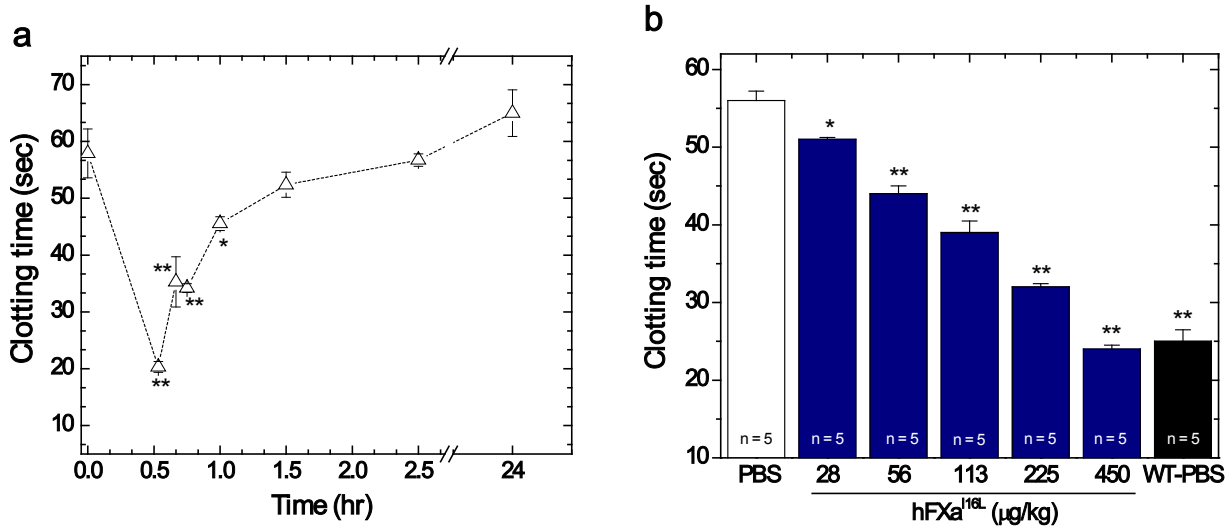
SUPPLEMENTAL FIGURES/TABLES:

Journal: Nature Biotechnology

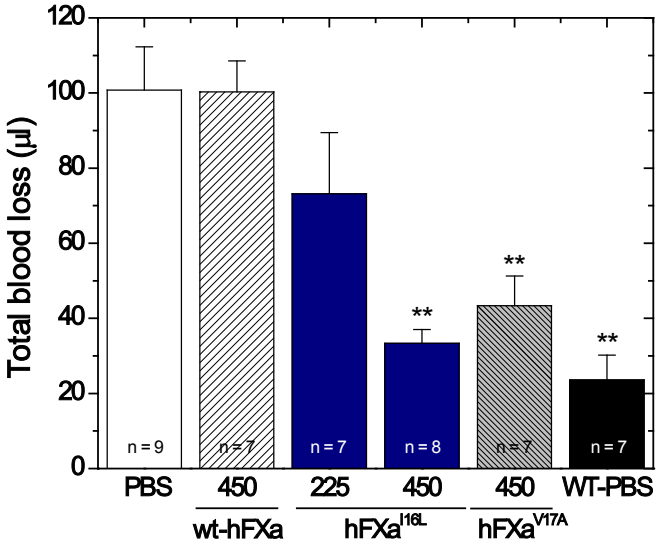
Article Title: Correction of the coagulation defect in hemophilia using a factor Xa variant with novel engineered protease function.

