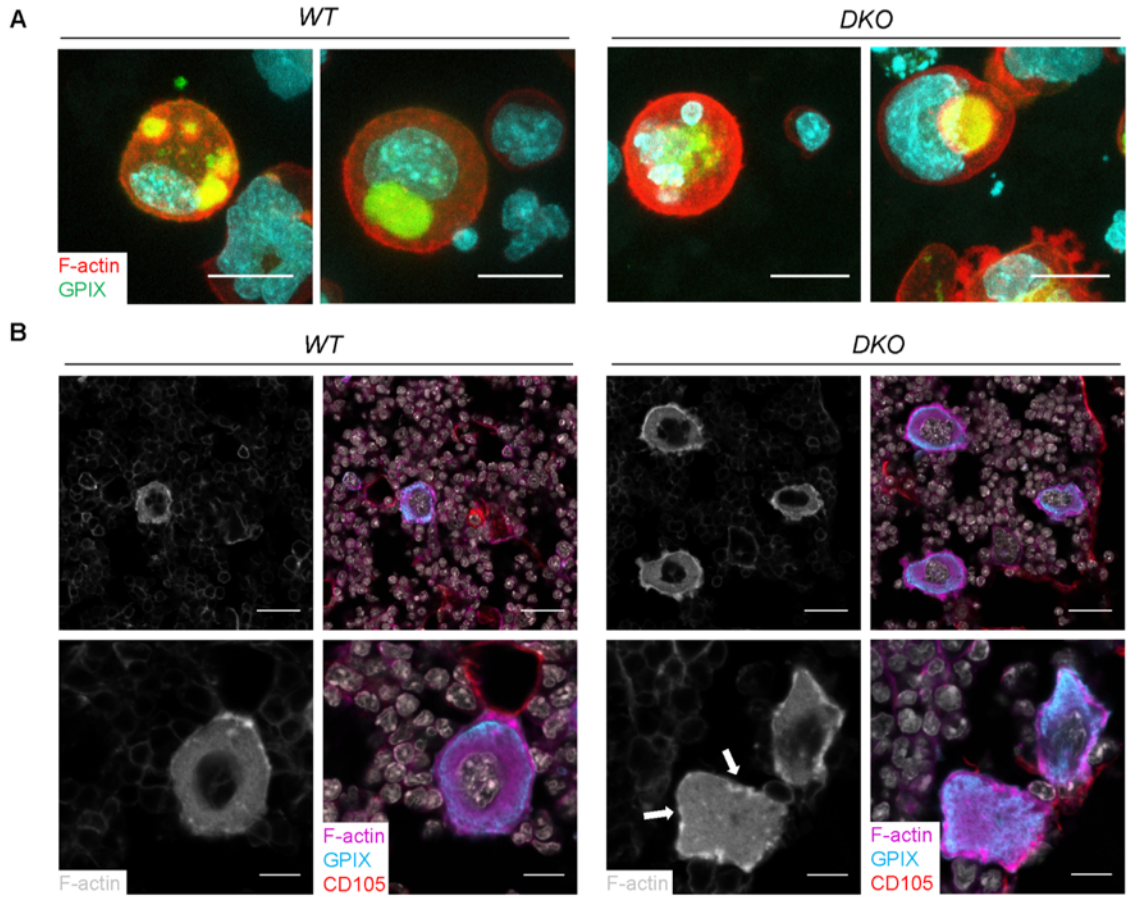


Supplemental Figure 7. Impaired spreading, podosome formation and increased MT stability in *DKO* MKs spread on fibrinogen. (**A**) Cultured BM MKs were allowed to spread on fibrinogen for 3 hours, imaged using confocal fluorescence microscopy (Leica TCS SP8,

