SUPPLEMENTARY INFORMATION

Profilin 1-mediated cytoskeletal rearrangements regulate integrin function in mouse platelets

Short running title: Pfn1 regulates platelet integrin function

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SUPPLEMENTARY METHODS

Animals

Animal studies were approved by the district government of Lower Franconia (Bezirksregierung Unterfranken). Conditional *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ mice have been described previously.[1] For all experiments 12- to 16-weeks old *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ and *Pfn1*$^{fl/fl}$ littermate controls, maintained on a C57Bl/6 background were used. All mice were derived from the following breeding strategy: *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ X *Pfn1*$^{fl/fl}$ yielding 50% *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ and 50% *Pfn1*$^{fl/fl}$ control mice.

Platelet preparation

Mice were bled under isoflurane anesthesia into heparin (20 U mL^{-1}, Ratiopharm) and blood was centrifuged twice for 6 minutes at 300 g. Platelet-rich plasma (PRP) was supplemented with 0.02 U mL^{-1} apyrase (A6410, Sigma-Aldrich) and 0.1 µg mL^{-1} PGI_2 (P6188, Sigma-Aldrich) and platelets were pelleted by centrifugation for 5 minutes at 800 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 0.02 U mL^{-1} apyrase and 0.1 µg mL^{-1} PGI_2 and allowed to rest for 30 minutes prior to experiments.

Flow cytometry

50 µL of blood were withdrawn under isoflurane anesthesia, washed twice with Tyrode-HEPES buffer and finally diluted (1:20) in Tyrode-HEPES buffer containing 2 mM Ca^{2+}.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 left untreated or pretreated with the calpain inhibitor MDL-28170 (200 µM; Sigma-Aldrich) or the actin polymerization inhibitor cytochalasin D (5 µM; ab143484, Abcam) and activated with the indicated platelet agonists and concentrations. Activation of αIIbβ3- (JON/A-PE, M023-2, Emfret)[2], β1- (9EG7, 554016, BD Biosciences), the surface exposure of β1- (HMβ1-1-FITC, 102206, Biolegend), β3-integrins (EDL1-FITC)[3] and P-selectin (WUG 1.9-FITC) were determined using fluorophore-conjugated antibodies (15 minutes at 37°C).

For determination of phosphatidylserine (PS) exposure, washed platelets (5 x 10^5 platelets per µL) remained resting or were activated with the indicated agonists for 15 minutes at 37°C in the presence of annexin-V–DyLight 488. The reaction was stopped by the addition of 500 µL of Tyrode-HEPES buffer containing 3 mM Ca^{2+}. Binding of fluorescently labeled fibrinogen (50 µg mL^{-1}) was assessed over time in resting or activated washed platelets (18.000 μL^{-1}). After the indicated time points the reactions were stopped by the addition of 500 µL of Tyrode-HEPES buffer containing 3 mM Ca^{2+}.[4,5] Irreversibly bound fibrinogen was determined as the percentage of platelet-bound fluorescently labeled fibrinogen 10 minutes after the addition of 10 mM EDTA f.c. compared to non-EDTA-treated controls. For assessment of CD62P-exposure, Itgb1 and Itgb3 activation, as well as for fibrinogen binding, the respective fluorophore-conjugated antibodies/substrates were present throughout the stimulation. Maximal α-granule release and integrin activation were measured after 5, 15 and 30 minutes. For integrin closure measurements, JON/A and Anxa5-FITC were added 5 minutes before the end of the indicated time points to allow determination of integrin inactivation dynamics. Analyses were performed on a FACSCalibur (BD Biosciences) flow cytometer.

Aggregometry

Light transmission of washed platelets or PRP (1.5 x 10^5 platelets per µL) supplemented with 100 µg mL^{-1} fibrinogen (Sigma-Aldrich) was monitored over time using a four-channel aggregometer (APACT, Laborgeräte und Analysensysteme, Hamburg). Aggregation studies with ADP were performed in PRP. Platelet aggregation was induced by the addition of the indicated agonists.

Platelet adhesion under flow

Anticoagulated whole blood was perfused at a shear rate of 1000 s^{-1} over collagen I-coated cover slips (100 µg mL^{-1}, Nycomed). Prior to perfusion, platelet counts in samples from *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ mice were adjusted to those of control mice by the addition of PRP isolated from

Pfn1$^{fl/fl\text{-}Pf4Cre}$ donor mice. Platelets were labeled with a Dylight-488-conjugated anti-GPIX derivative (0.2 µg mL^{-1}) for 5 minutes at 37°C. Time lapse phase-contrast and fluorescence images were recorded with a Zeiss Axiovert 200 inverted microscope (40x/0.60 objective) equipped with a CoolSNAP-EZ camera (Visitron) and analyzed off-line using Metavue 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 220 g for 5 minutes. Plasma was collected and platelets were resuspended in 1 mL Ca^{2+}-free Tyrode-HEPES buffer supplemented with apyrase [0.02 U mL^{-1}, f.c.] and PGI_2 [0.1 µg mL^{-1}, f.c.]. 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 5 U mL^{-1} thrombin (Sigma). Clot formation and retraction was recorded up to 3 h when the residual serum volume was determined.

Tail bleeding time

Mice were anesthetized 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 making contact with the wound site. Bleeding was determined to have ceased when no blood was observed on the paper. Experiments were stopped after 20 minutes by cauterization. Differences between the mean bleeding time was statistically assessed using the Student's t-test and differences between occluded and non-occluded wounds by Fisher's exact test.

