Supplemental information

Supplemental materials and methods

Preparation of murine plasma

Mice aged 6-9 weeks were anesthetized with pentobarbital (40 mg/kg), and whole blood was drawn from the inferior vena cava into 3.13% citrate (1 vol anticoagulant/9 vol blood). Blood was centrifuged at 1031 *g* for 10 min with the centrifuge pre-warmed to 26°C to obtain platelet rich plasma (PRP). Alternatively blood was centrifuged at 2400 *g* for 10 min at room temperature (RT), to obtain platelet-poor plasma (PPP). To obtain platelet-free plasma (PFP), an additional centrifugation at 10000 *g* for 10 min was performed.

Platelet count and measurement of coagulation parameters

Platelet counts were carried out with an automated cell counter (Procyte Dx Hematology Analyzer, IDEXX). Fibrinogen, FVIII and FIX activity were measured on an automated Sysmex CA-7000 coagulation analyzer (Sysmex Digitana). Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured on a coagulometer (*MC4plus*, Merlin Medical).

Measurement of murine PS antigen and TAT complexes by ELISA

Wells from 96-well plates (Maxisorb, Thermo) were coated with 50 µL per well of 10 µg/mL of rabbit polyclonal anti-human PS (DAKO Cytomation) and incubated overnight at 4°C. After 3 washes with TBS buffer (0.05 M tris(hydroxymethyl)aminomethane, 0.15 M NaCl, pH 7.5, 0.05% Tween 20), the plate was blocked with TBS-BSA 2%. Diluted plasma samples (dilution range: 1:300-1:600) were added to the wells and incubated at RT for 2h. After 3 washed, 50 µL of 1µg/mL biotinylated chicken polyclonal anti-murine protein S were added and incubated for 2h at RT. Signal was amplified by streptavidin-HRP conjugated horseradish peroxidase (Thermo) was added and plates incubated for 1h. The plates were washed 3 times and 100 µLTMB substrate (KPL) was added. Reactions were stopped by adding 100 µL HCl (1M). Absorbance was measure at 450 nm. Standard curves were set up by using serial dilution of pooled normal plasma obtained from 14 healthy mice (8 males and 6 females, 7–12 weeks old). Results were expressed in percentage relative to the pooled normal plasma.

TAT level was measured in duplicate for each plasma sample using a commercially available ELISA (Enzygnost TAT micro, Siemens), according to the manufacturer's instructions.

Mouse tissue processing and sectioning, immunohistochemistry and microscopy

Tissue sections (4 µm) with no pre-treatment were stained with hematoxylin/eosin or Masson

Trichrome or immunostained for insoluble fibrin, PS or TFPI. The following antibodies were used: fibrin (mAb clone 102-10)[1] final concentration 15.6 µg/mL, incubation for 30 min at RT, secondary antibody rabbit anti-human, (ab7155 Abcam, Cambridge, UK) 1:200 dilution, incubation for 30 min at RT; PS (MAB 4976, R&D, dilution 1:50) incubation for 30 min at RT, secondary antibody rabbit anti-rat, (ab7155 Abcam)–1:200 dilution, incubation for 30 min at RT; TFPI (PAHTFPI-S, Hematological Technologies) final concentration 18.6 µg/mL, incubation for 30 min at RT, secondary antibody rabbit anti-sheep IgG (ab7106, Abcam) 1:200 dilution, incubation for 30 min at RT. All the stainings were performed with the immunostainer BOND RX (Leica Biosystems, Muttenz, Switzerland) following manufacturer's instructions. Whole slides were scanned using 3D HISTECH Panoramic 250 Flash II, with 20x (NA 0.8), 40x (NA 0.95) air objectives. Images processing was done using Panoramic Viewer software.

In vivo* administration of FVIII to mice with complete genetic loss of *F8

Mice, aged 6-9 week, were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). We administered intravenously either 0.3 U/kg of recombinant FVIII (Advate®, Baxalta) to reach a FVIII level of 100% at 1h (normal dose) or an overdose of recombinant FVIII (2 U/kg) to reach >200% at 1h. Either the normal dose or the overdose was injected 1h before and 1h after the introduction of a jugular vein catheter (Mouse JVC 2Fr PU 10 cm, Instech) and then 4h, 8h and 16h after the placement of the central line. Mice were sacrificed 24h after the first injection. Blood was drawn and organs were harvested. FVIII, fibrinogen and thrombin-antithrombin complexes (TAT) were measured as described in the supplementary information. Lungs were isolated, fixed in 4% paraformaldehyde (PFA) and embedded in paraffin.