Authors: Lacramioara Ivanciu[1], Raffaella Toso[1], Paris Margaritis[1,2] , Giulia Pavani[1], Haein Kim[1], Alexander Schlachterman[1], Jian-Hua Liu[1], Valerie Clerin[3], Debra D. Pittman[3], Rosalind Rose-Miranda[3], Kathleen M. Shields[3], David V. Erbe[3], James F. Tobin[3], Valder R. Arruda[1,2] and Rodney M. Camire[1,2]

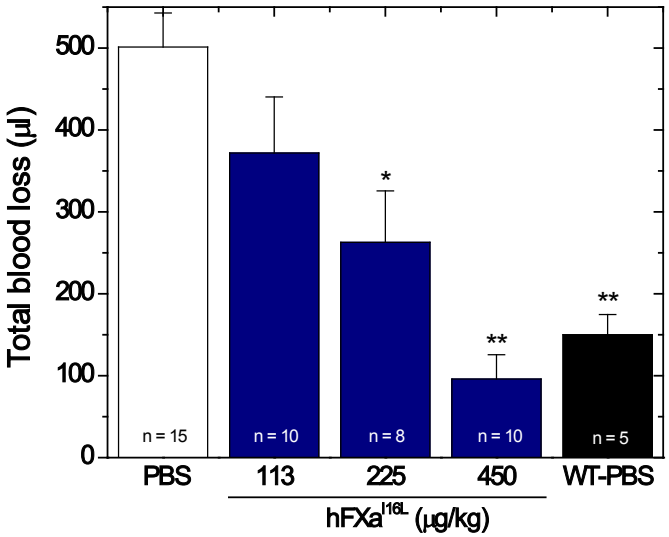
[1]Department of Pediatrics, Division of Hematology, The Children's Hospital of Philadelphia
[2]The University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA 19104.
[3]Pfizer Inc., Cambridge, MA 02140.



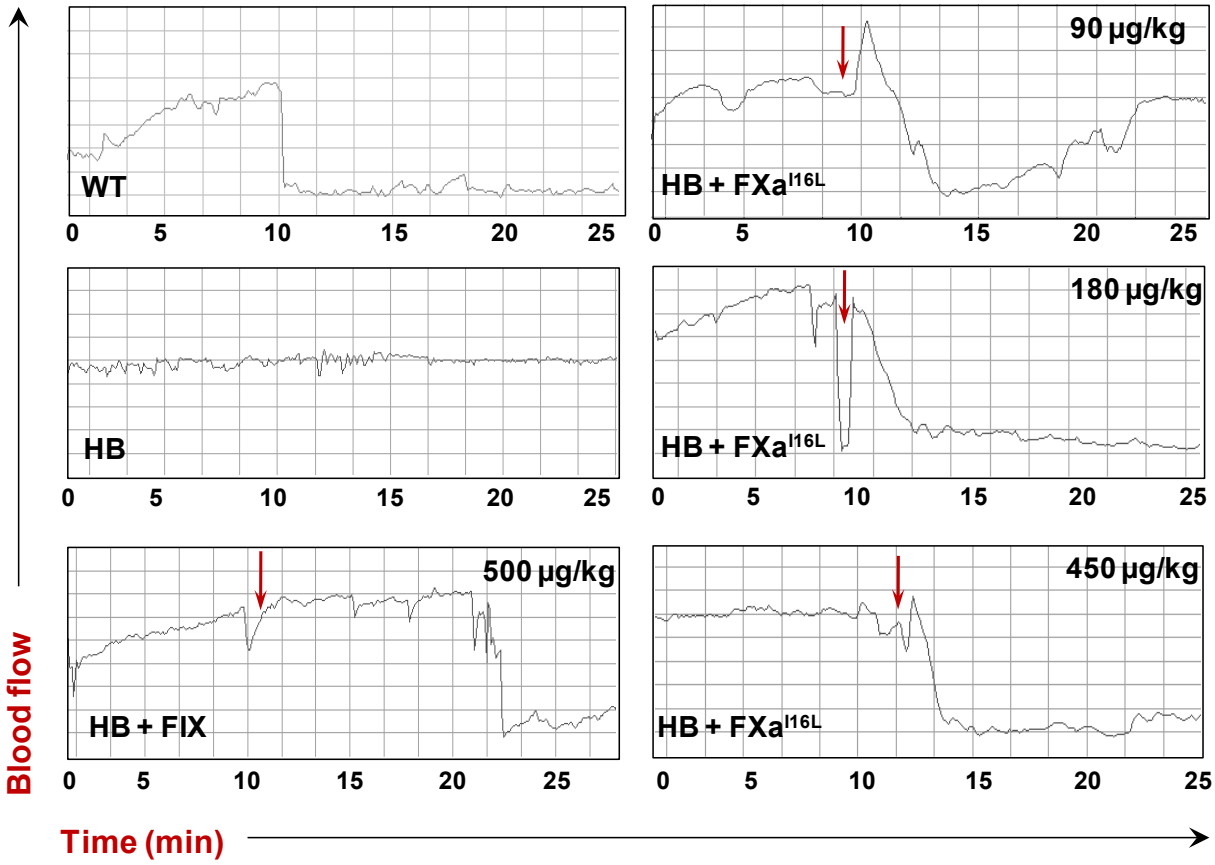
Supplemental Fig. 1. Characterization of hFXa^{I16L} in antibody induced HA mice. In ***a***, HA mice (C57BL/6; n = 6) were injected with 450 µg/kg hFXa^{I16L} (-△-) and at the indicated time intervals a modified one-stage aPTT was performed on processed mouse plasma. In ***b***, HA mice (C57BL/6) were injected with the indicated dose of hFXa^{I16L} (blue columns) and 35 min post-infusion a modified one-stage aPTT was performed on processed mouse plasma. The number of mice at each dose is shown. All values are presented as mean ± SEM. For statistical comparisons, treated animals are compared to untreated or PBS controls ** $p<0.001$; * $p<0.05$.