In vivo thrombus formation

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 Fisher's exact test.

Ca^{2+} measurements

For intracellular calcium measurements, washed platelets (0.6×10^6 μL^{-1}) were prepared in Ca^{2+}-free Tyrode-HEPES buffer and loaded with Fura-2/AM (5 µM) together with Pluronic F-127 (0.2 µg mL^{-1}; Invitrogen) for 30 minutes at 37°C. After labeling, platelets were washed once and resuspended in Tyrode-HEPES buffer containing 0.5 mM EGTA (store release) or 1 mM Ca^{2+} (Ca^{2+} entry). Platelets were activated with the indicated agonists and the fluorescence signal was measured with a fluorimeter (LS 55; PerkinElmer). Each measurement was calibrated using Triton X-100 (Roth) and EGTA.

Immunostaining of spread platelets

Coverslips were coated with fibrinogen (100 µg mL^{-1}; F4883, Sigma-Aldrich) overnight at 4°C. Washed platelets were either left untreated or pretreated (10 minutes at 37°C) with the calpain inhibitor MDL-28170 (200 µM) and subsequently stimulated with 0.01 U mL^{-1} thrombin (10602400001, Roche) and allowed to spread. 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 stained with phalloidin-Atto647N (170 nM, 65906, Fluka), anti-β3-integrin (1 µg mL^{-1}, 57B10)[3] and anti-Tln1 (1 µg mL^{-1}, sc-7534 (C-20), or sc-365460 (A-11), SantaCruz Biotechnology). Samples were mounted with Fluoroshield (F6182, Sigma-Aldrich) and images acquired using a Leica TCS SP5 confocal microscope (Leica Microsystems).

Atomic force microscopy (AFM)

Platelets were allowed to spread on a fibrinogen-coated (50 µg mL^{-1}, Merck 341576-100MG) glass bottom dish (World Precision Instruments) in Tyrode-HEPES buffer containing 1 mM Ca^{2+} and Mg^{2+}. Platelets were activated with 1 U mL^{-1} thrombin (Merck, 605190). The AFM (JPK-Instruments, Berlin) was operated in the force-volume (FV) mode. In the FV mode, force-distance (FD) curves were acquired on an array of 128 x 128 points along the platelet surface. The FD curves were acquired by indenting the platelet with a force of 500 pN using an approach-retract velocity of 50 μm s^{-1} and cantilevers (Mikromasch, HQ:CSC38/no Al) nominal spring constant 0.03 – 0.09 N m^{-1}). The spring constants of the cantilevers were calibrated using the in-built module in the JPK software before recording the FV map. The extension curves were fit to the Hertz model for conical tips,

$$F = \frac{E}{1-\nu^2}\frac{2\tan\alpha}{\pi}\delta^2$$

where, ν is the Poisson's ratio (assumed to be 0.5 for biological materials), E is the Young's modulus, δ is the tip indentation into the sample, and α is the half angle of the tip cone (20°).

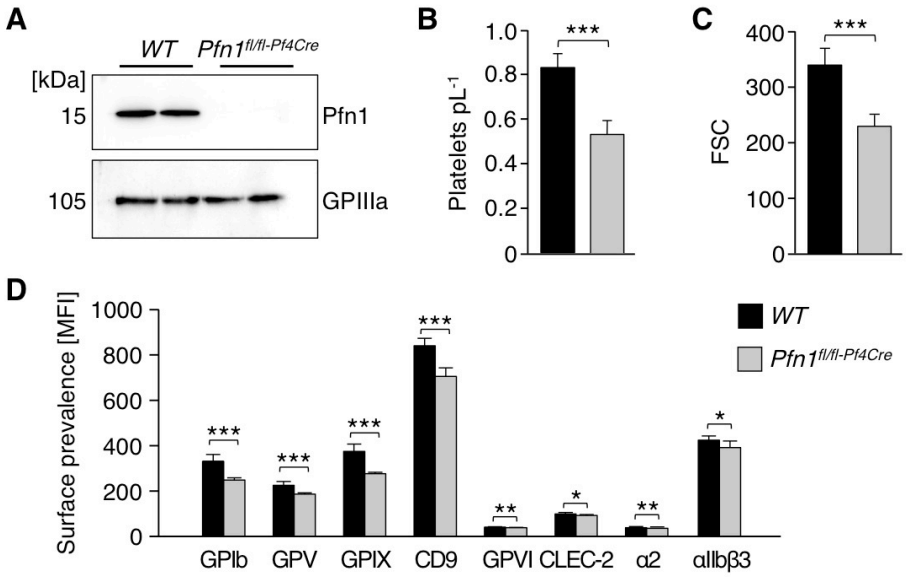
Immunoblotting

Platelets were left untreated or preincubated (10 minutes at 37°C) with the calpain inhibitor MDL-28170 (200 µM) and subsequently stimulated with the calcium ionophore A23187 (10 µM) or thrombin (0.1 U mL^{-1}) and CRP (10 µg mL^{-1}), lysed and processed for immunoblotting. Denatured lysates were separated by SDS-PAGE and blotted onto PVDF membranes. Full length (Ab762)[6] and cleaved (AbΔ747)[6] β3-integrin, as well as filamin A (1 µg mL^{-1}, EP2405Y, Epitomics), talin (1 µg mL^{-1}, sc-365460, SantaCruz Biotechnologies), and Gapdh (1 µg mL^{-1}, G5262, Sigma-Aldrich) were probed with the respective antibodies and detected using horseradish peroxidase-conjugated secondary antibodies (0.33 µg ml^{-1}) and enhanced chemiluminescence solution (JM-K820-500, MoBiTec). Images were recorded using a MultiImage® II FC Light Cabinet (Alpha Innotech cooperation) device. Ab762 as well as AbΔ747 antibodies were generated in the Du laboratory.[6]