In vivo* administration of hrFIX to mice with complete genetic loss of *F9

Mice, aged 7-10 week, were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). We administered intravenously 2 U/gr of recombinant FIX (Benefix®, Pfizer) to reach an overdose of recombinant FIX (300% at 1h) that was stable until 12 h. After 12 h mice received a second i.v. injection of 2U/gr FIX. Mice were monitored all over the duration of the experiment and sacrificed 24h after the first injection. Blood was drawn and organs were harvested. FIX, fibrinogen and thrombin-antithrombin complexes (TAT) were measured as described in the supplementary information. Lungs were isolated, fixed in 4% paraformaldehyde (PFA), embedded in paraffin and tested for Fibrin deposition (IHC) as described in the supplementary information.

$FeCl_3$ injury thrombosis model in mesenteric arteries

A model of thrombosis in mesenteric arteries using intravital microscopy was performed according to ref[2] with minor modifications. Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (16 mg/kg). Platelets were directly labeled *in vivo* by the injection of

100 µL rhodamine 6G (1.0 mM). After selection of the studied field, vessel wall injury was generated by a filter paper (1 mm diameter patch of 1M Whatmann paper) saturated with 10% $FeCl_3$ applied topically for 1 min. Thrombus formation was monitored in real time under a fluorescent microscope (IV-500, Micron instruments, San Diego, CA) with an FITC filter set, equiped with an affinity corrected water-immesion optics (Zeiss, Germany). The bright fluorescent labelled platelets and leucoytes allowed the observation of 1355µm X 965µm field of view through video triggered stroboscopic epi-illumination (Chadwick Helmuth, El Monte, CA). A 10X objective Zeiss Plan-Neofluar with NA0.3. was used. All scenes were recorded on video-tape using a customized low-lag silicon-intensified target camera (Dage MTI, Michigan city, IN), a time base generator and a Hi-8 VCR (EV, C-100, Sony, Japan). Time to vessel wall occlusion was measured, as determined by cessation of the blood cell flow.

Fibroblast-like synoviocytes (FLS) isolation, culture and flow cytometry

Murine FLS from 8-10 weeks old mice were isolated and cultured according to[3]. After three passages, phase contrast images of cells were taken, and cells were incubated with FITC-conjugated rat anti-mouse CD11b antibody (M1/70, Pharmingen, BD Biosciences), PE-conjugated rat anti-mouse CD90.2 antibody (30-H12, Pharmingen, BD Biosciences), FITC-conjugated rat anti-mouse CD106 antibody (429 MVCAM.A, Pharmingen, BD Biosciences), PE-conjugated hamster anti-mouse CD54 antibody (3E2, Pharmingen, BD Biosciences), and fluorochrome-conjugated isotype control antibodies for 30 min at 4 °C in the dark. After a final washing and centrifugation step, all incubated cells were analyzed on an LSR II flow cytometer (BD Biosciences) and FACS Diva 7.0 software (BD Biosciences). Human FLS from healthy individual and OA patient were purchased from Asterand, Bioscience and cultured according to manufacture instructions.

Western blotting

PS and TFPI were detected in human and mouse samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% gradient SDS-PAGE, Bio-Rad) under reducing conditions. The proteins were transferred to nitrocellulose membranes (Bio-Rad), and then visualized using: 2ug/mL monoclonal MAB-4976 (R&D system) for murine PS, 1µg/mL polyclonal AF2975 for murine TFPI (R&D system). Recombinant murine PS[4] (30 ng), recombinant human TFPI full length (provided by T. Hamuro, Kaketsuken, Japan), lysate of washed platelets, PFP from *F8*$^{-/-}$*Pros1*$^{+/+}$ mice and placenta lysates from *F8*$^{+/+}$*Pros1*$^{+/+}$ mice were used as PS, TFPIα controls. Samples from confluent murine and human FLS conditioned media were collected after 24h-incubation in a serum-free media (OptiMem) and concentrated 40 times using Amicon filter devices (Millipore, 10 kDa cut-off). For TFPI western blotting, samples were treated with a mixture of five protein deglycosidases (PNGase F, O-Glycosidase, Neuraminidase, β1-4 Galactosidase, β-N-Acetylglucosaminidase, Deglycosylation kit, V4931, Promega) for 12h at 37°C before being loaded on the gel. Final detection was completed by

using a horseradish peroxidase–conjugated secondary antibody (Dako) and the Supersignal West Dura Extended Duration Chemiluminescence Substrate (Pierce), monitored with a Fuji LAS 3000IR CCD camera.