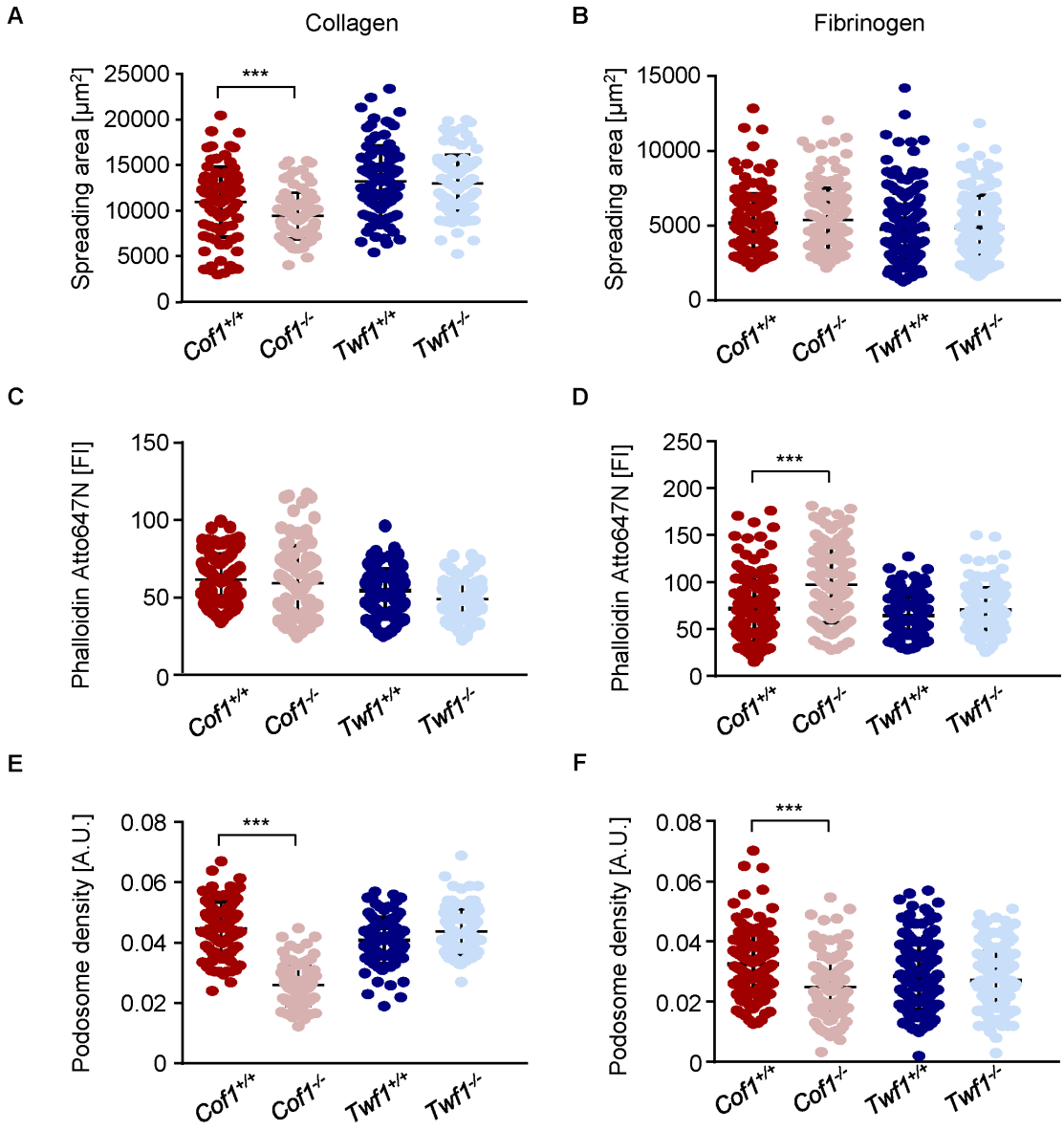
40x objective) and spreading area (**B**), actin MFI normalized to α-tubulin (**C**), density of podosome-like structures (**D**) and MT stability (detyrosination or acetylation) (**E**) were analyzed using ImageJ Software. At least 30 MKs were analyzed per animal. Values are mean ± SD (n = 3). Unpaired, two-tailed Student's t-test. *P < 0.05; **P < 0.01; ***P < 0.001. (**F**) MKs were left untreated or incubated with 500 µm colchicine for 30 min at 37°C and allowed to spread on fibrinogen for 3 hours. At least 30 MKs were analyzed per animal. Values are mean ± SD (n = 3). One-way ANOVA with Sidak correction for multiple comparisons. *P < 0.05; ***P < 0.001. A.U.: arbitrary unit; colch: colchicine; ns: non-significant.