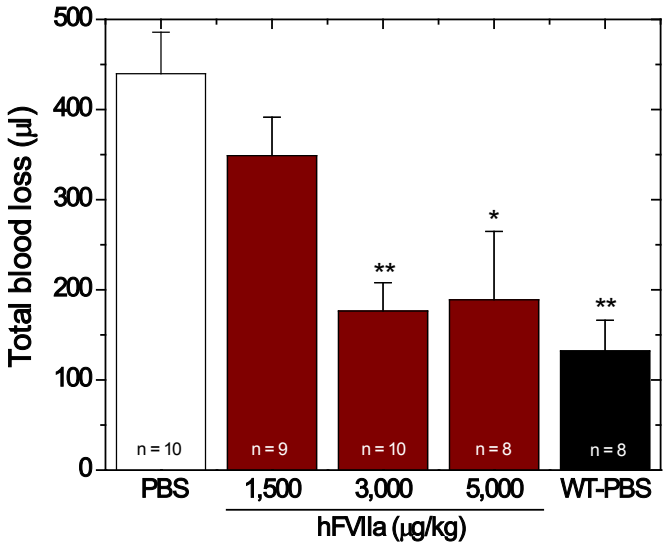
Supplemental Fig. 2. Blood loss following tail clip in HB mice. Five minutes prior to injury, PBS (white column), wt-hFXa (light hatch column), $hFXa^{I16L}$ (blue columns), or $hFXa^{V17A}$ (dark hatch column) was administered to HB mice (Balb/c) via tail vein at the indicated dosage. Total blood loss (µl) was then measured following tail transection. Hemostatically normal mice infused with PBS (WT-PBS, black column) served as a control. The number of animals per group is indicated and all measurements are presented as mean ± SEM. For statistical comparisons, treated animals are compared to HB-PBS controls ** $p<0.001$; * $p<0.05$.



