

File Name: Supplementary Information

Description: Supplementary Figures, Supplementary Tables and Supplementary Reference

File Name: Peer Review File

24 **Supplementary Figures and Legends**

25

		99	143 146	174	189 - 192 195	213 215 217 219	227 228
1	<i>H. sapiens</i>	< H ₉₁ NRFT-----KETYDFD>	< GFGR T HE KGR>	< SSS F IIT>	< KQ E D A C Q GD S GGP>	< TGI V S W GE G C A R K G K Y G I Y TKV ₂₃₁ >	
2	<i>M. musculus</i>	< H ₉₁ NKFQ-----RDTYDYD>	< GFGR T HE KGR>	< STS F SIT>	< KLE D A C Q G D S GGP>	< TGI V S W GE G C A R K G K Y G I Y TKV ₂₃₁ >	
3	<i>B. taurus</i>	< H ₉₁ SRFV-----KETYDFD>	< GFGR T HE KGR>	< SSS F IT>	< QP E D A C Q GD S GGP>	< TGI V S W GE G C A R K G K F G V Y TKV ₂₃₁ >	
4	<i>S. scrofa</i>	< H ₉₁ SKFV-----RETYDFD>	< GFGR T HE RGR>	< SSS F LIT>	< QP E D A C Q GD S GGP>	< TGI V S W GE G C A R R G K Y G V Y TKV ₂₃₁ >	
5	<i>G. gallus</i>	< H ₉₁ SKYI-----AETYDND>	< GFGR E F E AGR>	< STN F AIT>	< EQ K D A C Q GD S GGP>	< TGI V S W GE G C A R K G K Y G V Y TKL ₂₃₁ >	
6	<i>M. gallopavo</i>	< H ₉₁ SKYI-----AETYDND>	< GFGR E F E AGR>	< STN F AIT>	< EQ K D A C Q GD S GGP>	< TGI V S W GE G C A R K G K Y G I Y TKL ₂₃₁ >	
7	<i>X. tropicalis</i>	< H ₉₁ PRFV-----KSTYDYD>	< GFGR I H E RGR>	< SST F AIT>	< EV K D A C Q GD S GGP>	< TGI V S W GE G C A R K G K F G V Y TKV ₂₃₁ >	
8	<i>A. carolinensis</i>	< H ₉₁ QKFV-----LATYDYD>	< GFGR L H E RGR>	< SSN F VIT>	< LA Q D A C Q GD S GGP>	< TGI V S W GE G C A R E D K Y G I Y TKV ₂₃₁ >	
9	<i>P. textilis</i>	< H ₉₁ QKFV-----ATYDYD>	< GFGR T R E RGK>	< SSN F PIT>	< LP Q D A C Q GD S GGP>	< TGI V S W GE G C A Q T G K Y G V Y TKV ₂₃₁ >	
10	<i>T. carinatus</i>	< H ₉₁ QKFV-----STYDYD>	< GFGR T R E RGQ>	< SSN F PIT>	< LP Q D A C Q GD S GGP>	< TGI I S W GE G C A Q T G K Y G A Y TKV ₂₃₁ >	
11	<i>P. textilis 1</i>	< H ₉₁ KKFVPPQKAY---KFDLAA Y DYD>	< GFGR I F E KGP>	< SSE T PIT>	< LPR D A C Q G D S GGP>	< TGI V S S GE G C A R N G K Y G I Y TKL ₂₃₁ >	
12	<i>P. textilis 2</i>	< H ₉₁ KKFVPPKKSQEFY E KFDL V S Y DYD>	< GF G G I F E RGP>	< SSN F PIT>	< LP Q D A C Q GD S GGP>	< TGI V S W GE G C A R K G R Y G I Y TKL ₂₃₁ >	
13	<i>T. carinatus 3</i>	< H ₉₁ TKFVPPNY Y VH Q N-FDRVA Y DYD>	< GFGR I Q F KQP>	< SSD F RIT>	< LP Q D A C Q GD S GGP>	< TGI I S W GE G C A R K G K Y G V Y TKV ₂₃₁ >	
14	<i>D. rerio</i>	< H ₉₁ KNYQP-----DTYHND>	< GFGR V R E GGL>	< SSN F KIS>	< EE K D A C Q GD S GGP>	< TGI V S W GE G C A R K G K Y G V Y TQV ₂₃₁ >	
15	<i>T. rubripes</i>	< H ₉₁ YNYKP-----NTYHND>	< GFGR L L G G NRQ>	< STS L RIS>	< IA K D A C Q GD S GGP>	< TGI V S W GE G C A Q K G K Y G V Y TQV ₂₃₁ >	