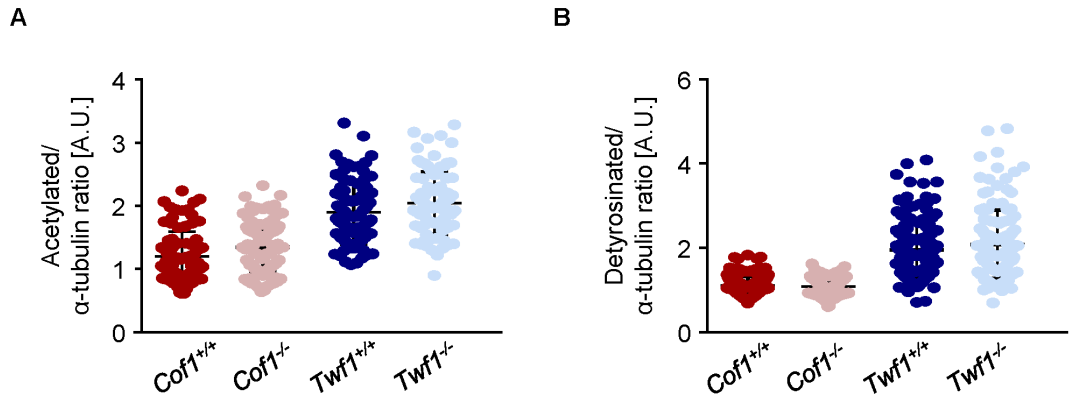
Supplemental Figure 8. Altered MK morphology of spread *DKO* MKs. (A) TPO- and rHirudin-conditioned MKs were allowed to spread on either collagen **(A)** or fibrinogen **(B)**. Distribution of α-tubulin and F-actin was analyzed by confocal immunofluorescence (Leica TCS SP8, 40x objective). Scale bars: 25 µm.