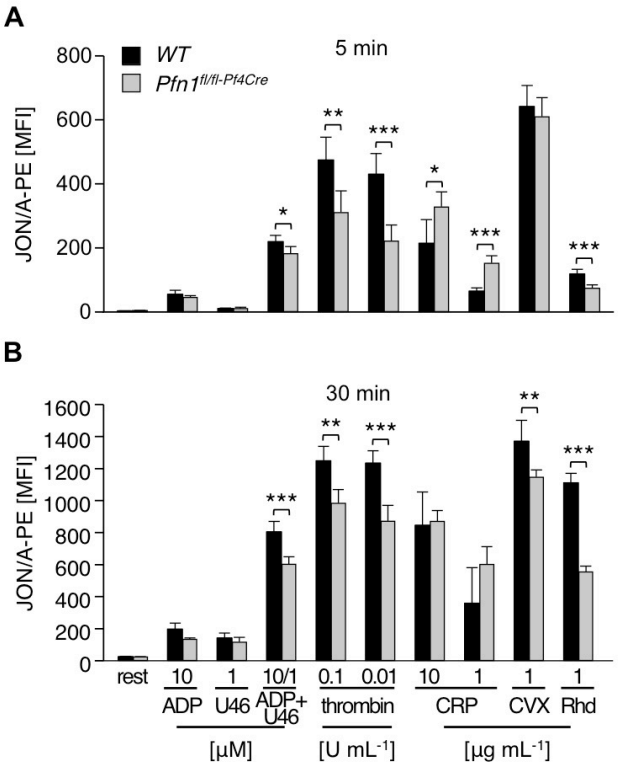
Data analysis

The presented results are mean ± standard deviation (SD) from at least three independent experiments per group, if not stated otherwise. Data distribution was analyzed using the Shapiro-Wilk-test and differences between control and knockout mice were statistically analyzed using Student's-t- or Wilcoxon-Mann-Whitney-test, respectively. Variance between non-occluded vessels was assessed using Fisher's exact test. *P*-values < .05 were considered as statistically significant **P*< .05; ***P*< .01; ****P*<. 001. Results with a *P*-value > .05 were considered as not significant (NS).

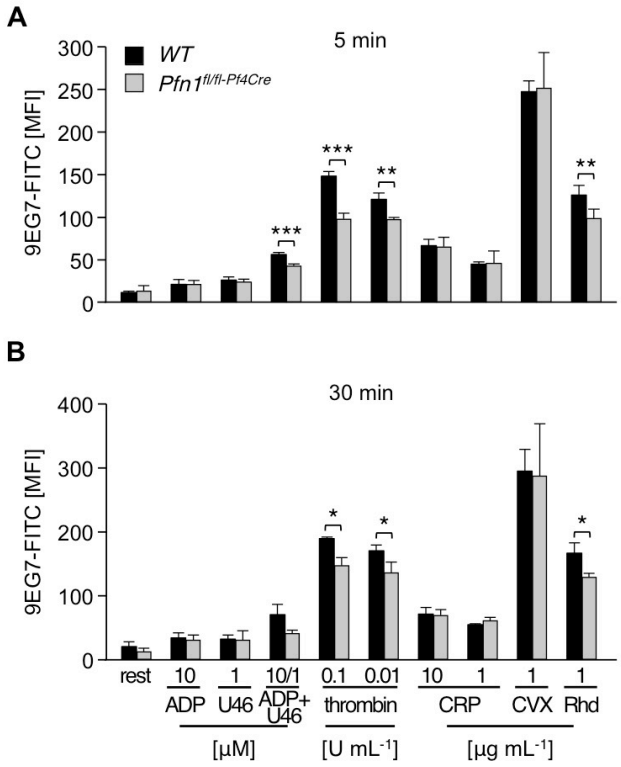
SUPPLEMENTARY FIGURES



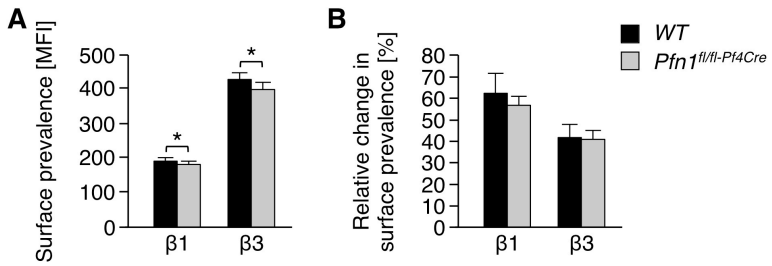
Supplementary Figure 1. *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ mice display microthrombocytopenia. (**A**) Immunoblot on platelet lysates proved the efficiency of the targeting strategy. (**B-D**) Platelet count (**B**), size (**C**) and surface prevalence of major platelet glycoproteins (**D**) were assessed by flow cytometry. Values are mean, forward scatter signal (FSC), or mean fluorescence intensity (MFI) ± SD (n = 6 vs 6). Unpaired Student's t-test: ***P< .001; **P< .01; *P< .05; NS, non-significant.



