# Supplemental methods and data

## Supplemental materials and methods

### ***Reagents and buffers***

Recombinant human TF (Innovin, Siemens Healthineers AG, Germany) and synthetic phospholipids (a mixture of 20 mol% phosphatidylserine, 20 mol% phosphatidylethanolamine and 60 mol% phosphatidylcholine, Avanti Polar Lipids Inc., Alabama, USA) were provided by Synapse Research Institute (Maastricht, the Netherlands). Human TF was used as a trigger because a previous study showed murine FVIIa binds with high affinity to both murine and human TF.[1] Thrombin calibrator (i.e. thrombin-$\alpha_2$-macroglobulin complex) was from Synapse Research Institute. The contact pathway activator silica particulate (Pacific Hemostasis KONTACT #100312) was from Thermo Fisher Scientific, VA, USA. Thrombin fluorogenic substrate (Z-Gly-Gly-Arg-7-amino-4-methylcoumarin [ZGGR-AMC], #4002155) was from Bachem (Bubendorf, Switzerland).

Corn trypsin inhibitor (CTI; CAT#IAFCTILY1MG) was from Molecular Innovations (MI, USA). Anti-FXI antibody 14E11 was described before[2] and was provided by Dr Andras Gruber (Aronora, Inc, OR, USA). Activated human FXII was from Enzyme Research Lab, (CAT# HFXIIa 1212a). Platelet glycoprotein VI agonist snake venom toxin convulxin was a generous gift from Drs. David S. Paul and Wolfgang Bergmeier (UNC-Chapel Hill).

A buffer (referred to as BSA5 buffer) containing 20 mM HEPES, 140 mM NaCl and 5 mg/mL bovine serum albumin (BSA), pH 7.35 was used for dilution of TF, phospholipids or mouse whole blood. Buffer (BSA60 buffer) containing 20 mM HEPES and 60 mg/mL BSA, pH 7.35 was used to dilute the thrombin substrate.

### ***Ethical***

This study complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was performed with the approval of the Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

### ***Mice***

*F12*$^{-/-}$, *F11*$^{-/-}$ and *F9*$^{-/-}$ mice were described previously [3-5] and backcrossed 10 generations onto the C57BL/6J background. Appropriate age- and sex-matched sister-line wild-type C57BL/6J mice were used as control mice for comparisons with *F12*$^{-/-}$ and *F11*$^{-/-}$ mice. Age- and sex-matched wild-type C57BL/6J were used as background control for *F9*$^{-/-}$ mice. Both male and female mice used in this study were aged between 8 to 14 weeks.

### ***Blood sample handling***

Platelet rich plasma (PRP) was prepared by centrifuging WB collected with citrate and CTI was centrifuged twice at 150 xg for 5 minutes each and used fresh. Washed RBCs were prepared as previously described[6]. RBC fractions were taken from the bottom of the tube (approximately 140 µL to avoid disturbing the buffy coat) after centrifuging CTI-containing mouse WB at 150 xg for 5 minutes. This RBC fraction was then washed with washing buffer (1.29 mM sodium citrate, 3.33 mM glucose, 124 mM NaCl, pH 7.2) and then centrifuged at 500 xg, for 5 minutes and the supernatant was discarded

together with the top 1/5 of the RBC fraction. After 2 more washing steps, RBCs were resuspended in HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4) and centrifuged (400 xg, 10 minutes) to packed RBCs. Packed RBCs of 2 mice of the same genotype were pooled together to have enough materials for TG measurements. PRP of 2 mice with the same genotype were also pooled together. The RBC counts of packed RBCs were adjusted to physiological levels of $7*10^6$/ µL using HEPES-buffered saline. Reconstitution of PRP with packed RBCs (PRP+RBCs) were done by mixing 50 µL of PRP with 50 µL of packed RBCs, together with 100 µL HEPES-buffered saline. PRP were diluted 4-fold as well so that the platelet counts are the same in PRP and PRP+RBCs.

Cell counts of WB, PRP and PPP were measured with Hemavet HV950FS (Drew Scientific, Miami Lakes, FL, USA).

### ***Measurement of mouse fibrinogen, prothrombin and factor X***

Plasma fibrinogen level was measured using a mouse fibrinogen ELISA kit (CAT#E-90FIB) from Immunology Consultants Laboratory (OR, USA).