26

27

28 **Supplementary Fig. 1. Alignment of factor X serine protease domain**

29 **sequences.** Serine protease domain regions of FX were aligned to corresponding

30 FX regions from several selected vertebrates. The His91-Val231 sequence is shown

31 with the non-conserved 99-loop residues specific for *P. textilis* isoform FX¹, *P. textilis*

32 venom FX², and *T. carinatus* venom FX³ in bold ¹. Residues with the shortest

33 Minimal Interatomic distances from their side chain to apixaban as observed in the

34 MD simulation of apixaban-bound human FXa and as indicated in **Fig. 1A** are

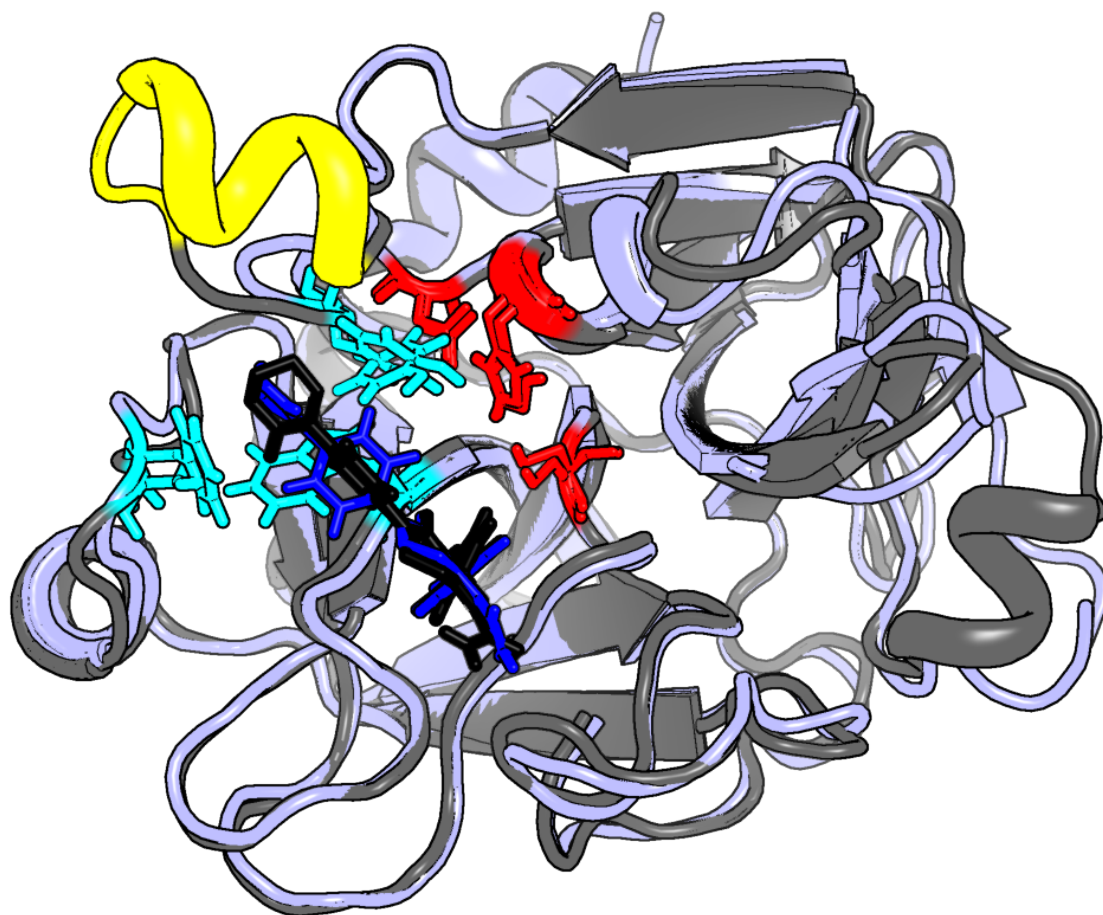
35 numbered and indicated in bold (Y99, R143, E146, F174, D189, A190, C191, Q192,

36 S195, V213, W215, E217, C219, I227, Y228).

37

38

39



40

41 **Supplementary Fig. 2. Apixaban binding orientation in human and snake factor**

42 **Xa.** Apixaban (blue sticks) bound to human FXa (light blue; PDB ID 2P16) was