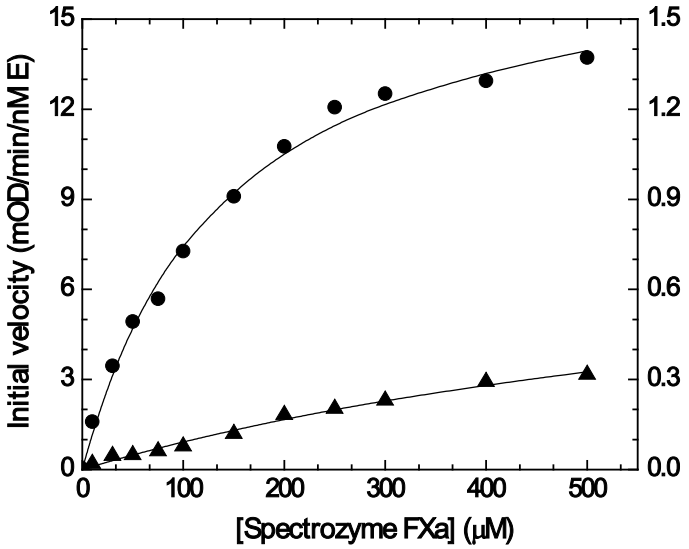
Supplemental Fig. 3. Blood loss following tail clip in antibody induced HA mice. Five minutes prior to injury, PBS (white column) or $hFXa^{I16L}$ (blue columns) was administered to HA mice (C57BL/6) via tail vein at the indicated dosage. Total blood loss (µl) was then measured following tail transection. Hemostatically normal mice infused with PBS (WT-PBS, black column) served as a control. The number of animals per group is indicated and all measurements are presented as mean ± SEM. For statistical comparisons, treated animals are compared to HA-PBS controls ** $p<0.001$; * $p<0.05$.



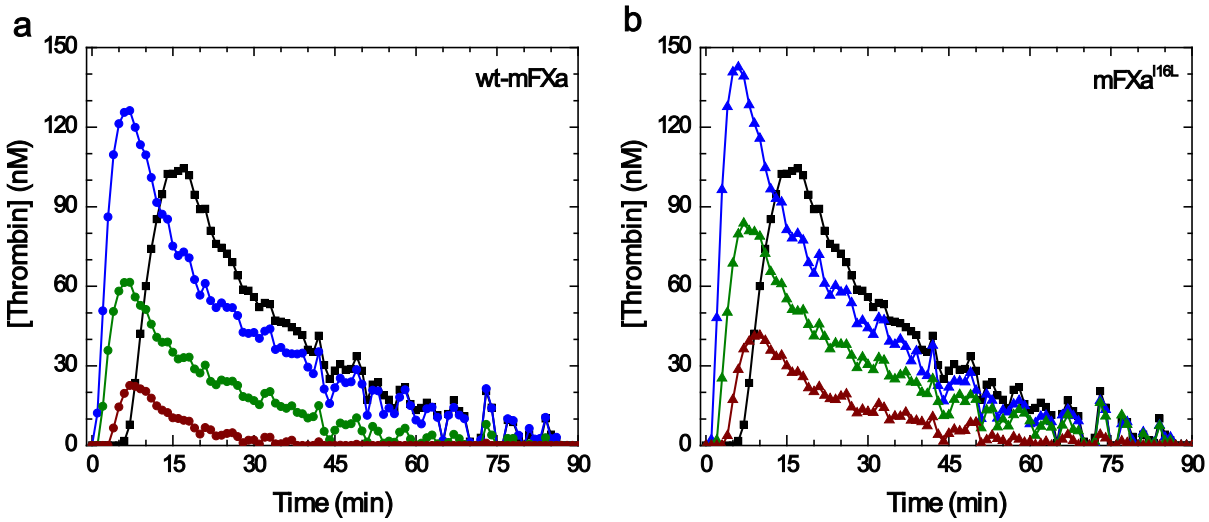
Supplemental Fig. 4. Thrombus formation upon $FeCl_3$-injury of the carotid artery. Representative recordings of Doppler blood flow (blood flow *versus* time) downstream of clots induced following application of $FeCl_3$ (15%) to the carotid artery in normal and HB animals (Balb/c). In each tracing, the injury was made 2 min prior to data collection. Control recordings for untreated wt and HB mice are indicated. For protein treated HB animals, blood flow was monitored for approximately10 min post-injury to ensure a stable response after which hFIX or $hFXa^{I16L}$ at the indicated dosage were infused denoted by an arrow. Blood flow was monitored for an additional 10-20 min. A summary of the collected data is found in **Table 1**.