Comparisons of plasma prothrombin and FX levels between FXII, FXI or FIX knock-out mice and wild-type controls were done with western blot. Sheep anti-mouse FX immune-adsorbed polyclonal antibody (CAT#PAMFX-SIA) and rat anti-mouse prothrombin monoclonal antibody (CAT#AMP-9013) was from PROLYTIX (VT, USA) were used at 0.5 µg/mL (f.c.) each for the detection of mouse prothrombin and FX, respectively. Fluorescently labelled secondary antibodies (0.5 µg/mL) were used for signal output and the membrane was visualized on an Odyssey imaging system (Li-Cor Biosciences). Densitometric analysis was conducted using ImageJ software (version 1.52, National Institutes of Health) as previously described [7]

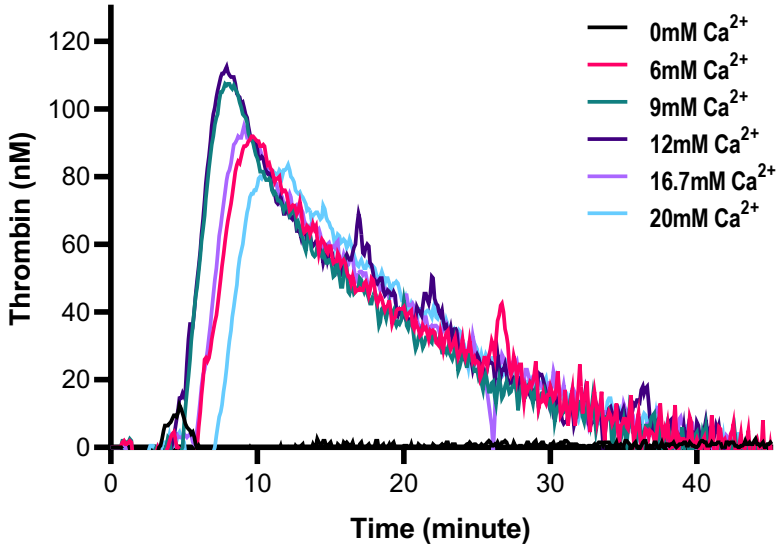
## Supplemental References


1. L C Petersen, P L Nørby, S Branner, B B Sørensen, T Elm, H R Stennicke et al. *Characterization of recombinant murine factor VIIa and recombinant murine tissue factor: a human-murine species compatibility study*. **Thromb Res**, 2005; 116: 75-85.
2. D V Kravtsov, A Matafonov, E I Tucker, M F Sun, P N Walsh, A Gruber et al. *Factor XI contributes to thrombin generation in the absence of factor XII*. **Blood**, 2009; 114: 452-458.
3. D Gailani, N M Lasky, G J Broze, Jr. *A murine model of factor XI deficiency*. **Blood Coagul Fibrinolysis**, 1997; 8: 134-144.
4. H U Pauer, T Renné, B Hemmerlein, T Legler, S Fritzlar, I Adham et al. *Targeted deletion of murine coagulation factor XII gene-a model for contact phase activation in vivo*. **Thromb Haemost**, 2004; 92: 503-508.
5. H F Lin, N Maeda, O Smithies, D L Straight, D W Stafford. *A coagulation factor IX-deficient mouse model for human hemophilia B*. **Blood**, 1997; 90: 3962-3966.
6. B L Walton, M Lehmann, T Skorczewski, L A Holle, J D Beckman, J A Cribb et al. *Elevated hematocrit enhances platelet accumulation following vascular injury*. **Blood**, 2017; 129: 2537-2546.
7. S P Grover, T M Olson, B C Cooley, N Mackman. *Model-dependent contributions of FXII and FXI to venous thrombosis in mice*. **J Thromb Haemost**, 2020; 18: 2899-2909.