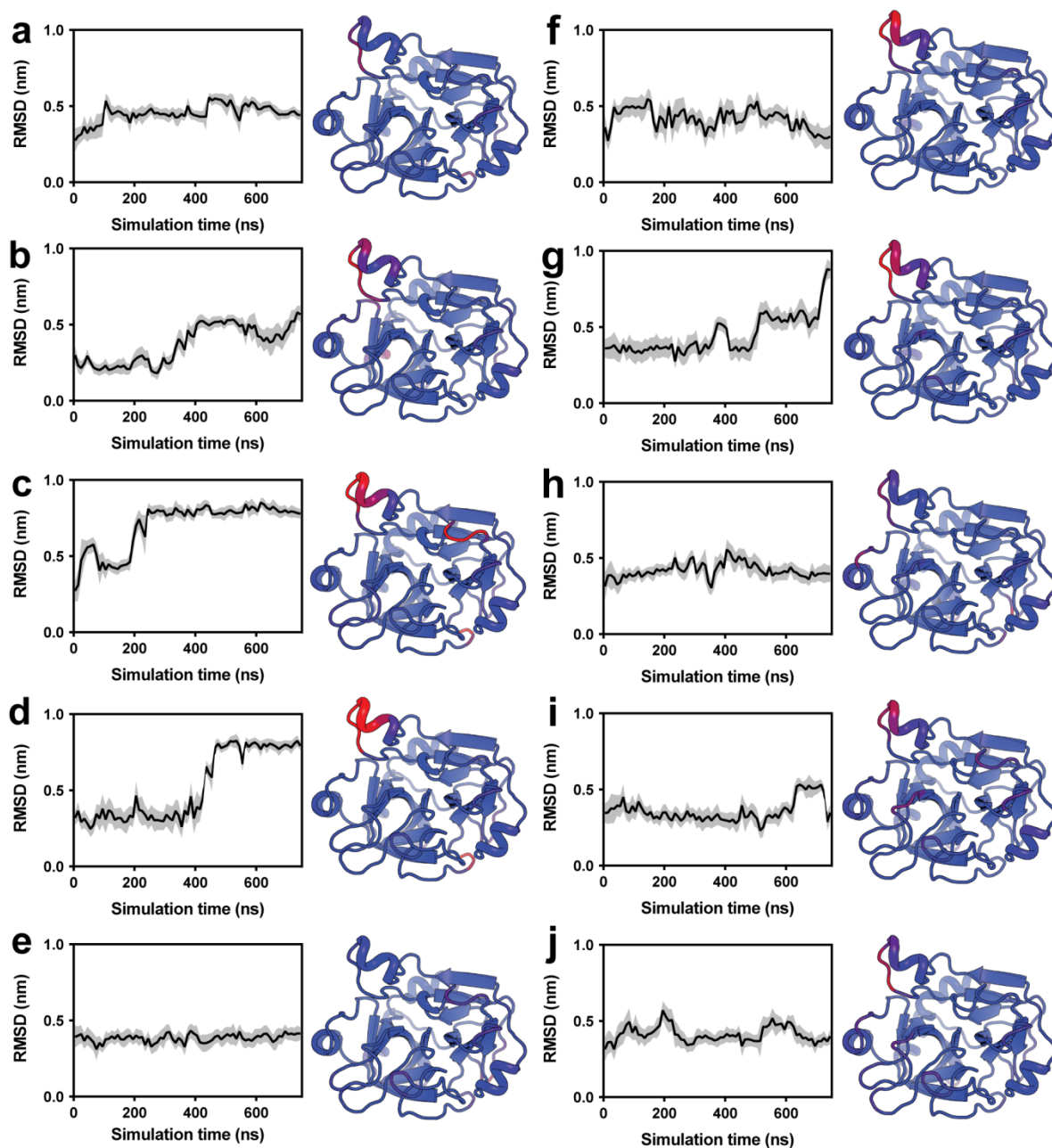
43 overlaid with apixaban (black sticks) docked into *P. textilis* isoform FXa (grey; PDB

44 ID 4BXW). The isoform FXa insertion region PQKAYKFDL is highlighted in yellow,

45 red residues indicate the position of the catalytic triad in both proteases, and cyan

46 residues indicate the position of the S4 subsite residues Tyr99, Phe174, and Trp215.