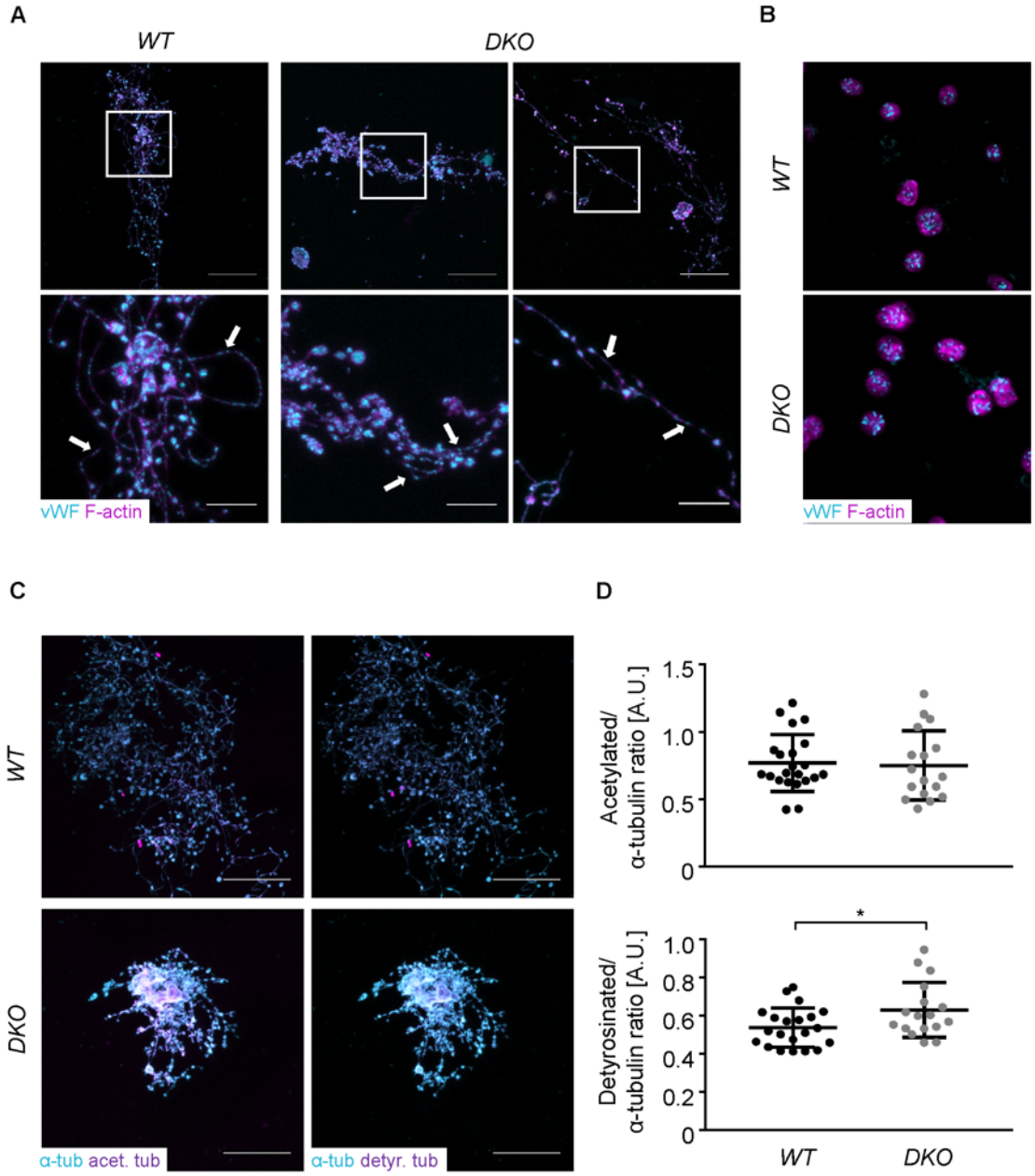
Supplemental Figure 9. Irregular F-actin distribution in *DKO* BM MKs in vitro and in situ. (**A**) Confocal images of *WT* and *DKO* MKs stained for α-tubulin and F-actin and analyzed in suspension at a Leica TCS SP8 (40x objective). Scale bars: 20 µm. (**B**) Femoral cryosections of *WT* and *DKO* mice stained for endothelial cells (CD105), megakaryocytes (GPIX) and F-actin (phalloidin) analyzed by confocal microscopy (Leica TCS SP8; 40x objective). Arrows point to irregular F-actin structures (n = 3). Scale bars: upper panel: 25 µm; lower panel: 10 µm.