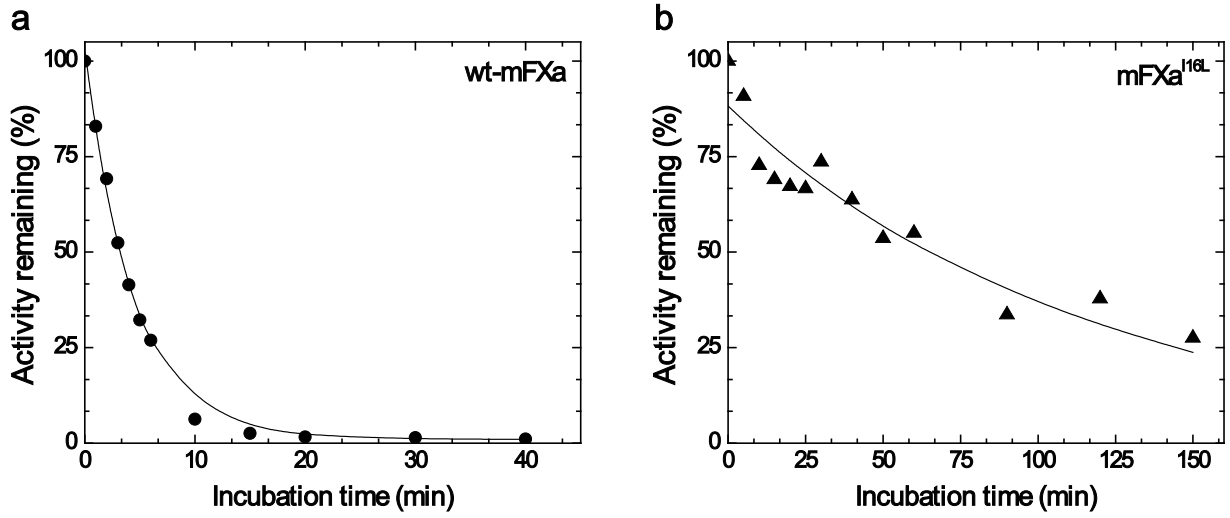
Supplemental Fig. 5. Blood loss following tail clip in antibody induced HA mice. Five minutes prior to injury, PBS (white column) or hFVIIa (red columns) was administered to HA mice (C57BL/6) via tail vein at the indicated dosage. Total blood loss (µl) was then measured following tail transection. Hemostatically normal mice (C57BL/6) infused with PBS (WT-PBS, black column) served as a control. The number of animals per group is indicated and all measurements are presented as mean ± SEM. For statistical comparisons, treated animals are compared to HA-PBS controls ** $p<0.001$; * $p<0.05$.



Supplemental Fig. 6. Characterization of $mFXa^{I16L}$: Peptidyl substrate cleavage. The initial rate of chromogenic substrate cleavage by wt-mFXa (-●-) or $mFXa^{I16L}$ (-▲-) was determined using increasing concentrations of Spectrozyme FXa. Note that $mFXa^{I16L}$ is plotted on the right hand axis due to its low activity compared to wt-mFXa. The lines are drawn following analysis of all data sets to a rectangular hyperbola with the following fitted parameters: wt-mFXa, K_m, 136 ± 11 μM , k_{cat}, 18 ± 0.6 min^{-1}; and $mFXa^{I16L}$, K_m, 860 ± 200 μM , k_{cat}, 0.89 ± 0.15 min^{-1}. The K_m and k_{cat} values for $mFXa^{I16L}$ should be considered estimates as the activity of the variant is too low to accurately determine a value. The data are representative of three similar experiments.