47



48

49 **Supplementary Fig. 3. Molecular Dynamics simulations of apixaban-bound and**

50 **unbound snake isoform factor Xa.** Root-mean-square deviations (RMSD) of the

51 backbone positions in the isoform FXa extended 99-loop region PQKAYKFDL during

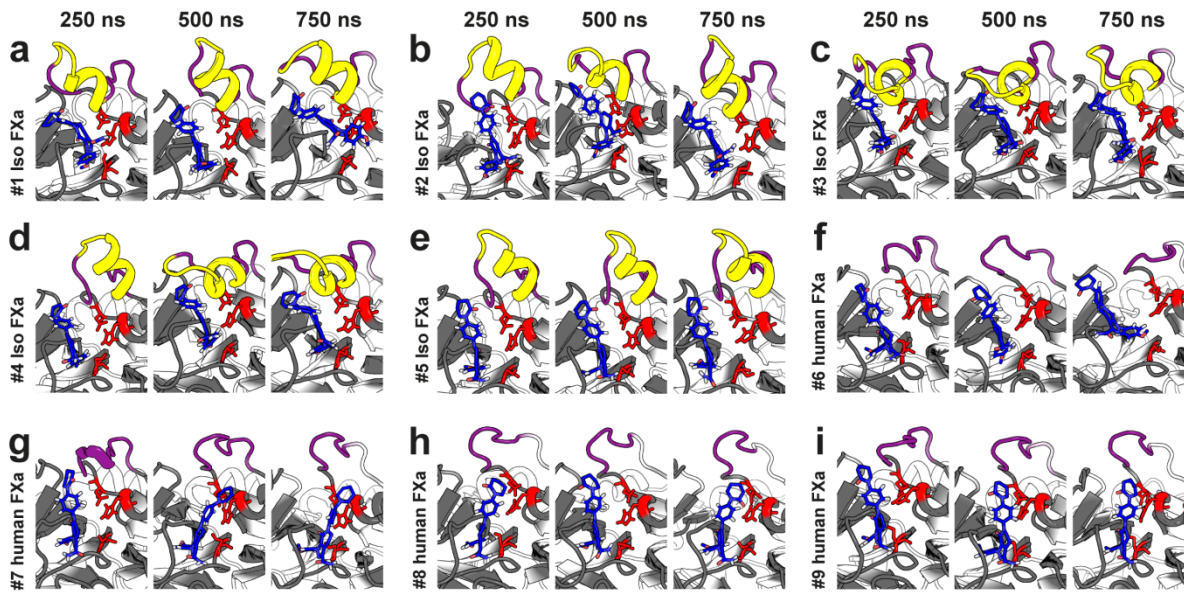
52 five independent 750 ns MD simulations of apixaban-bound (APX bound) (a-e) or

53 unbound (apo) (f-j) isoform FXa are presented as block averages over 10 ns

54 intervals. The corresponding single standard deviation interval is indicated (grey

55 density). The local flexibility of the *P. textilis* isoform FXa main-chain atoms was
56 evaluated and calculated using GROMACS tool *g_rmsf* from atomic root-mean-
57 square fluctuations. The resulting Debye-Waller factors (or B-factors) are projected
58 onto the initial conformation of *P. textilis* isoform FXa (PDB ID 4BXW) as a blue-red
59 gradient representing a $\leq 10 \text{ \AA}^2$ to $\geq 300 \text{ \AA}^2$ range of mobility. The B-factors are
60 displayed for independent MD simulations starting from the same atomic coordinates
61 but different randomly assigned velocities. The 750 ns MD simulations of apixaban-
62 bound isoform FXa (a-e) correspond to simulations #1-5 in **Fig. 3a-e** and
63 **Supplementary Fig. 4a-e**.

64



65

66 **Supplementary Fig. 4. Factor Xa conformations during Molecular Dynamics**

67 **simulations.** The conformations of the isoform FXa-apixaban complex (simulations

68 #1-5; *a-e*) and human FXa (simulations #6-9; *f-i*) at 250, 500, and 750 ns of MD

69 simulations are depicted for each independent simulation. Apixaban (blue), the 99-