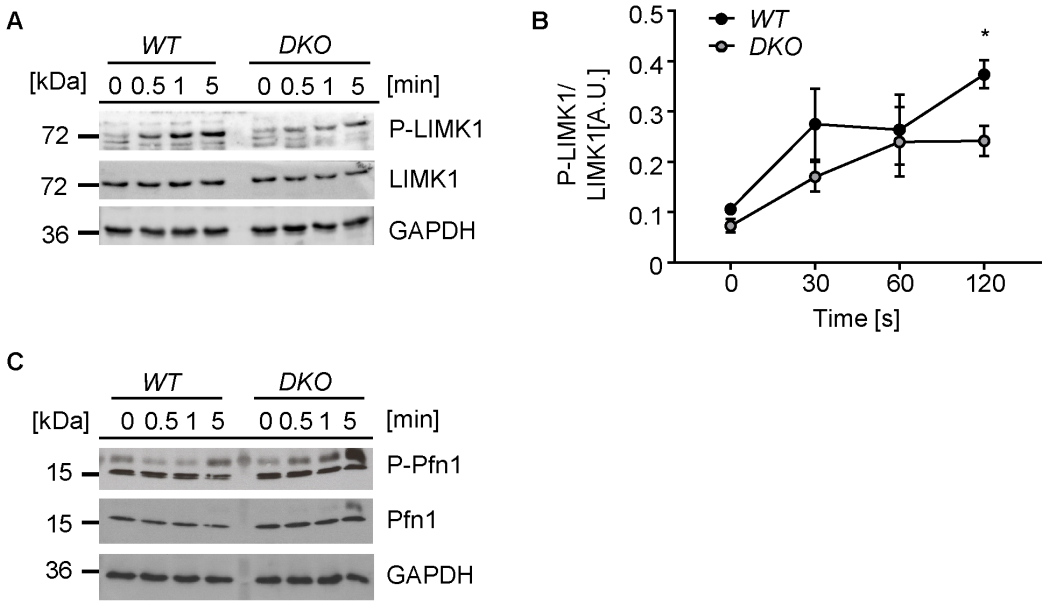
Supplemental Figure 10. Spreading ability and podosome formation of *Cof1*$^{-/-}$, but not *Twf1*$^{-/-}$ MKs are mildly impaired. Quantification of spreading area (**A, B**), F-actin MFI (**C, D**) as well as density of podosome-like structures (**E, F**) of BM MKs derived from *Twf1*$^{-/-}$ or *Cof1*$^{-/-}$ mice or the respective littermate control spread on Horm collagen or fibrinogen and visualized by confocal microscopy (Leica TCS SP8, 40x objective). At least 30 MKs were analyzed per animal. Quantification was done using ImageJ Software (NIH). Values