**Supplemental Table 1. Blood cell counts and plasma coagulation factor levels of mice with different genotypes.**

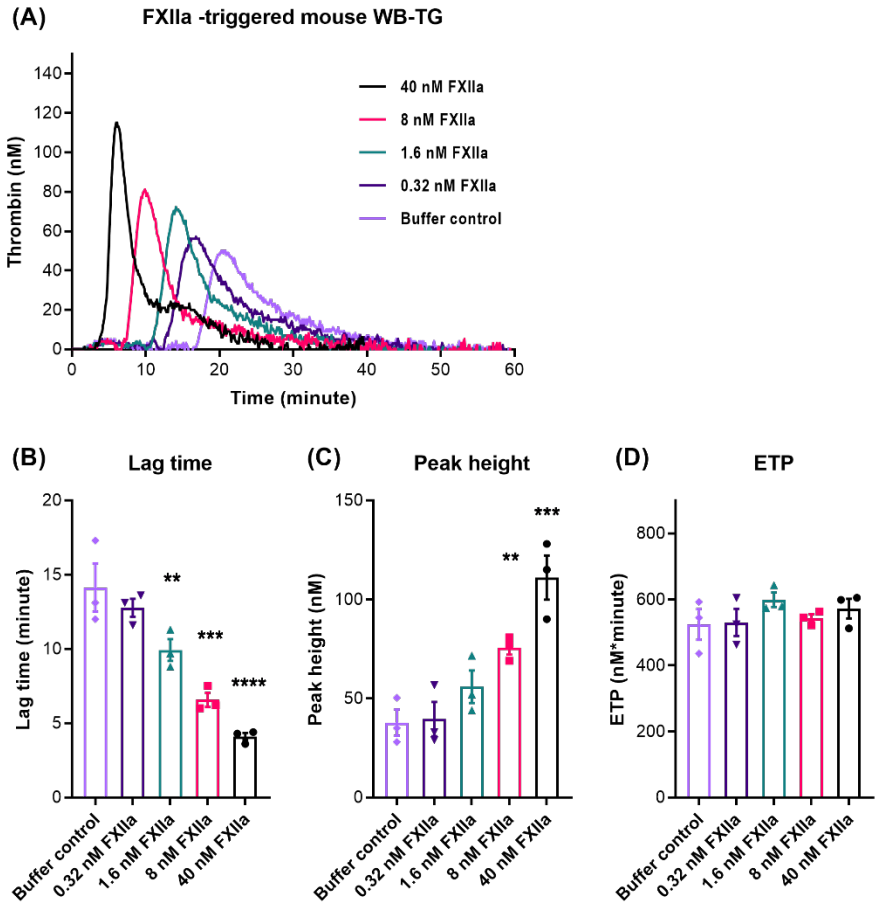
| | $F12^{+/+}$ | $F12^{-/-}$ | p value | $F11^{+/+}$ | $F11^{-/-}$ | p value | $F9^{+/+}$ | $F9^{-/-}$ | p value |
|---|---|---|---|---|---|---|---|---|---|
| **Cell counts** | | | | | | | | | |
| **Platelet (10^3/μL)** | 806 (675-845) | 950 (744-1022) | 0.138 | 1161 (1078-1190) | 1016 (977-1056) | 0.132 | 970 (955-1057) | 980 (891-1124) | 0.945 |
| **RBC (10^6/uL)** | 7.5 (7.2-7.7) | 7.1 (6.9-7.3) | 0.073 | 7.5 (7.3-7.6) | 7.3 (7.2-7.4) | 0.31 | 7.3 (7.2-7.5) | 7.3 (6.6-7.6) | 0.836 |
| **Hemoglobin (g/dL)** | 11.8 (11.8-11.9) | 11.5 (11.3-11.7) | 0.014 | 11.3 (11.2-11.3) | 11.3 (11-11.4) | 0.818 | 11.3 (11.1-11.5) | 11.2 (10.4-11.9) | 0.731 |
| **Hematocrit (%)** | 34.6 (33.5-35.2) | 33.5 (32.7-33.8) | 0.101 | 33.8 (33.3-34) | 32.6 (31.8-32.8) | 0.065 | 33.8 (33.2-34.7) | 33.3 (30.3-34.6) | 0.534 |
| **WBC (10^3/uL)** | 6.8 (6.6-9.2) | 9.5 (6-9.9) | 0.731 | 5.3 (4.4-6.6) | 7.2 (6.8-8) | 0.132 | 7.9 (7.6-8.6) | 8.6 (7.1-11.2) | 0.628 |
| **Neutrophil (10^3/uL)** | 0.7 (0.6-0.8) | 0.9 (0.6-0.9) | 0.628 | 0.6 (0.4-0.8) | 1.8 (1.5-2.2) | 0.015 | 1 (0.8-1.1) | 0.7 (0.5-1.2) | 0.731 |
| **Lymphocyte (10^3/uL)** | 6 (5.7-8.1) | 8.2 (5.2-8.5) | 0.836 | 4.5 (3.9-5.5) | 4.7 (4.6-5.9) | 0.699 | 6.8 (6.6-7.3) | 8.1 (5.2-10.3) | 0.731 |
| **Monocyte (10^3/uL)** | 0.17 (0.13-0.17) | 0.23 (0.1-0.35) | 0.534 | 0.13 (0.1-0.22) | 0.27 (0.24-0.33) | 0.065 | 0.2 (0.14-0.25) | 0.16 (0.12-0.21) | 0.445 |
| **Eosinophil (10^3/uL)** | 0.05 (0.04-0.065) | 0.075 (0.048-0.088) | 0.534 | 0.05 (0.043-0.058) | 0.035 (0.03-0.048) | 0.485 | 0.05 (0.04-0.07) | 0.04 (0.04-0.08) | 0.945 |
| **Basophil (10^3/uL)** | 0 (0-0.005) | 0.01 (0.01-0.01) | 0.101 | 0 (0-0) | 0.005 (0-0.018) | 0.31 | 0.01 (0.01-0.01) | 0.01 (0.01-0.02) | 0.138 |
| **Coagulation factor levels** | | | | | | | | | |
| **FX (% of control)** | 101.3 (96.3-104.1) | 110 (97.6-119.9) | 0.421 | 107.4 (77.3-115.6) | 95 (78.2-122.3) | 1.000 | 94 (90.1-104.4) | 115 (114.6-142.6) | 0.421 |
| **FII (% of control)** | 99.5 (97.4-103.8) | 92.8 (90.1-97) | 1.000 | 109.1 (88.9-114.2) | 82.5 (76.3-88.2) | 0.151 | 90.8 (84.4-100.9) | 128.3 (122.3-131.7) | 0.095 |
| **Fibrinogen (% of control)** | 102.8 (96.2-103.6) | 104.4 (103.7-119.6) | 0.222 | 101.2 (86.2-110.8) | 118.6 (86.7-137.7) | 0.421 | 91 (90.2-96.4) | 136.6 (129.1-175) | 0.032 |