70 loop (magenta), the isoform FXa extended 99-loop region PQKAYKFDL (yellow),

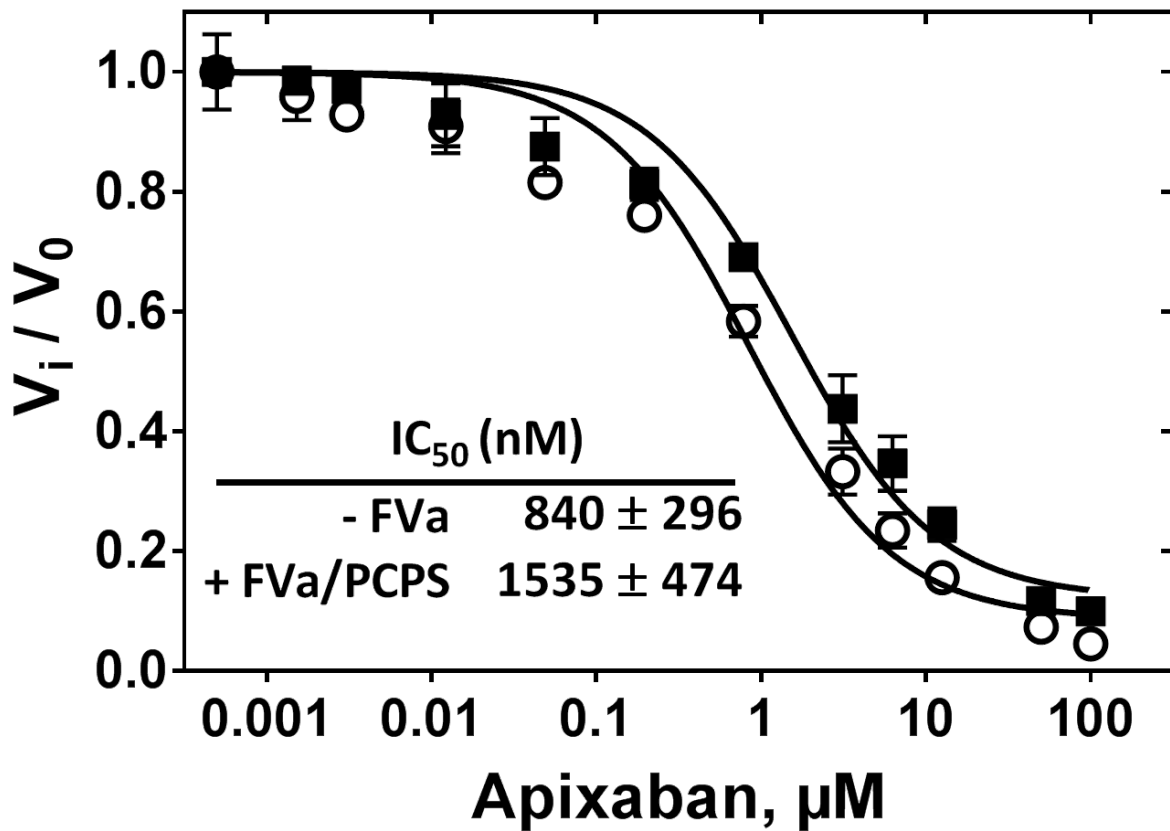
71 and the catalytic triad residues (red) are highlighted. The five independent

72 simulations of apixaban-bound isoform FXa (*a-e*) correspond to simulations #1-5 in

73 **Fig. 3a-e** and **Supplementary Fig. 3a-e**. The four independent simulations of

74 human FXa (*F-I*) correspond to simulations #6-9 in **Fig. 3f-i**.