are mean ± SD (n = 3). Unpaired, two-tailed Student's t-test. **P < 0.01, ***P < 0.001. A.U.: arbitrary unit.



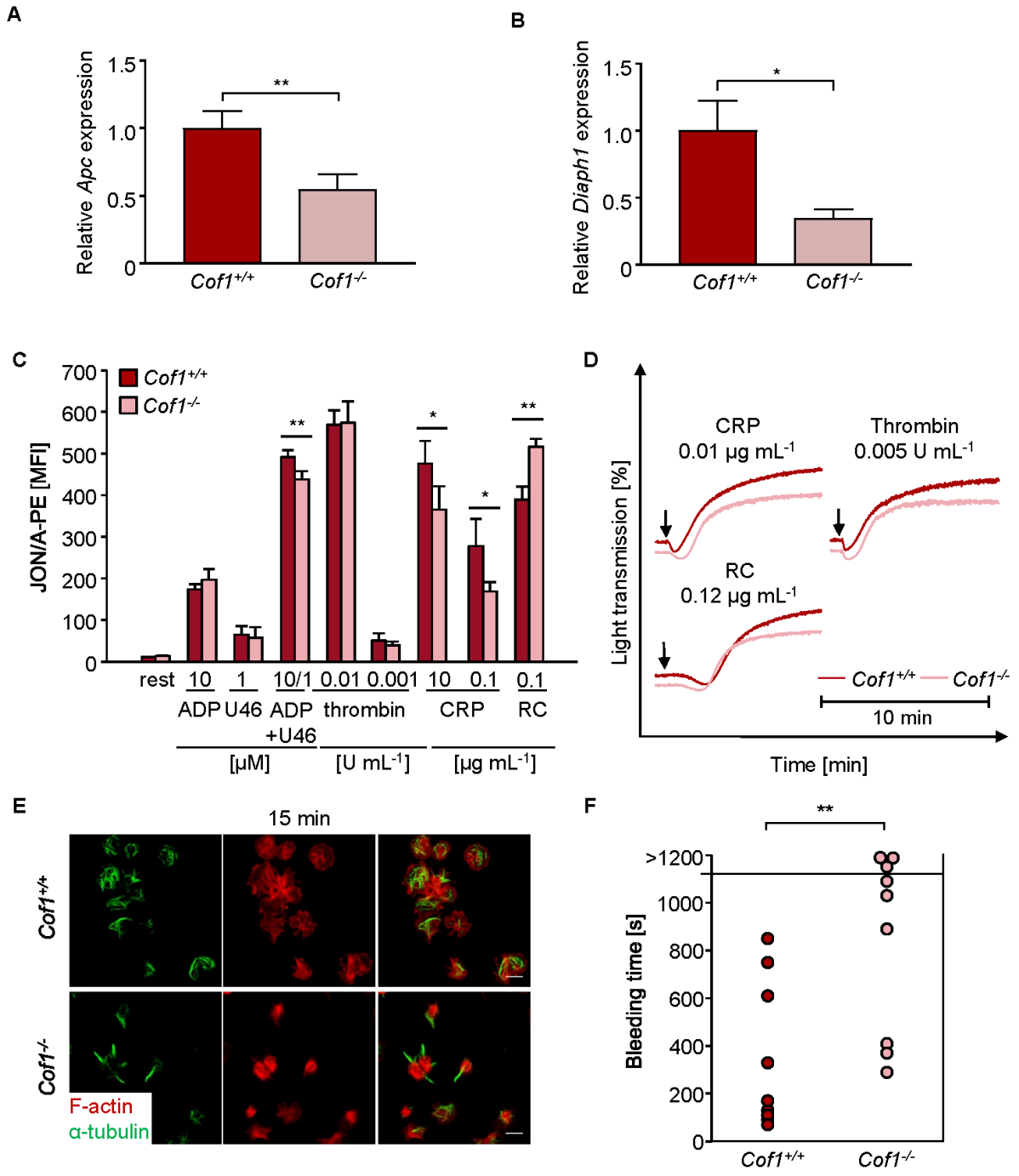
Supplemental Figure 11. Microtubule modifications of *Twf1*$^{-/-}$ as well as *Cof1*$^{-/-}$ MKs are unaltered. (**A, B**) Assessment of microtubule modifications of cultured BM MKs derived from *Twf1*$^{-/-}$ or *Cof1*$^{-/-}$ mice or the respective littermate control spread on Horm collagen. At least 30 MKs were analyzed per animal. Quantification was done using ImageJ Software (NIH). Values are mean ± SD (n = 3). A.U.: arbitrary unit.