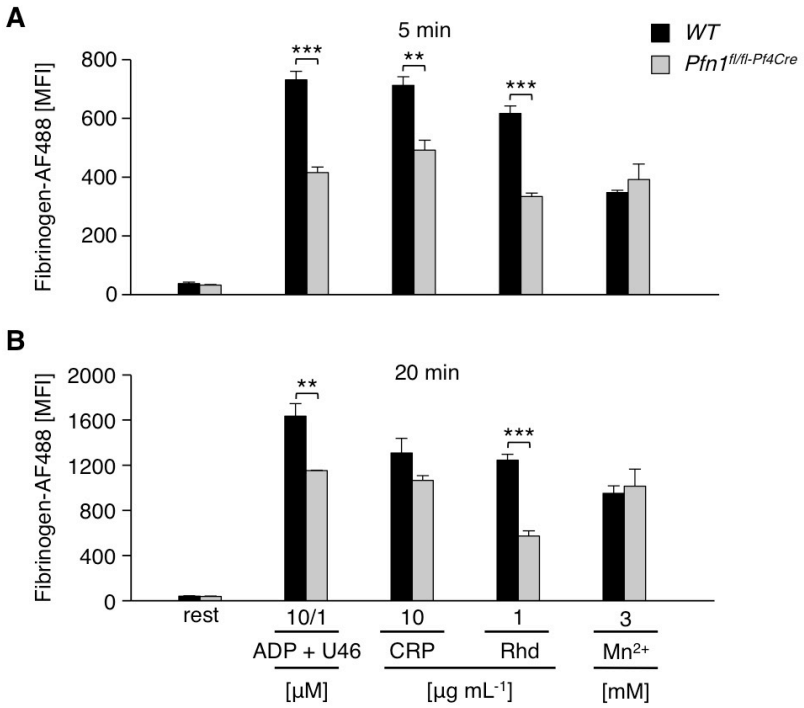
Supplementary Figure 2. Impaired αIIbβ3-integrin activation in *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets. Activation of platelet αIIbβ3-integrins (JON/A-PE) in response to different agonists was assessed by flow cytometry at the indicated time points. Rest, resting; ADP, adenosine diphosphate; U46, stable thromboxane A_2 analog U46619; CRP, collagen-related peptide; CVX, convulxin; Rhd, rhodocytin. Antibodies were present throughout the stimulation to assess maximal integrin activation at the respective time points**.** Values are mean ± SD (n = 6 vs 6). Unpaired Student's *t*-test: ***P< .001; **P< .01; *P< .05, NS, non-significant.



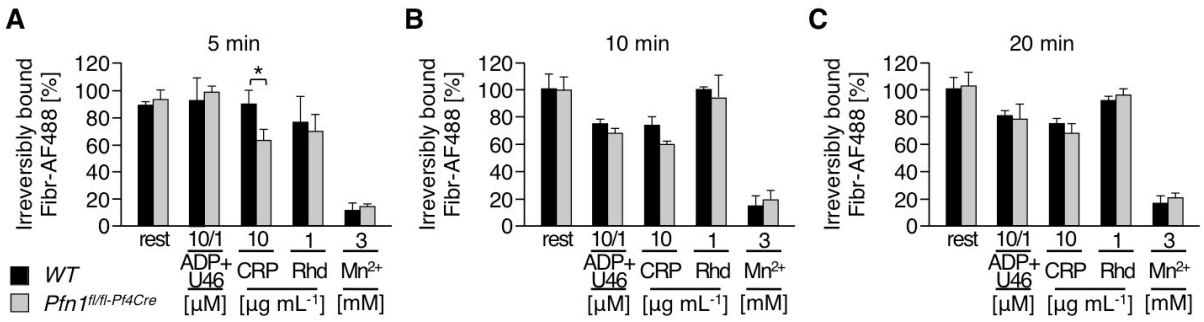
Supplementary Figure 3. Impaired β1-integrin activation in *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets. Activation of platelet β1-integrins (9EG7-FITC) in response to different agonists was assessed by flow cytometry at the indicated time points. Rest, resting; ADP, adenosine diphosphate; U46, stable thromboxane A_2 analog U46619; CRP, collagen-related peptide; CVX, convulxin; Rhd, rhodocytin. Antibodies were present throughout the stimulation to assess maximal integrin activation at the respective time points**.** Values are mean ± SD (n = 6 vs 6). Unpaired Student's *t*-test: ***P< .001; **P< .01; *P< .05, NS, non-significant.