Footnote: Blood was collected from inferior vena cava of mice (n=6-7 mice per group) into citrate (1/10 volume) and mixed with corn trypsin inhibitor (f.c. 50 μg/mL, 6.2% of total volume). Blood cell counts were measured on Hemavet HV950FS (Drew Scientific, FL, USA). Western blot was used to assess plasma coagulation factor levels in wild-type and knock-out mice. Plasma fibrinogen levels were measured using a mouse fibrinogen ELISA kit. Data are shown as median and interquartile range. Comparisons between wild-type and knock-out mice were done with Mann-Whitney test.



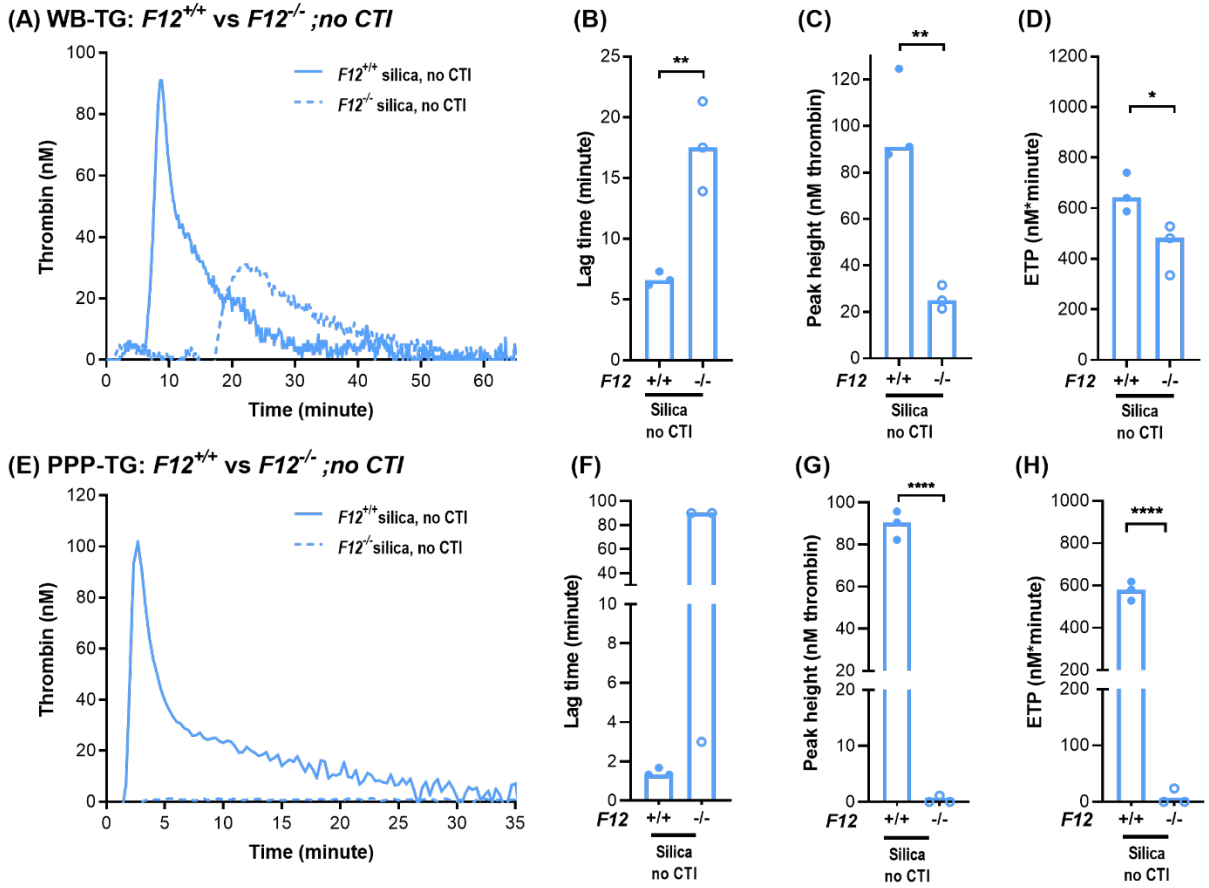

**Supplemental Figure 1. Effect of calcium concentration on mouse WB-TG.**

Citrated mouse WB was triggered by 120x diluted silica and varying doses of $CaCl_2$(0, 6, 9, 12, 16.7 or 20mM, final concentrations) in presence of 416.7 μM thrombin substrate ZGGR-AMC.