Real Time PCR

RNA was isolated from *F8*$^{+/+}$*Pros1*$^{+/+}$, *F8*$^{-/-}$*Pros1*$^{+/+}$ and *F8*$^{-/-}$*Pros1*$^{-/-}$ synoviocytes using peqGOLD Total RNA Kit following the manufacturer's instructions. *Pros1* and *Tfpi* mRNA were determined by real time quantitative polymerase chain reaction (RT-PCR) analysis. To synthetize cDNA and detect the expression of *Pros1* and *Tfpi* genes, SuperScript® III Platinum® One-Step qRT-PCR Kit (Invitrogen) was used. The reaction components for each probe contained: 6µL of Mastermix (4µL reaction mix, 0.16µL superscript RT/Taq, 0.16µL rox dye, 1.28µL RNase free water and 0.4µL of TaqMan gene expression assays for mouse or human: *Pros1*: Mm01343429_m1, Hs00165590_m1. *Tfpi*: Mm01334601_m1, Hs00409207_m1. Endogenous controls: *Actb* Mm00607939_s1, Hs99999903_m1. *P4htm*: Mm00512331_m1, Hs00977922_m1. *Gapdh*: Mm99999915_g1, Hs99999905_m1) and 5 ng of RNA). Real-time PCR reactions were amplified and analyzed in duplicate using an Applied Biosystem ViiA7 System. PCR reaction conditions were as follows: step 1: 50°C for 15 min; step 2: 95°C for 2 min; step 3: 45 cycles of 95°C for 3 sec followed by 60°C for 30 sec. Expression relative to endogenous controls was calculated using $2^{-\Delta\Delta CT}$ and levels were normalized to control untreated cells.

Immunohistochemistry on human knee synovium

Paraffin-embedded specimens of synovial tissue from twelve HA patients and four HB patients who underwent arthroplasty for severe knee arthropathy were collected at the archives of the Section of Anatomy and Histology, Department of Experimental and Clinical Medicine, University of Florence, as described elsewhere[5,6]. Seven HA patients were treated on demand and five with secondary prophylaxis. All four HB patients were treated on demand. Synovial samples from seven osteoarthritis (OA) patients were used as controls[5,6]. For immunohistochemistry analysis, synovial tissue sections (5 µm thick) were deparaffinized, rehydrated, boiled for 10 minutes in sodium citrate buffer (10 mM, pH 6.0) for antigen retrieval and subsequently treated with 3% H_2O_2 in methanol for 15 min at room temperature to block endogenous peroxidase activity. Sections were then washed in PBS and incubated with Ultra V block (UltraVision Large Volume Detection System Anti-Polyvalent, HRP, catalog number TP-125-HL, LabVision) for 10 min at RT according to the manufacturer's protocol. After blocking non-specific site binding, slides were incubated overnight at 4°C with rabbit polyclonal anti-human Protein S/PROS1 antibody (1:50 dilution, catalog number NBP1-87218, Novus Biologicals) or sheep polyclonal anti-human Tissue Factor Pathway Inhibitor (TFPI) antibody (1:500 dilution, catalog number PAHTFPI-S, Haematologic Technologies) diluted in PBS. For PS immunostaining, tissue sections were then incubated with biotinylated secondary antibodies followed

by streptavidin peroxidase (UltraVision Large Volume Detection System Anti-Polyvalent, HRP; LabVision) according to the manufacturer's protocol. For TFPI immunostaining, tissue sections were instead incubated with HRP-conjugated donkey anti-sheep IgG (1:1000 dilution; catalog number ab97125; Abcam) for 30 min. Immunoreactivity was developed using 3-amino-9-ethylcarbazole (AEC kit, catalog number TA-125-SA; LabVision) as chromogen. Synovial sections were finally counterstained with Mayer's hematoxylin (Bio-Optica), washed, mounted in an aqueous mounting medium and observed under a Leica DM4000 B microscope (Leica Microsystems). Sections not exposed to primary antibodies or incubated with isotype-matched and concentration-matched non-immune IgG (Sigma-Aldrich) were included as negative controls for antibody specificity. Light microscopy images were captured with a Leica DFC310 FX 1.4-megapixel digital colour camera equipped with the Leica software application suite LAS V3.8 (Leica Microsystems).