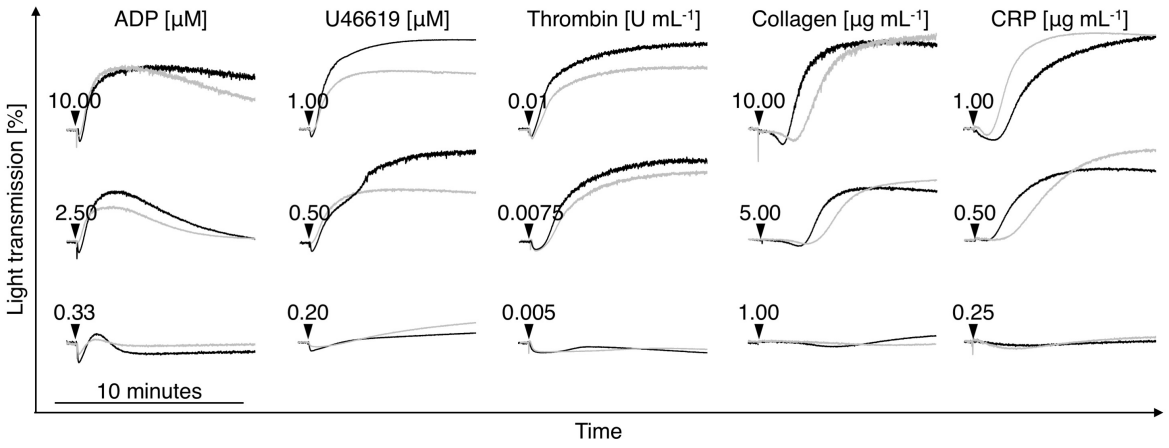
Supplementary Figure 4. Unaltered integrin surface recruitment upon stimulation of *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets. (**A**) Surface expression of β1- and β3-integrins on resting platelets and (**B**) the relative change above resting levels upon stimulation with thrombin (0.1 U mL^{-1}) was determined by flow cytometry. Values are mean ± SD (n = 6 vs 6). Unpaired Student's *t*-test: **P*< .05, non-significant.



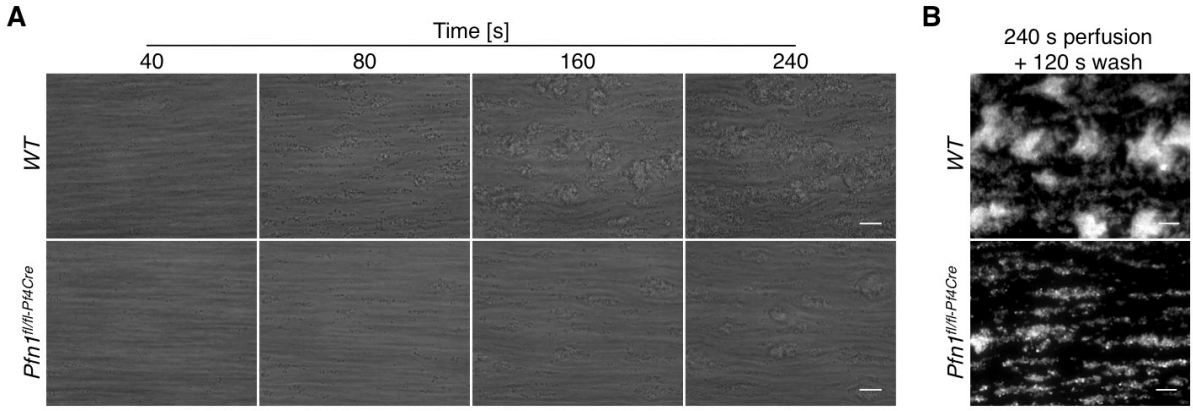
Supplementary Figure 5. Reduced fibrinogen binding to activated *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets. Binding of Alexa F488-conjugated fibrinogen (50 µg mL^{-1}) to resting and stimulated platelets was assessed after 5 or 20 minutes by flow cytometry. Mn^{2+} served as activation-independent positive control. Rest, resting; ADP, adenosine diphosphate; U46, stable thromboxane A_2 analog U46619; CRP, collagen-related peptide; Rhd, rhodocytin; Mn^{2+}, manganese. Values are mean ± SD (n = 6 vs 6). Unpaired Student's *t*-test: ***P< .001; **P< .01; NS, non-significant.



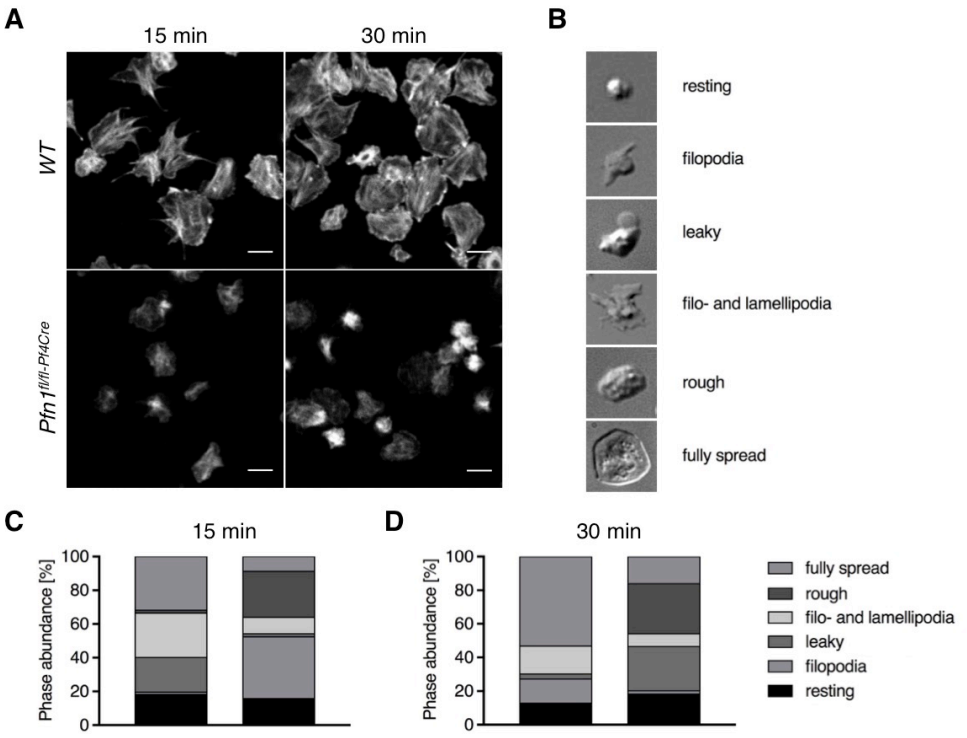
Supplementary Figure 6. Normal irreversible fibrinogen binding to *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets. Irreversible binding (% bound after 10 minutes in the presence of 10 mM EDTA f.c. as compared to non-EDTA-treated controls) of Alexa F488-conjugated fibrinogen (50 µg mL^{-1}) to resting and stimulated platelets was assessed after 5, 10 and 20 minutes by flow cytometry. Mn^{2+} served as positive control. Rest, resting; ADP, adenosine diphosphate; U46, stable thromboxane A_2 analog U46619; CRP, collagen-related peptide; Rhd, rhodocytin; Mn^{2+}, manganese. Values are mean ± SD (n = 4 vs 4). Unpaired Student's *t*-test: **P*< .05; NS, non-significant.