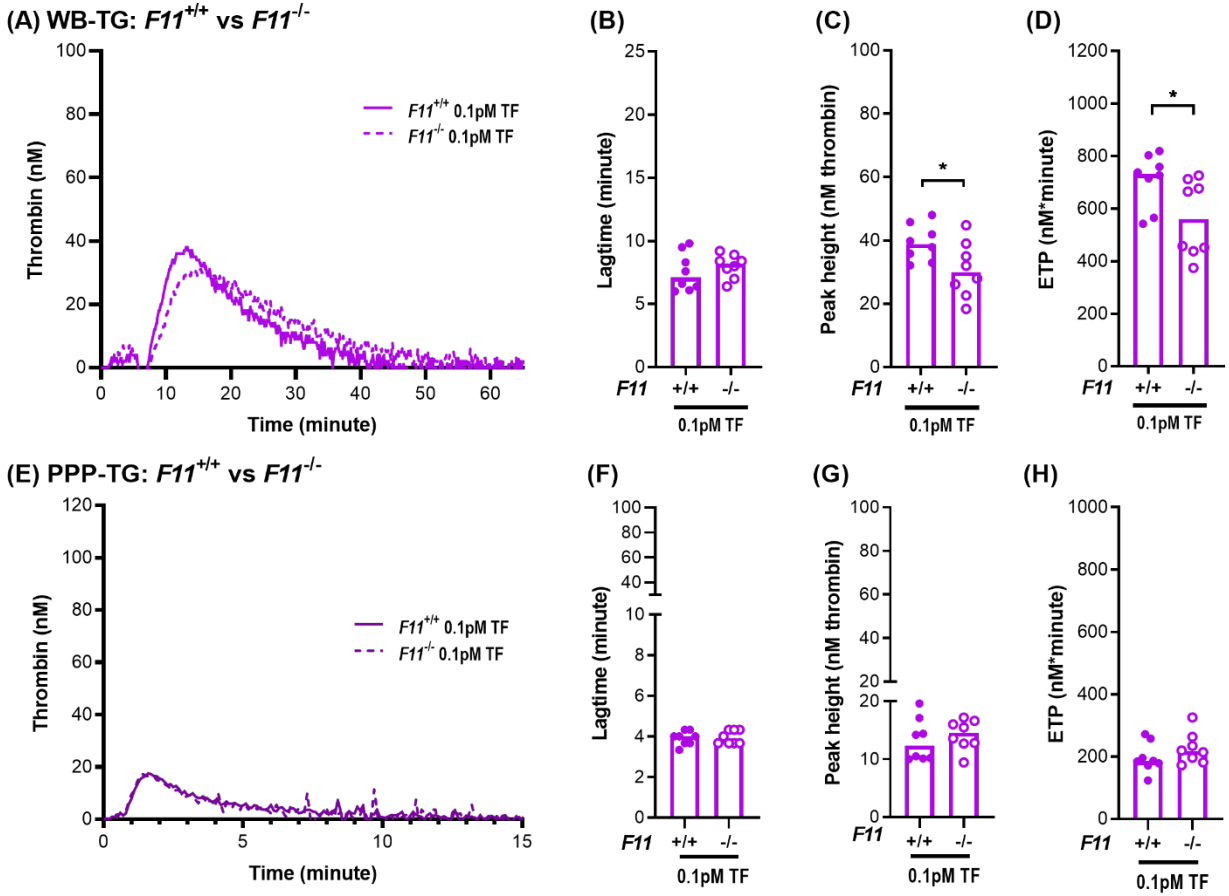

**Supplemental Figure 2. Effect of FXIIa trigger concentrations on mouse WB-TG.**

Mouse WB was collected from inferior vena cava into citrate and 50μg/mL corn trypsin inhibitor. TG was triggered by 9 mM (f.c.) CaCl2 and varying doses of human FXIIa (0, 0.32, 1.6, 8 or 40nM). (A) Representative WB-TG curves. TG parameters are shown as lag time (B), peak height (C) and ETP (D), respectively. Bars represents mean±SD (n=3 mice per group). Comparisons of different groups with the buffer control group were done with repeated measures ANOVA and Dunnett's multiple comparisons test against buffer control group. **p<0.01, ***p<0.001, ****p<0.0001.