Fibrin clot ultrastructure investigation

Fibrin clots were prepared at 37°C from PFP by the addition of ~5 nM TF (Dade Innovin, Siemens). They were then fixed in 2% glutaraldehyde, dehydrated, dried and sputter-coated with gold palladium for visualization using scanning electron microscopy, accordingly to Zubairova et al[7]. Semi quantitative evaluation of network density and fibers branching were performed using STEPanizer software (www.stepanizer.com).

Calibrated automated thrombography assays in murine samples

Thrombin generation in PFP and PRP was determined using the calibrated automated thrombogram (CAT) method.

TFPI dependent PS activity was assessed in PRP (150 G/L), as follows. Briefly, 10 µL mouse PRP (150 G/L) was mixed with 10 µL PRP reagent (Diagnostica Stago), and 30 µL of buffer A (25 mm Hepes, 175 mm NaCl, pH 7.4, 5 mg/mL BSA). Thrombin generation was initiated at 37°C with 10 µL of a fluorogenic substrate/$CaCl_2$ mixture. Final concentrations were as follows: 16.6% mouse plasma, 1 pM hrTF, 4 µM phospholipids, 16 mM $CaCl_2$, and 0.42 mM fluorogenic substrate.

APC dependent PS activity was assessed in a CAT-based APC resistance test in mouse PFP and PRP in accordance to Dargaud Y et al[8]. PRP (150 G/L) was previously activated using 40 µM Ca^{2+} ionophore (A23187) for 5 min at 37C. Final concentrations were as follows: 16.6% mouse plasma, 22 µM A23187, 1 pM hrTF, 4 µM phospholipids, 2nM (for PFP) or 8 nM (for PRP) wild type recombinant mouse APC (wt-rmAPC)[5] or mutated recombinant mouse APC (rmAPC L38D),16 mM $CaCl_2$, and 0.42 mM fluorogenic substrate. The generation and characterization of rmAPC L38D was performed according to ref [9,10] and the purification according to ref[11,12].

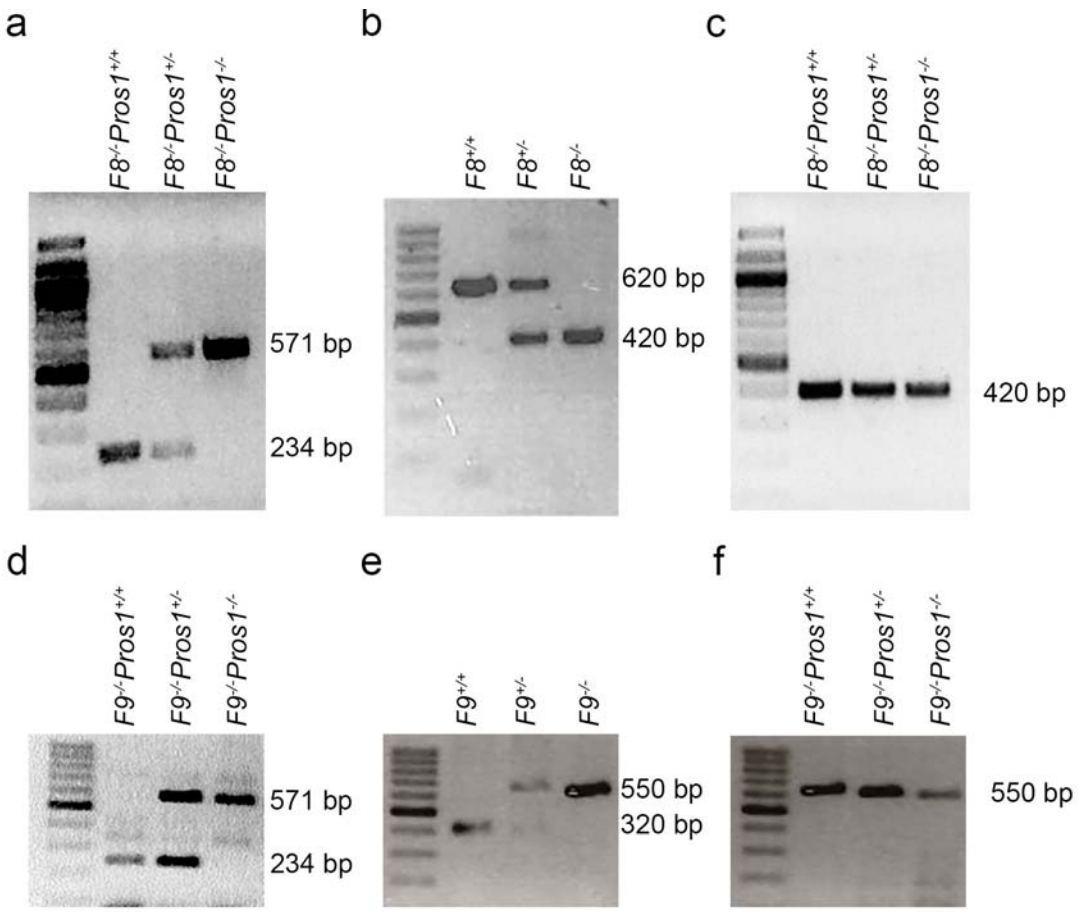
For TF titration on PFP, the following reagents were used: PPP reagent and MP reagent (Diagnostica Stago).