Supplemental Fig. 7. Characterization of $mFXa^{I16L}$: Thrombin Generation Assay. Thrombin generation was measured for 90 minutes at 33°C in dilute murine HB plasma supplemented with increasing concentrations (dark red 0.5; green, 1.5; and blue, 5.0 nM) of wt-mFXa (circles; **panel a**) or $mFXa^{I16L}$ (triangles; **panel b**) in the presence of 2.0 pM TF/4 μM phospholipid (reagent RB, Technoclone). Thrombin generation was initiated with $CaCl_2$ and a thrombin fluorogenic substrate as detailed in Methods. In both panels, the black squares are a representative run of pooled murine plasma; murine HB plasma gave no detectable signal under these conditions. Each curve is the average of four independent experiments.



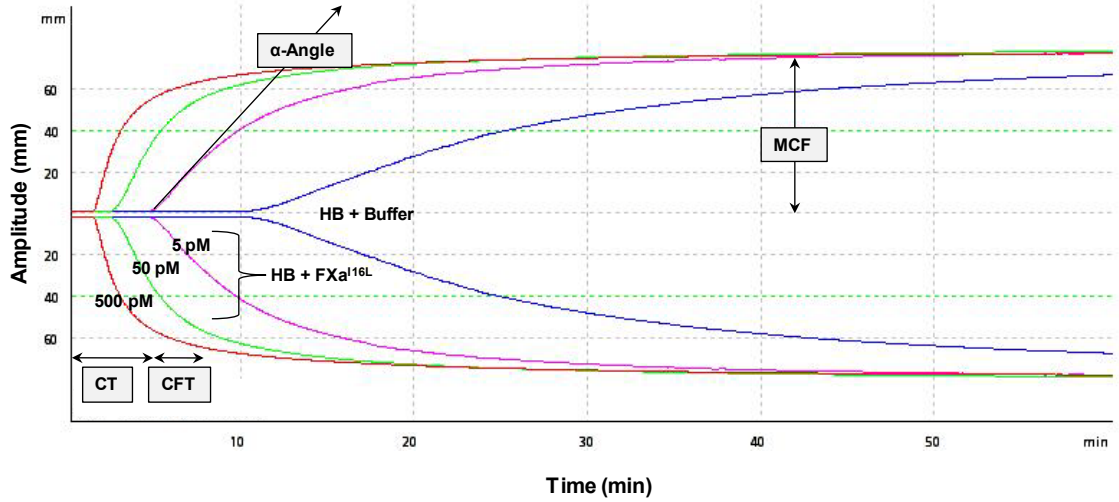
Supplemental Fig. 8. Characterization of mFXa^{I16L}: *Ex vivo* half-life studies in murine plasma. (***a***) wt-mFXa (-●-, 20 nM) or (***b***) mFXa^{I16L} (-▲-, 20 nM) were incubated in diluted HB (Balb/c) mouse plasma. At the indicated time points, residual activity was assessed by clotting assay. The solid lines were drawn following analysis of data sets to an exponential decay function with the following fitted parameters for half-life: wt-mFXa, 0.6 ± 0.2 min; mFXa^{I16L}, 17.2 ± 2.3 min. These values have been appropriately adjusted for sample dilution (1:5). The data are representative of two similar experiments.

Supplemental Table 1.
Effect of hFXa^{I16L} on murine whole blood thromboelastography (ROTEM).