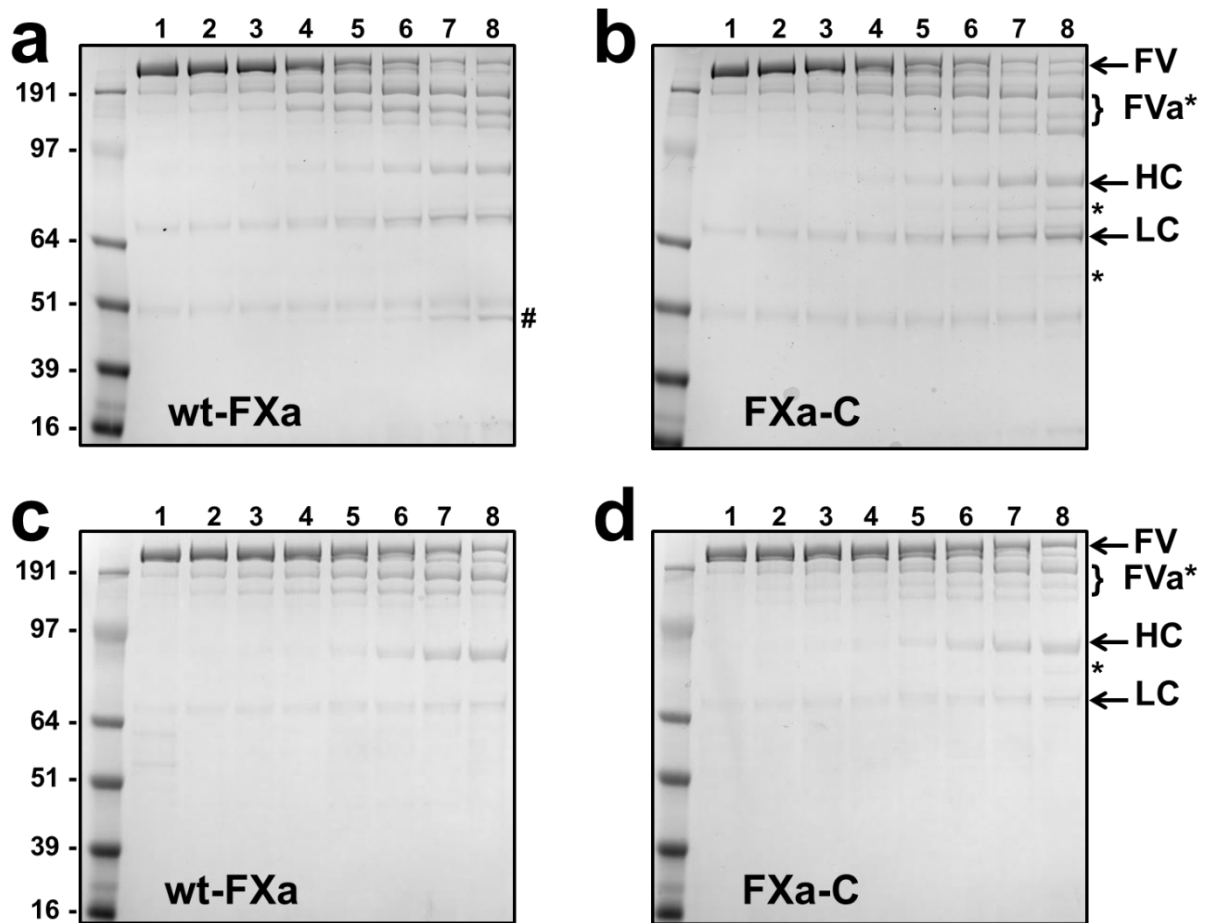
75



76

77 **Supplementary Fig. 5. Inhibition of factor Xa-C by apixaban in the absence and**
 78 **presence of the cofactor Va.** The rate of peptidyl substrate conversion (250 μM
 79 SpecXa) by free (open circles) or prothrombinase-assembled (PCPS, 50 μM ; FV-
 80 810 (FVa, 30 nM; closed squares) purified recombinant human FXa-C (5 nM) was
 81 determined in the absence (V_0) or presence (V_i) of increasing concentrations (0.002
 82 – 100 μM) of the inhibitor apixaban. The lines were drawn following nonlinear
 83 regression analysis of the data sets, and the fitted parameters for $IC_{50} \pm 1$ standard
 84 deviation of the induced fit are shown in the inset. The data are the means of three
 85 independent experiments, and are normalized for activity in the absence of inhibitor
 86 (V_0).