Fluorescence was measured using a Fluoroscan Ascent® fluorometer, equipped with a dispenser. Fluorescence intensity was detected at wavelengths of 390 nm (excitation filter) and 460 nm (emission filter). A dedicated software program, Thrombinoscope® version 3.0.0.29 (Thrombinoscope bv) enabled the calculation of thrombin activity against the calibrator (Thrombinoscope bv) and displayed thrombin activity with the time. All experiences were carried out in duplicate at 37 °C and the measurements usually lasted 60 min.

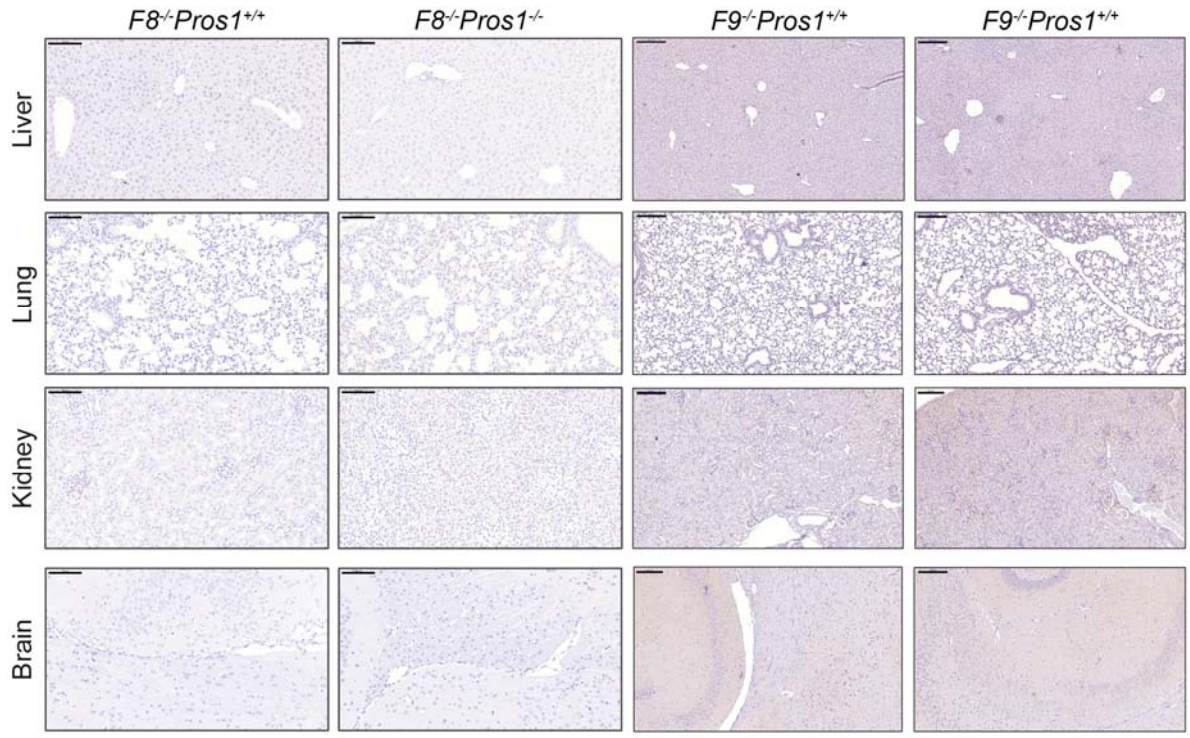
CAT assay in human samples

Written informed consent was obtained from patients. Venous blood was drawn by venipuncture in 3.2% sodium citrate (vol/vol) and centrifuged at 2000g for 5 min. Platelet-poor plasma (PPP) was then centrifuged at 10000g for 10 min to obtain PFP. PFP was aliquoted, snap-frozen, and stored at −80°C until use. For PRP, blood was centrifuged at 180 g x 10 min. All subjects gave informed consent to participation. Thrombin generation was assessed in human PFP and PRP, according to ref[13] with minor changes. Briefly, 68 µL PFP or PRP (150 G/L) was incubated for 15 min at 37 °C with 12 µL of either a polyclonal rabbit anti-human PS-IgG antibody (0.42 mg/mL, Dako) or monoclonal antibodies against TFPI (0.66 µm, MW1848, Sanquin) or buffer A. Coagulation was initiated with 20 µL of a 7 : 1 mixture of the PPP low and PPP 5 pm reagents (Diagnostica Stago) for PFP samples or with PRP reagent (Diagnostica stago) for PRP samples. After addition of 20 µL of $CaCl_2$ and fluorogenic substrate (I-1140; Bachem), the thrombin generation was followed in a Fluoroskan Ascent reader (Thermo Labsystems).