Supplemental Figure 12. Unaltered granule distribution but increased microtubule stability in proplatelet-forming *DKO* MKs. (**A**) Analysis of α-granule distribution by staining of proplatelet-forming MKs and platelet with anti-vWF-FITC and phalloidin Atto647N. Images were acquired at a confocal microscope (Leica TCS SP8) using a 40x objective. Scale bars: 25 µm. (**B, C**) Visualization (**B**) and quantification (**C**) of acetylated and detyrosinated microtubules in proplatelet-forming BM MKs. Images were acquired at a confocal microscope (Leica TCS SP8) using a 40x objective. Scale bars: 25 µm. Values are mean ± SD (n = 3). Unpaired, two-tailed Student's t-test. *P < 0.05.

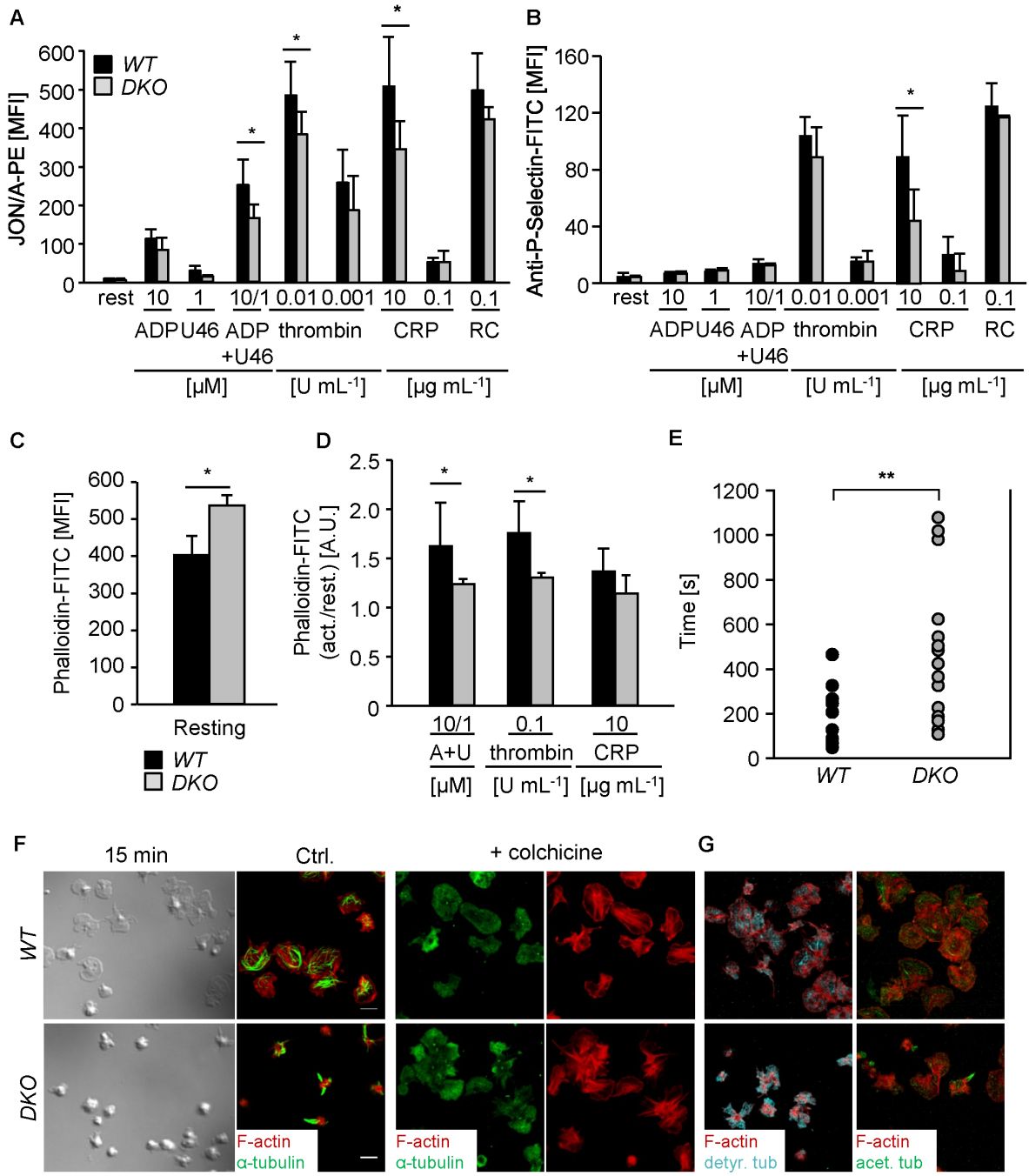


Supplemental Figure 13. Impaired LIM Kinase 1, but unaltered Profilin1 phosphorylation in Twf1/Cof1-deficient platelets. Immunoblot (**A**) and densitometric analysis (**B**) of LIM Kinase 1 (LIMK1) phosphorylation (Thr506) upon stimulation of platelets with 0.1 U mL^{-1} thrombin for 5 min. Values are mean ± SD (n = 3). Unpaired, two-tailed Student's t-test. *P < 0.05. (**C**) Profilin1 (Pfn1) phosphorylation (Tyr129) was assessed in thrombin-stimulated platelets in the presence of 2 mM Ca^{2+}. Images were acquired at an Amersham Image 680 (GE Healthcare). GAPDH served as loading control.