	CT (min)	*CFT (min)*	*α-Angle (°)*	*MCF (mm)*
INTEM				
WT	1.7 ± 0.01	0.5 ± 0.02	84 ± 0.3	77 ± 0.6
HB	8.9 ± 1.5	3.1 ± 0.4	47 ± 9.2	71 ± 7.0
HB + 5 pM hFXa^{I16L}	4.4 ± 0.8	1.6 ± 0.2	72 ± 2.5	79 ± 4.5
HB + 50 pM hFXa^{I16L}	N.D.	N.D.	N.D.	N.D.
HB + 500 pM hFXa^{I16L}	1.2 ± 0.01	0.4 ± 0.06	85 ± 0.5	83 ± 4.0
ADP (25 µM)				
WT	4.5 ± 0.8	1.3 ± 0.3	76 ± 3.5	76 ± 7.5
HB	10.7 ± 0.2	5.1 ± 1.4	50 ± 15	73 ± 0.5
HB + 5 pM hFXa^{I16L}	4.6 ± 0.2	2.1 ± 0.1	66 ± 1.2	78 ± 1.3
HB + 50 pM hFXa^{I16L}	2.3 ± 0.1	1.1 ± 0.1	77 ± 1.7	79 ± 0.6
HB + 500 pM hFXa^{I16L}	1.3 ± 0.1	0.6 ± 0.05	82 ± 0.3	83 ± 2.5
Collagen (30 µg/mL)				
WT	3.3 ± 0.4	1.2 ± 0.4	78 ± 4.0	79 ± 5.0
HB	9.1 ± 0.2	3.1 ± 0.05	58 ± 3.2	75 ± 3.4
HB + 5 pM hFXa^{I16L}	3.6 ± 0.2	1.5 ± 0.4	72 ± 3.5	77 ± 3.9
HB + 50 pM hFXa^{I16L}	1.8 ± 0.06	0.7 ± 0.05	82 ± 0.6	77 ± 3.3
HB + 500 pM hFXa^{I16L}	1.1 ± 0.03	0.5 ± 0.04	84 ± 0.5	75 ± 1.0

WT, hemostatically normal mice; HB, hemophilia B mice; CT, coagulation time; CFT, clot formation time; MCF, maximum clot firmness; ADP, adenosine diphosphate; N.D., not determined.
Details of whole blood thromboelastography can be found in Methods.
Values are presented as mean ± SEM from three determinations.

Scheme below is a representative example of a reaction curve with whole blood from an HB mouse stimulated with ADP (25 µM) in the presence of buffer or hFXa^{I16L} at different concentrations. The various coagulation parameters (CT, CFT, α-angle, and MCF) are shown on the scheme and correlate with the 5 pM curve (pink):



Supplemental Movie 1. Representative movie depicting thrombus formation following laser injury in a wild-type mouse(Balb/c). Brightfield images of the cremaster muscle along with accumulating platelets and fibrin are shown. Platelets (red) were detected by an $Alexa_{555}$-labeled rat anti- CD41 $F(ab)_2$ and fibrin (green) with $Alexa_{488}$-labeled anti-fibrin antibody; areas of overlap are depicted by yellow. A 10 μm scale bar is shown in the lower left corner and a time stamp in the upper right corner. For convenience, the speed of the movie is increased by 5-fold. Further details of the methodology can be found in Methods.

Supplemental Movie 2. Representative movie depicting thrombus formation following laser injury in an HB mouse (Balb/c) treated with FXa^{I16L} (10 μg/kg) prior to laser injury. Platelets and fibrin accumulation were detected as described in Supplemental Movie 1 and in Methods.

Supplemental Movie 3. Representative movie depicting thrombus formation following laser injury in an HB mouse (Balb/c) treated with FXa^{I16L} (30 μg/kg). In this experiment, the animal was injured and monitored for approximately 3.5 min. After this observation period, FXa^{I16L} was infused via a jugular vein cannulus and thrombus formation was immediately monitored. The animal was not repositioned or reinjured. Platelets and fibrin accumulation were detected as described in Supplemental Movie 1 and in Methods.