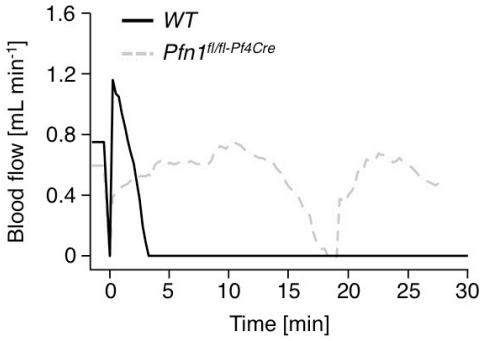
Supplementary Figure 7. Slightly altered aggregation responses of *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets. Light transmission of washed platelets or platelet-rich plasma (PRP; 1.5 x 10^5 platelets per µL) supplemented with 100 µg mL^{-1} fibrinogen was monitored over time using a four-channel aggregometer. Aggregation studies with ADP were performed in PRP. Platelet aggregation was induced by the addition of the indicated agonists. ADP, adenosine diphosphate; U46619, stable thromboxane A_2 analog; CRP, collagen-related peptide. Aggregation traces are representative of at least 6 animals per group.



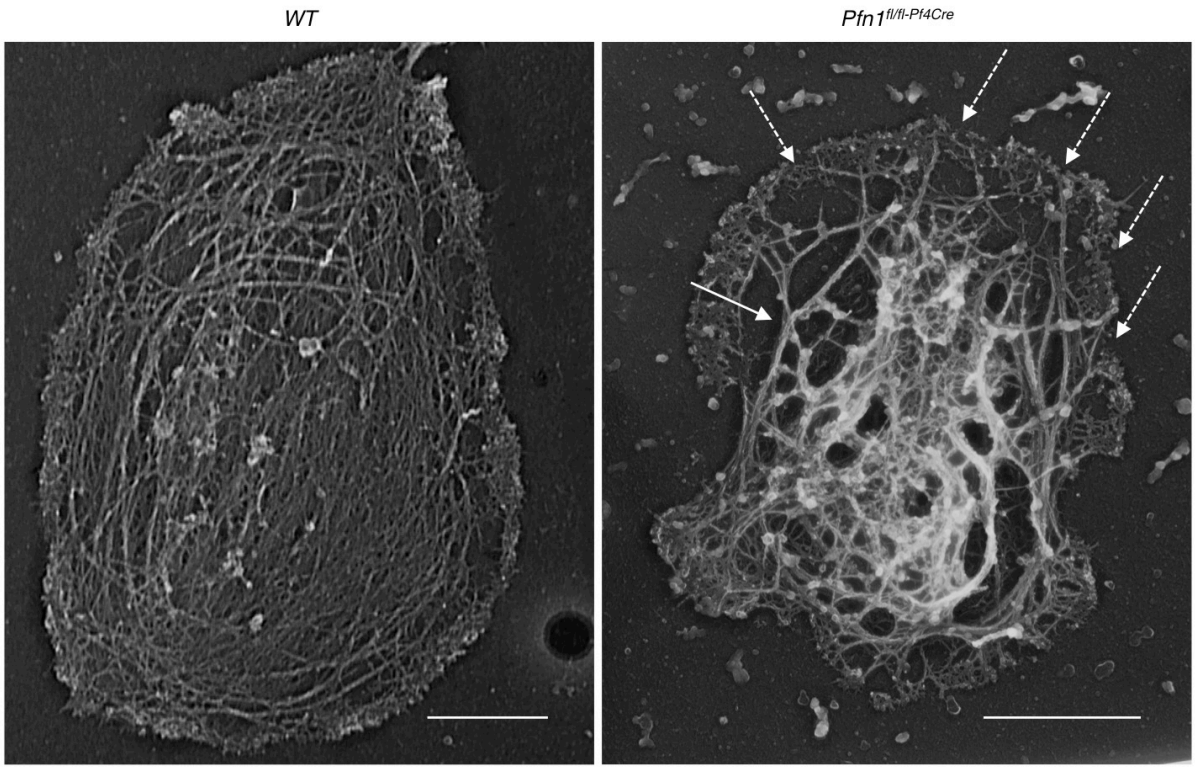
Supplementary Figure 8. *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets show reduced adhesion and aggregate formation under flow. Thrombus formation of control and *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets was assessed in platelet count-adjusted whole blood under flow (1000 s^{-1}) on collagen I (70 µg mL^{-1}). (**A**) Brightfield images were acquired during the perfusion period (4 minutes) and (**B**) fluorescence microscopy images were acquired after subsequent washing for 2 minutes with Tyrode-HEPES buffer containing Ca^{2+}. 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 bars, 25 µm.