Supplemental Figure 14. Analysis of MK and platelet function in *Cof1*$^{-/-}$ mice. (**A, B**) Quantification of *Apc* (**A**) and *Diaph1* (**B**) mRNA content in *Cof1*$^{-/-}$ and control MKs by qPCR. Values are mean ± SD (n = 3). Unpaired, two-tailed Student's t-test. *P < 0.05. **P < 0.01. (**C**) Integrin αIIbβ3 activation (JON/A-PE) in response to different agonists was assessed by flow cytometry. Values are mean ± SD (n = 5). Unpaired, two-tailed Student's t-test. *P < 0.05; **P < 0.01. (**D**) Aggregation of washed platelets in response to different agonists. Arrows indicate addition of the agonist. Values are mean ± SD (n = 3). (**E**) Fibrinogen-spread *WT* and *Cof1*$^{-/-}$ platelets were stained for α-tubulin and F-actin (Phalloidin Atto647N) and analyzed by confocal

microscopy (Leica TCS SP5; 100x objective). Values are mean ± SD (n = 3). (**F**) Hemostasis was assessed in a tail bleeding time assay on filter paper (n = 10). Each dot represents on individual. Unpaired, two-tailed Student's t-test and Fisher's exact test. **P < 0.01. Rest: resting; U46: U46619; CRP: collagen-related peptide; RC: rhodocytin.



Supplemental Figure 15. Analysis of platelet function in Twf1/Cof1-deficient mice. Integrin αIIbβ3 activation (**A**) (JON/A-PE) and P-selectin exposure (**B**) in response to different agonists was assessed by flow cytometry. Values are mean ± SD (n = 5). Unpaired, two-tailed

Student's t-test. *P < 0.05. (**C**) F-actin content of resting platelets and (**D**) agonist-induced F-actin assembly were measured by flow cytometry after incubating platelets with phalloidin-FITC. The MFI ratio of activated and resting platelets ± SD is shown (n = 4). Unpaired, two-tailed Student's t-test. *P < 0.05. (**E**) Hemostasis was assessed in a tail bleeding time assay on filter paper (n = 10). Unpaired, two-tailed Student's t-test and Fisher's exact test. **P < 0.01. (**F**) Untreated or colchicine-treated washed platelets were allowed to spread on fibrinogen and analyzed by differential interference microscopy or were immunostained for F-actin (red) and α-tubulin (green) and analyzed using a Leica TCS SP5 confocal microscope (100x STED WHITE objective). Scale bars: 3 µm. Values are mean ± SD (n = 6). (**G**) Washed platelets were spread on fibrinogen for 15 min, stained for detyrosinated and acetylated tubulin and analyzed by confocal microscopy (n = 3). Rest: resting; U46: U46619; CRP: collagen-related peptide; RC: rhodocytin; CVX: convulxin.

Supplemental Video 1. *WT* BM MKs forming proplatelets in vitro. MKs were allowed to adhere to poly-L-lysine and imaged over 24h at 37°C at a Leica TCS SP8 (10x objective). Scale bars: 100 µm.

Supplemental Video 2. Aberrant in vitro proplatelet formation of BM MKs derived from *DKO* mice. MKs were imaged over 24h at 37°C at a Leica TCS SP8 (10x objective). Scale bars: 100 µm.

Supplemental Video 3. Visualization of in vivo PPF in *WT* mice. Two-photon intravital microscopy of PPF in a control animal. MKs were labeled using an anti-GPIX antibody derivative coupled to Alexa F546. Vessels were visualized using BSA-FITC as well as an anti-CD105 antibody coupled to Alexa F488. (20x objective; Frame: 5450.52 ms; 1017x1017 pixel). Scale bar: 80 µm.

Supplemental Video 4. Aberrantly thickened proplatelet protrusions in *DKO* mice in vivo. Increased amount of BM MKs in the skull of *DKO* mice and altered proplatelet morphology visualized by two-photon microscopy. MKs and vessels were stained as described above. (20x objective; Frame: 5568.42 ms; 1039x1039 pixel). Scale bar: 80 µm.

Supplemental Video 5. Increased proplatelet shaft stability in *DKO* mice. MKs and vessels were visualized as already described. (20x objective; Frame: 845.66 ms; 525x525 pixel). Scale bar: 50 µm.

Supplemental Video 6. Unaltered appearance of proplatelet shafts in *Cof1*$^{-/-}$ mice. MKs and vessels were visualized as already described. (20x objective; Frame: 5450.52 ms; 1017x1017 pixel). Scale bar: 50 µm.