87

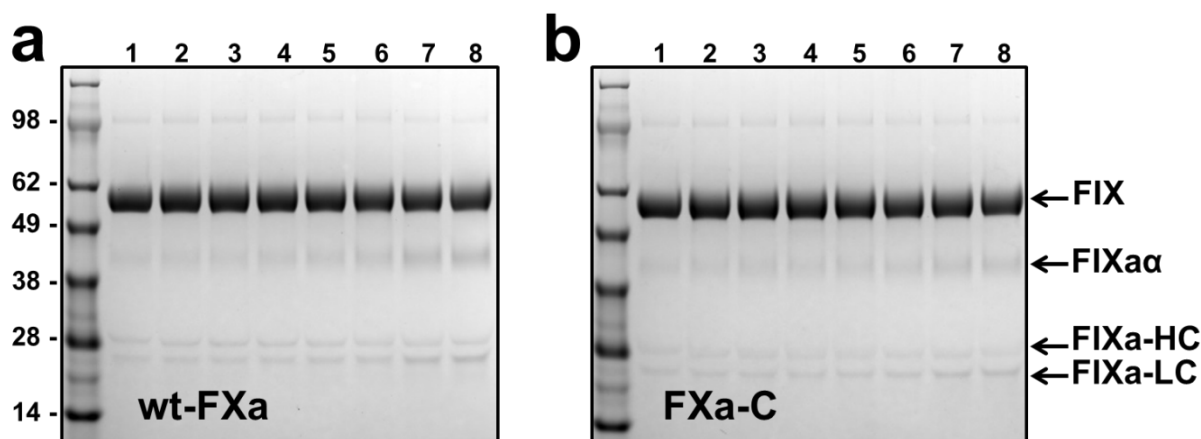


88

89 **Supplementary Fig. 6. Activation of factor V by factor Xa variants.** Plasma-
 90 derived (pd)-FV (400 nM) was incubated at 25°C either with increasing
 91 concentrations (0 – 100 nM) of wild-type FXa (wt-FXa; a,c) or FXa variant C (FXa-C;
 92 b,d) in the presence of 50 μM PCPS for 10 minutes (a,b) or with 5 nM wt-FXa or
 93 FXa-C in the presence of 25 μM PCPS for 1 – 60 minutes (c,d). Samples (3 μg per
 94 lane) were subjected to SDS-PAGE under reducing conditions using the MOPS
 95 buffer system and visualized by staining with Coomassie Brilliant Blue (CBB). (a,b)
 96 Lane 1: pd-FV, no FXa; lanes 2-8: pd-FV, wt-FXa or FXa-C at 0.5, 1, 5, 10, 20, 50,
 97 and 100 nM. (c,d) Lane 1: pd-FV, no FXa; lanes 2-8: pd-FV, incubated for 1, 2, 4, 10,
 98 20, 40, and 60 minutes with wt-FXa or FXa-C. The protein bands indicative of single
 99 chain uncleaved pd-FV (FV), partially activated FV (FVa*), FVa heavy chain (HC),

100 FVa light chain (LC), FV activation fragment specific to wt-FXa (#), and FV activation
101 fragments specific to FXa-C (*) are indicated. The apparent molecular weights of the
102 standards (kDa) are indicated. The data are representative of two independent
103 experiments.

104

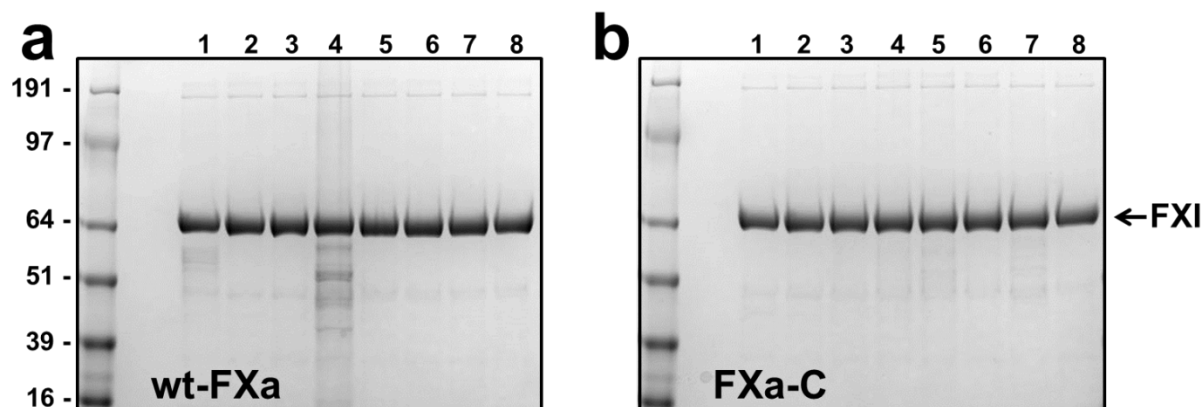


105

106 **Supplementary Fig. 7. Cleavage of factor IX by factor Xa variants.** Plasma-
 107 derived factor IX (2.7 μ M; pd-FIX) was incubated for 1 – 60 minutes with 5 nM
 108 recombinant human wt-FXa (a) or FXa-C (b) in the presence of 50 μ M PCPS at 25°C
 109 in assay buffer. Samples (3 μ g per lane) were subjected to SDS-PAGE under
 110 reducing conditions using the MES buffer system and visualized by staining with
 111 CBB. Lane 1: pd-FIX, no FXa; lanes 2-8: pd-FIX, incubated for 1, 2, 4, 10, 20, 40,
 112 and 60 minutes with wt-FXa or FXa-C. The protein bands corresponding to full-
 113 length pd-FIX, partially activated FIX (FIX α ; 45 kDa), the heavy chain of fully
 114 activated pd-FIX (FIXa-HC; 28 kDa), or the light chain of activated pd-FIX (FIXa-LC;
 115 17 kDa) are indicated. The apparent molecular weights of the standards (kDa) are
 116 indicated. The data suggest that both FXa variants are capable of partially activating
 117 FIX into FIX α (\geq 40 minutes), and wt-FXa may be slightly more efficient in doing so.
 118 FIX α is known to display catalytic activity toward synthetic substrates only and does
 119 not have clotting activity. The data represents one experiment.

