Supplementary Material to Subramaniam et al. “Defective thrombus formation in mice lacking endogenous factor VII activating protease (FSAP)” (Thromb Haemost 2015; 113.3)

Suppl. Figure 1: TFPI, FVII and FVIIa in carotid artery thrombosis model: (A) Control arteries or those with thrombosis were immediately embedded in OCT compound (Tissue-Tek, Miles Inc, USA), and snap-frozen. 5 μm cross sections were made through the whole frozen tissue and stained with TFPI (H-120, 200 μg/ml, 1:500; Santa Cruz Biotech). 4 mice in each group were analyzed. Fluorescence microscope images were performed in Leica Microsystems, Wetzlar, Germany equipped with a High speed VISitron systems camera using MetaMorph imaging software version series 7.0. Mouse plasma from WT and FSAP-/− mice subjected to FeCl3-induced carotid artery thrombosis was analyzed for TFPI and FSAP by quantitative Western blotting (B), TFPI activity by Actichrome assay (C), FVII (D) and FVIIa (E). Data were expressed as mean (n=7 to 10). Statistical significance was determined using the Mann-Whitney U test and there were no differences between WT and FSAP-/− mice.
Suppl. Figure 2: Integrin activation and α-granule release in platelets from WT and FSAP⁻/⁻ mice: (A) Flow cytometric analysis of integrin αIIbβ3 activation (binding of JON/A-PE). (B) Degranulation-dependent P selectin exposure in response to the indicated agonists in WT (white bars) and FSAP⁻/⁻ (grey bars) platelets. Results were expressed as mean fluorescence intensities (MFI) ± SD, n=6. Abbreviations: U46-U4619; CVX-convulxin; RC- rhodocytin; CRP-collagen related peptide. In detail: Heparinized whole blood was diluted 1:20 with modified Tyrode-HEPES buffer (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid], pH 7.0) containing 5 mM glucose, 0.35% bovine serum albumin (BSA), and 1 mM CaCl₂. Activation studies were performed with blood samples washed twice with modified Tyrode-HEPES buffer, which then were activated with various agonists, stained with fluorophore-labeled antibodies, for 15 minutes at room temperature, and directly analyzed on a FACScalibur (Becton Dickinson, Heidelberg, Germany). Statistical analysis was performed using the Students ‘t’ test, there were no significant differences between WT and FSAP⁻/⁻ mice.
Suppl. Figure 3: Ex-vivo analysis of platelet adhesion under flow in platelets of WT and FSAP<sup>−/−</sup> mice: (A) Adhesion and thrombus formation of FSAP<sup>−/−</sup> platelets on collagen under flow rate of 7.53 ml/h for 4 min at a shear rate of 1000<sup>s</sup>-1. Representative phase-contrast (bright field [BF]) and fluorescence images are shown as well as mean surface coverage (B) and relative thrombus volume (C), as measured by integrated fluorescent intensity (IFI) per mm<sup>2</sup> ± SD (n = 6 mice). Scale bar: 50 μm. There were no significant differences between WT and FSAP<sup>−/−</sup> mice, n.s. indicates not significant. In detail: Rectangular coverslips (24mm by 60 mm) were coated with fibrillar type I collagen (0.2 mg/ml; Nycomed, Munich, Germany) for 1 hour at 37°C and were blocked with 1% bovine serum albumin (BSA). Heparinized whole blood was labeled with a DyLight 488-conjugated antibody against GPIX immunoglobulin derivative at 0.3 mg/ml, and during perfusion, microscopic phase-contrast images were recorded in real time and phase-contrast pictures were taken from at least 5 different microscopic fields (63× objectives). Image analysis was performed off-line with Metamorph software (Visitron, Germany). Thrombus formation was expressed as the mean percentage of total area covered by thrombi and as the mean integrated fluorescence intensity per square millimeter. Statistical analysis was performed using the Students ‘t’ test, there were no significant differences between WT and FSAP<sup>−/−</sup> mice.
Suppl. Figure 4: Levels of platelet glycoproteins in WT and FSAP\textsuperscript{-/-} mice. Expression of glycoproteins on the platelet surface was determined by flow cytometry. Diluted whole blood was incubated with FITC-labeled antibodies at saturating concentrations for 15 min at RT and platelets were analyzed directly. Results were expressed as mean fluorescence intensity (MFI) ± SD (n=6). α2, integrin α2; β1, integrin β1; αIIbβ3, integrin αIIbβ3. In detail: Heparinized whole blood was diluted 1:20 with modified Tyrode-HEPES buffer (134 mM NaCl, 0.34 mM Na\textsubscript{2}HPO\textsubscript{4}, 2.9 mM KCl, 12 mM NaHCO\textsubscript{3}, 20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.0) containing 5 mM glucose, 0.35% bovine serum albumin (BSA), and 1 mM CaCl\textsubscript{2}. For glycoprotein expression and platelet count, blood samples were incubated with appropriate fluorophore-conjugated monoclonal antibodies for 15 minutes at room temperature and were directly analyzed on a FACScalibur (Becton Dickinson, Heidelberg, Germany). Statistical analysis was performed using the Students ‘t’ test, there were no significant differences between WT and FSAP\textsuperscript{-/-} mice.