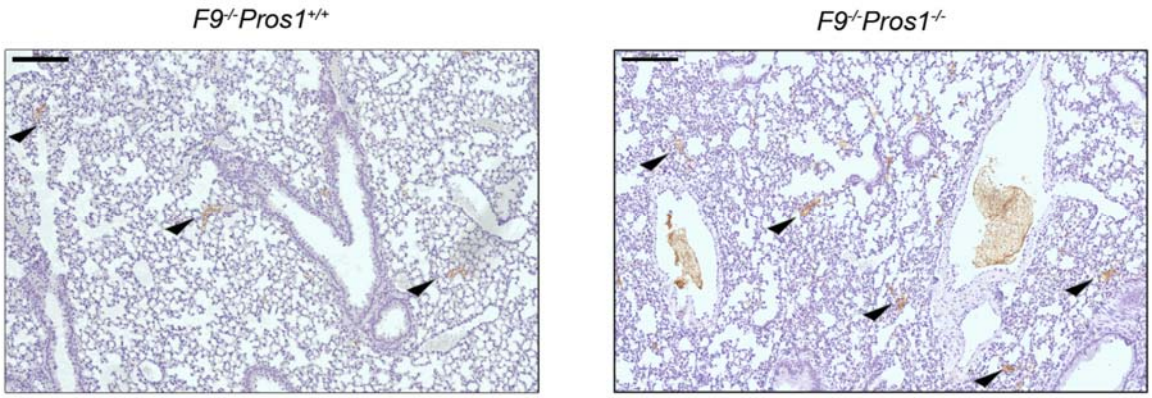
Supplemental Figures



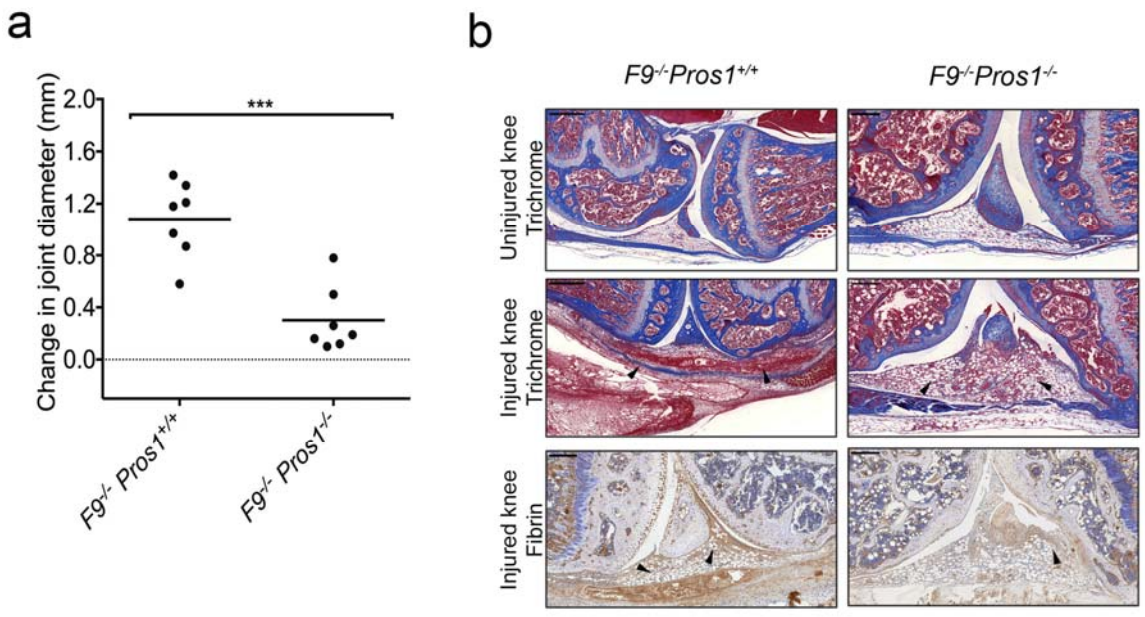
Supplementary Figure 1. Genotyping approaches. Genotypes obtained by crossing *F8*$^{-/-}$*Pros1*$^{+/-}$ (**a-c**) and *F9*$^{-/-}$*Pros1*$^{+/-}$ (**d-f**) mice. a, *Pros1* alleles were amplified by a multiplex PCR. PCR products were then subjected to electrophoresis; the *wt* band has a lower molecular weight (234 bp) compared to the *null* band (571 bp), in accordance to Saller, 2009. **b,** Set-up of multiplex PCR to amplify the *wt* band (620 bp) and the *null* band (420 bp) of *F8* alleles from genomic DNA. **c,** PCR products of *F8* alleles amplification (*null* band: 420 bp) on the same samples than in (**a**). **d,** *Pros1* alleles were amplified by a multiplex PCR. PCR products were then subjected to electrophoresis; the *wt* band has a lower molecular weight (234 bp) compared to the *null* band (571 bp), in accordance to Saller, 2009. **e**, Set-up