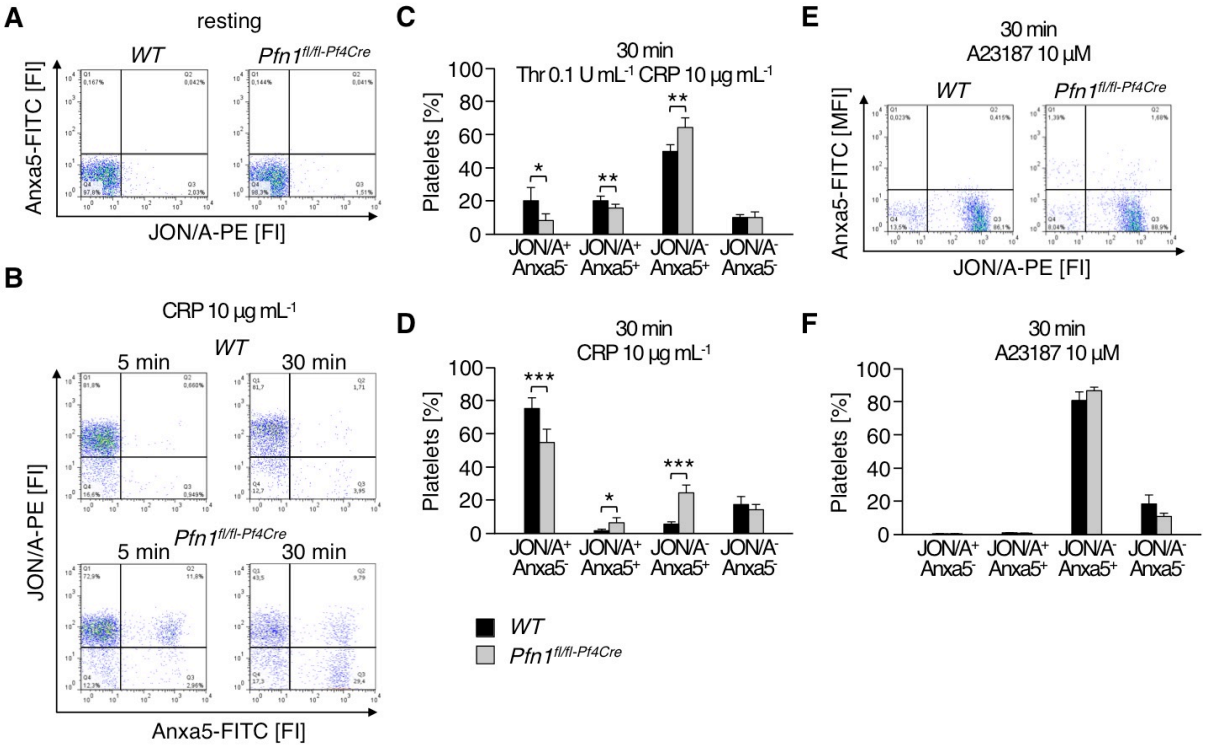
Supplementary Figure 9. Impaired filopodia and lamellipodia formation of spread *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets. Washed control and *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets were allowed to spread on fibrinogen-coated cover slips (100 µg mL^{-1}) after stimulation with 0.01 U mL^{-1} thrombin. (**A**) Representative confocal microscopic images of phalloidin stained platelets were acquired with a Leica TCS SP5 confocal microscope (100x/1.4 oil STED WHITE objective, Leica Microsystems). Scale bars, 3 µm. (**B**) Overview of defined spreading phases. Please note the newly defined stages. (**C, D**) Quantification of the different spreading phases after 15 minutes and 30 minutes from differential interference contrast images (Zeiss Axiovert 200 inverted microscope (100x/1.4 oil objective, Zeiss)). Images are representative of at least 6 animals per group. Values are mean (n = 6 vs 6).