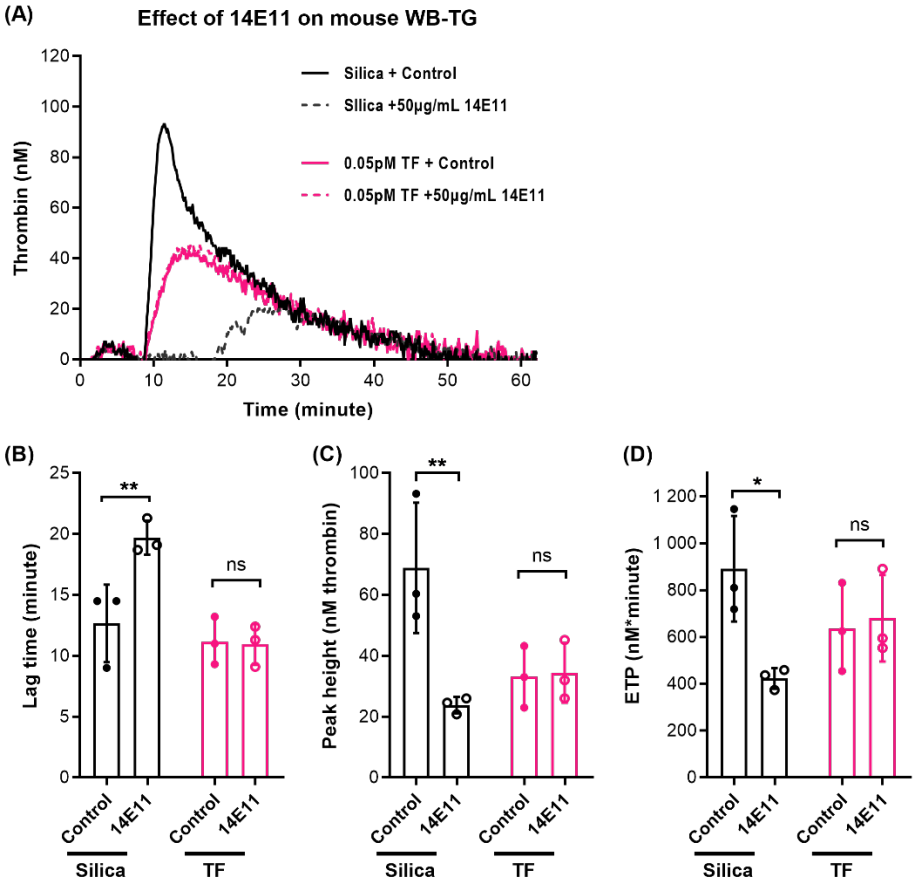

**Supplemental Figure 3. Effect of FXII deficiency on mouse TG in WB and PPP.**

Whole blood (WB) samples of *F12*$^{-/-}$ or *F12*$^{+/+}$ mice were collected in the absence of CTI and a portion was centrifuged at 4500 xg for 15 minutes to generate platelet poor plasma (PPP). Thrombin generation (TG) in both WB and PPP were triggered by 120x diluted silica and 9 mM $CaCl_2$ (4 μM phospholipids were added in PPP). Bar graphs are shown as mean (n=3 mice per group). In the samples that yielded no TG in the duration of the 90-minute measurement, their lag time were marked as 90 minutes. Comparisons between groups were done with student t test. * p<0.05; ** p<0.01; **** P<0.0001.