of multiplex PCR to amplify the *wt* band (320 bp) and the *null* band (550 bp) of *F9* alleles from genomic DNA. **f**, PCR products of *F9* alleles amplification (*null* band: 550 bp) on the same samples than in (**d**).



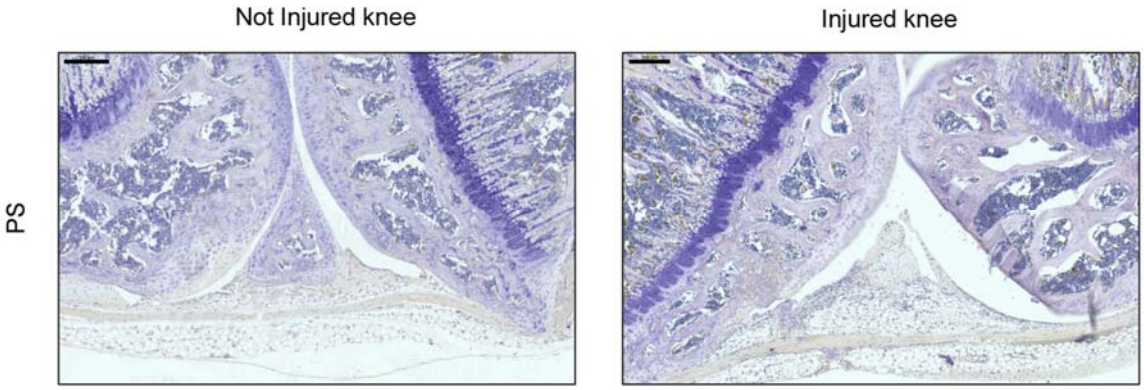
Supplementary Figure 2: Histology in physiologic condition. Immunostaining for insoluble fibrin on liver, lung, kidney, brain sections in *F8*$^{-/-}$*Pros1*$^{-/-}$ and in *F8*$^{-/-}$*Pros1*$^{+/+}$ mice as well as in *F9*$^{-/-}$*Pros1*$^{+/+}$ and *F9*$^{-/-}$*Pros1*$^{-/-}$. Scale bar: 100 µm.



Supplementary Figure 3: Microscopic evaluation of fibrin clots in lung section after rFIX administration in *F9*$^{-/-}$*Pros1*$^{-/-}$ mice. *F9*$^{-/-}$*Pros1*$^{-/-}$ mice received 2 injections of an overdose of rFIX administered 12h apart in *F9*$^{-/-}$*Pros1*$^{-/-}$ mice (FIX plasma level was 200% in *F9*$^{-/-}$*Pros1*$^{-/-}$ at 24h), Thrombi localization is indicated by arrow heads.