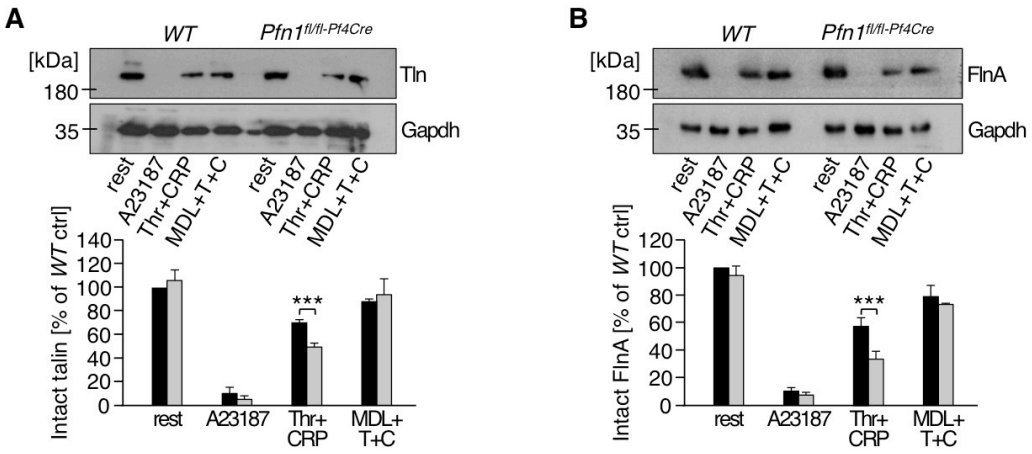
Supplementary Figure 10. *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets do not form stable arterial thrombi. Representative blood flow curves upon induction of a mechanical injury in the abdominal aorta. Blood flow was monitored with an ultrasonic flow probe (0.5PSB699; Transonic Systems, USA). Flow profiles are representative of at least 6 animals per group.



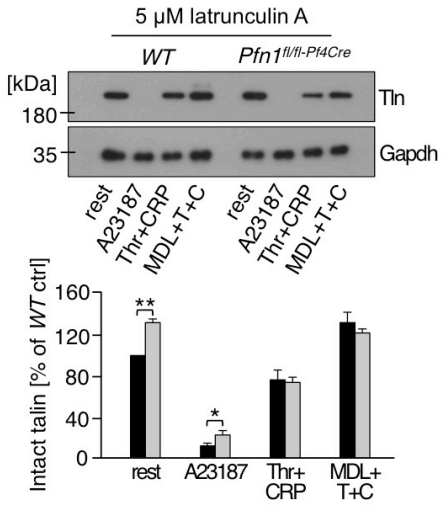
Supplementary Figure 11. Disrupted cortical actin cytoskeleton in spread *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets. Control and *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets were allowed to spread for 15 minutes on fibrinogen-coated surfaces and processed for rapid-freezing electron microscopic analysis of the cytoskeleton. Dashed arrows highlight defective cortical F-actin meshwork and the arrow indicates disorganized microtubules in spread *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets. Images are representative of at least 5 animals per group and were acquired with a JEOL 1200-EX transmission electron microscope at 80 kV. Scale bars, 1 µm.



Supplementary Figure 12. Accelerated integrin inactivation in *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets. (**A-F**) Washed platelets remained resting (**A**) or were stimulated with 10 µg mL^{-1} collagen-related peptide (CRP; **B, D**), 0.1 U mL^{-1} thrombin and 10 µg mL^{-1} CRP (**C**), or 10 µM A23187 (**E, F**). Activation of αIIbβ3-integrins (JON/A-PE) and phosphatidylserine exposure (Anxa5-FITC) on the outer leaflet of the platelet membrane were determined by adding the fluorescently labeled antibody and protein 5 minutes prior to the end of the indicated time points. Analysis was performed by flow cytometry. (**A, B, E**) Flow cytometry plots are representative of at least 6 animals per group. (**C, D, F**) Percentage of platelets per quadrant (Q). Q1, JON/A$^+$ Anxa5$^-$ (upper left); Q2, JON/A$^+$ Anxa5$^+$ (upper right); Q3, JON/A$^-$ Anxa5$^+$ (lower right); Q4, JON/A$^-$ Anxa5$^-$ (lower left). Values are mean ± SD (n = 6 vs 6). Unpaired Student's *t*-test: ***P< .001; **P< .01; *P< .05; NS, non-significant.



Supplementary Figure 13. Increased calpain-mediated cleavage of talin and filamin A in *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets. (**A-B**) Platelets were left untreated or pre-incubated for 10 minutes in the presence of the calpain inhibitor MDL-28170 (200 µM). Subsequently, samples were stimulated with the calcium ionophore A23187 (10 µM) or thrombin (0.1 U mL^{-1}) and CRP (collagen-related peptide; 10 µg mL^{-1}), lysed and processed for immunoblotting. Full-length talin (Tln) and filamin A (FlnA) were probed with the respective antibodies and analyzed by densitometry. Gapdh served as control. Immunoblots are representative of 3 animals per group. Values are mean ± SD (n = 3 vs 3 animals). Unpaired Student's *t*-test: ***P<.001; NS, non-significant.



Supplementary Figure 14. Inhibition of actin polymerization restores talin cleavage in *Pfn1*$^{fl/fl\text{-}Pf4Cre}$ platelets. Latrunculin A-pretreated platelets (2.5 µM, 10 minutes) were left untreated or pre-incubated for 10 minutes in the presence of the calpain inhibitor MDL-28170 (200 µM). Subsequently, samples were stimulated with the calcium ionophore A23187 (10 µM) or thrombin (0.1 U mL^{-1}) and CRP (collagen-related peptide; 10 µg mL^{-1}), lysed and processed for immunoblotting. Full-length talin (Tln) was probed with the respective antibody and analyzed by densitometry (n = 3 vs 3 animals). Gapdh served as control. Immunoblots are representative of 3 animals per group. Values are mean ± s.d. Unpaired Student's *t*-test: $^{**}P<.01$; $^{*}P<.05$; NS, non-significant.

SUPPLEMENTARY REFERENCES

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