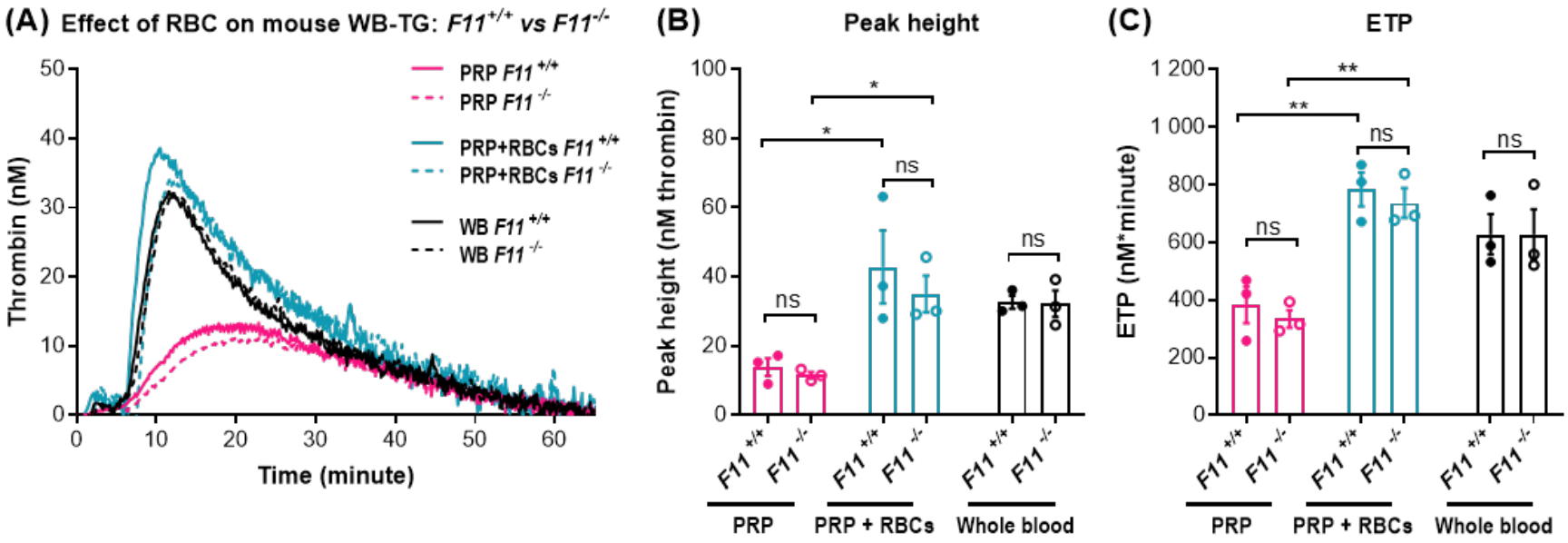

**Supplemental Figure 4. Effect of FXI deficiency on mouse TG in WB and PPP.**

Whole blood (WB) samples of *F11*$^{-/-}$ or *F11*$^{+/+}$ mice were collected in citrate and CTI and a portion was centrifuged at 4500 xg for 15 minutes to generate platelet poor plasma (PPP). Thrombin generation (TG) in both WB and PPP were triggered by recalcification and 0.1 pM tissue factor (TF) and 4 μM phospholipids were added for PPP-TG. Representative curves of WB- and PPP-TG are shown in (A) and (E), respectively. Bar graphs of lag time (B, F), peak height (C, G) and ETP (D, H) are shown as median (n=8 mice per group). Comparisons between groups were done with Mann-Whitney test. *p<0.05.



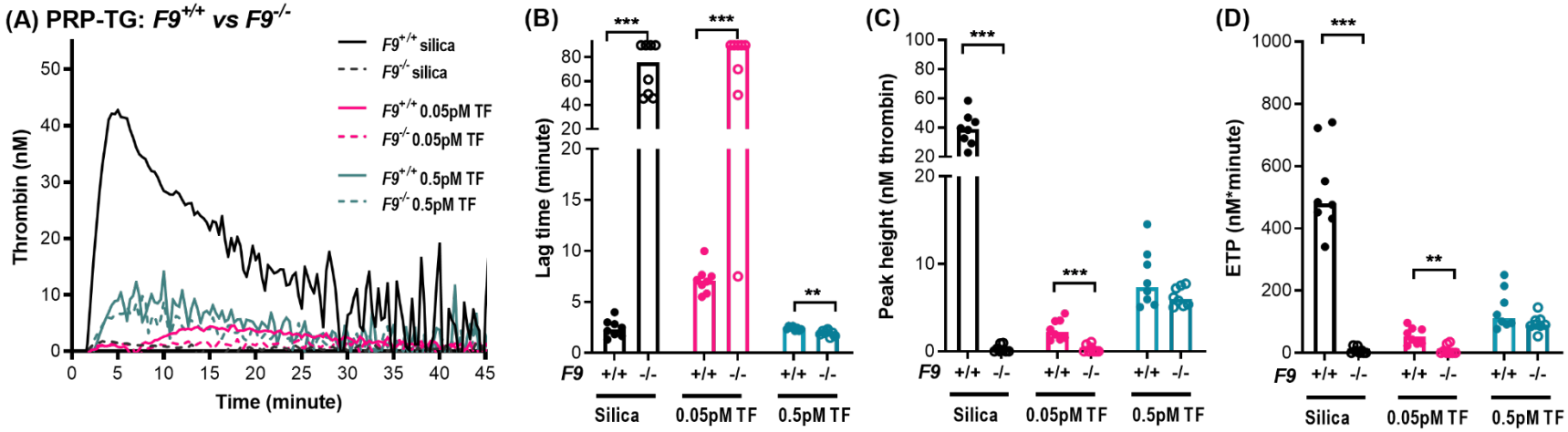

**Supplemental Figure 5. Effect of FXI inhibition by 14E11 on mouse WB-TG.**

Whole blood (WB) of wild-type C57BL/6J mice were collected in presence of citrate and 50 µg/mL CTI. Thrombin generation (TG) were triggered by 120x diluted silica or 0.05 pM tissue factor (TF) and $CaCl_2$, in the absence or presence of an anti-FXI antibody 14E11 (final dose 50µg/mL). Bars represent mean (n=3 mice per group). Comparisons between with and without 14E11 were done with one-way ANOVA and Holm-Sidak's multiple comparisons test, *p<0.05, **p<0.01.