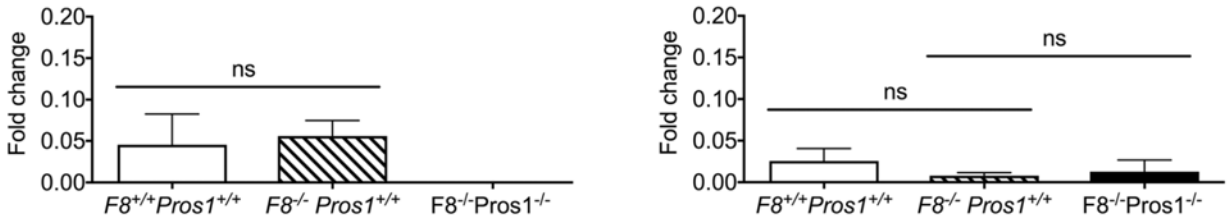


Supplementary Figure 4: Genetic loss of *Pros1* prevents hemarthrosis in mice with hemophilia B. a, Difference between the knee diameter 72 h after the injury and before the injury in *F9*$^{-/-}$*Pros1*$^{+/+}$, *F9*$^{-/-}$*Pros1*$^{+/-}$, *F9*$^{-/-}$*Pros1*$^{-/-}$ and *F9*$^{+/+}$*Pros1*$^{+/+}$ mice. **b,** Microscopic evaluation (Masson's trichrome stain and staining for insoluble fibrin, mAb clone 102-10) of the knee intra-articular space of a representative not injured and injured legs after 72 h in *F9*$^{+/+}$*Pros1*$^{+/+}$, *F9*$^{-/-}$*Pros1*$^{+/+}$ and *F9*$^{-/-}$*Pros1*$^{-/-}$ mice. Scale bar: 500 μm. Measurements are presented as mean±s.e.m. ***, P<0.0005.

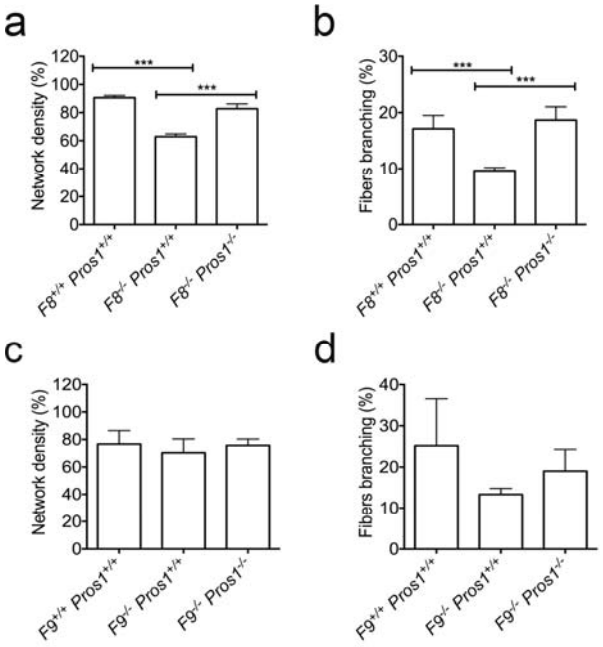


Supplemental Figure 5: Lack of PS expression in hemophilic mice synovium after recombinant human FVIII infusion. Immunostaining for PS in the knee intra-articular space of injured and not injured knees from F8-/-Pros1-/- mice previously treated with hrFVIII (0.3U/g body weight). Scale bars: 200 µm.

a b



Supplementary Figure 6: Expression of Pros1 and Tfpi in murine fibroblast-like synoviocytes (FLS). Real time PCR on murine FLS from *F8*$^{+/+}$*Pros1*$^{+/+}$, *F8*$^{-/-}$*Pros1*$^{+/+}$ and *F8*$^{-/-}$*Pros1*$^{-/-}$ mice to investigate *Pros1* (a) and *Tfpi* (b) expression.



Supplementary Figure 7: Quantification of fibrin network density and fibers branching. a-b, Fibrin network from *F8*$^{+/+}$ *Pros1*$^{+/+}$, *F8*$^{-/-}$ *Pros1*$^{+/+}$ and *F8*$^{-/-}$ *Pros1*$^{-/-}$ mice. **c-d,** Fibrin network from *F9*$^{+/+}$ *Pros1*$^{+/+}$, *F9*$^{-/-}$ *Pros1*$^{+/+}$ and *F9*$^{-/-}$ *Pros1*$^{-/-}$. Quantification of fibrin network density (a and c). Quantification of fibers branching (b and d). Measurements are presented as mean±s.e.m. ***, *P*<0.0005

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