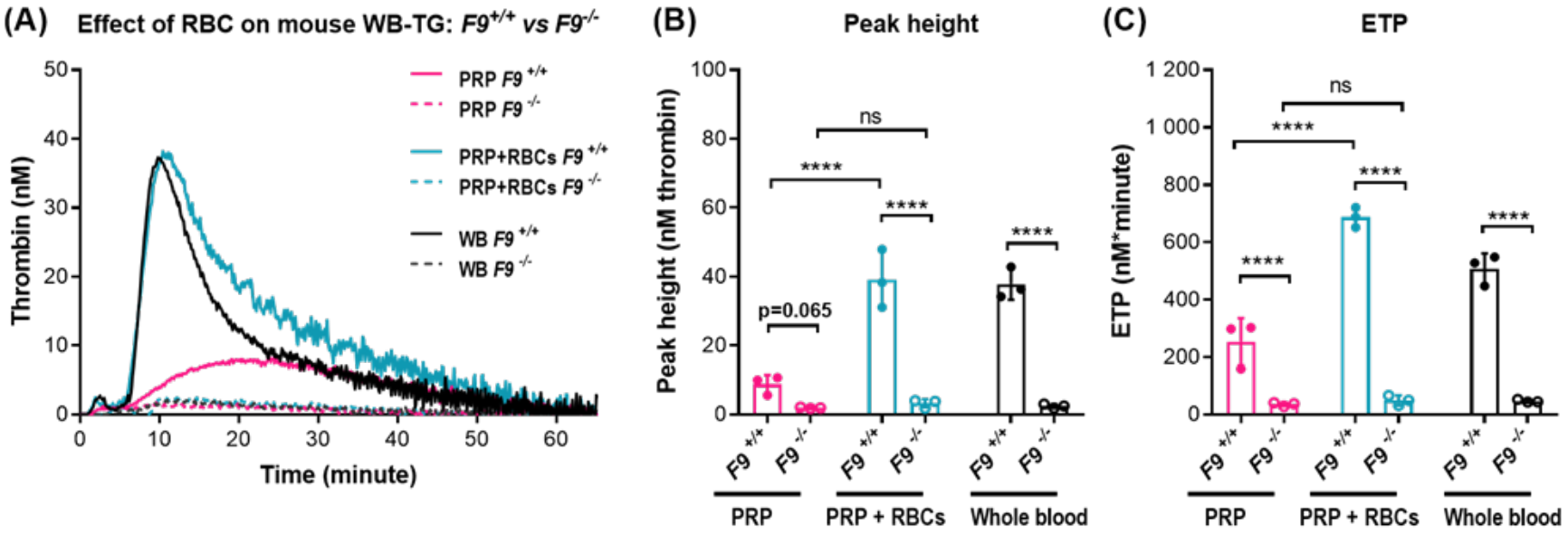

**Supplemental Figure 6. Influence of WB pre-dilutions and RBCs supplementation on *F11*$^{-/-}$ *or F11*$^{+/+}$ mice mouse TG.**

Washed RBCs were prepared from WB collected with citrate and CTI from *F11*$^{-/-}$ or *F11*$^{+/+}$ mice and mixed with autologous PRP (PRP+RBCs). PRP and WB were diluted 4-fold using HEPES buffered saline and PRP+RBCs were prepared to contain same platelet count as in diluted PRP (details in supplemental method). Thrombin generation (TG) were triggered by 0.05 pM tissue factor (TF) and recalcification. Bar graphs are shown as mean±SD (n=3 samples prepared from 6 mice per group). Comparisons between groups were done with One-way ANOVA with Holm-sidak's multiple comparison test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.




**Supplemental Figure 7. Effect of FIX deficiency on mouse PRP-TG.**

Whole blood (WB) samples of *F9*$^{-/-}$ or *F9*$^{+/+}$ mice were collected in citrate and CTI and were then double centrifuged at 150 xg for 5 minutes each to generate platelet rich plasma (PRP). Thrombin generation (TG) were triggered by recalcification and 120x diluted silica or 0.05 / 0.5pM tissue factor (TF). Bar graphs are shown as median (n=8 mice per group). Comparisons between groups were done with Mann-Whitney test. ** p<0.01; *** p<0.001.




**Supplemental Figure 8. Influence of RBCs supplementation on *F9*$^{-/-}$ or *F9*$^{+/+}$ mice mouse TG.**

Washed RBCs were prepared from WB collected with citrate and CTI from *F9*$^{-/-}$ or *F9*$^{+/+}$ mice and mixed with autologous PRP (PRP+RBCs). PRP and WB were diluted 4-fold using HEPES buffered saline and PRP+RBCs were prepared to contain same platelet count as in diluted PRP (details in supplemental method). Thrombin generation (TG) were triggered by 0.05 pM tissue factor (TF) and recalcification. Bar graphs are shown as mean±SD (n=3 samples prepared from 6 mice per group). Comparisons between groups were done with One-way ANOVA with Holm-sidak's multiple comparison test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.