120

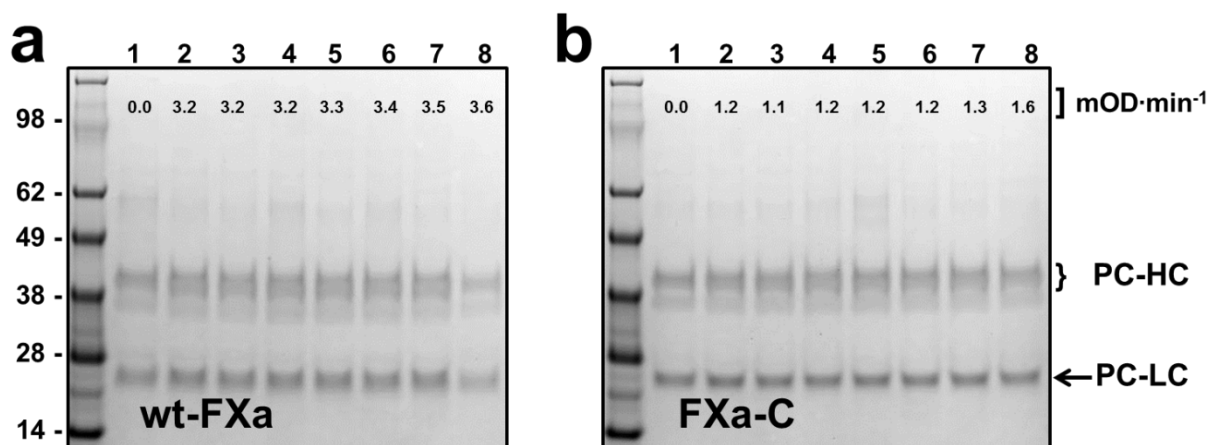
120



121

122 **Supplementary Fig. 8. Cleavage of factor XI by factor Xa variants.** Plasma-
123 derived factor XI (0.9 μ M; pd-FXI) was incubated for 1 – 60 minutes with 5 nM
124 recombinant human wt-FXa (a) or FXa-C (b) in the presence of 50 μ M PCPS at 25°C
125 in assay buffer. Samples (3 μ g per lane) were subjected to SDS-PAGE under
126 reducing conditions using the MOPS buffer system and visualized by staining with
127 CBB. Lane 1: pd-FXI, no FXa; lanes 2-8: pd-FXI, incubated for 1, 2, 4, 10, 20, 40,
128 and 60 minutes with wt-FXa or FXa-C. The protein bands corresponding to full-
129 length pd-FXI are indicated. The apparent molecular weights of the standards (kDa)
130 are indicated. Conversion of the 80 kDa FXI zymogen subunit to the 50 and 30 kDa
131 heavy and light chains of FXIa was not observed. The data represents one
132 experiment.

133



134

135 **Supplementary Fig. 9. Cleavage of protein C by factor Xa variants.** Plasma-

136 derived protein C (2.4 μM ; pd-PC) was incubated for 1 – 60 minutes with 5 nM

137 recombinant human wt-FXa (a) or FXa-C (b) in the presence of 50 μM PCPS at 25°C

138 in assay buffer. Samples (3 μg per lane) were subjected to SDS-PAGE under

139 reducing conditions using the MES buffer system and visualized by staining with

140 CBB. Lane 1: pd-PC, no FXa; lanes 2-8: pd-PC, incubated for 1, 2, 4, 10, 20, 40, and

141 60 minutes with wt-FXa or FXa-C. The protein bands corresponding to the heavy

142 chain of pd-PC (PC-HC; 41 kDa) and the light chain of pd-PC (PD-LC; 21 kDa) are

143 indicated. The apparent molecular weights of the standards (kDa) are indicated.

144 Conversion of the 41 kDa pd-PC heavy chain to the 35 kDa light chain of activated

145 protein C was not observed. These findings were corroborated by assessment of

146 peptidyl substrate S2366 (250 μM final) conversion (indicated as mOD min^{-1}) by time

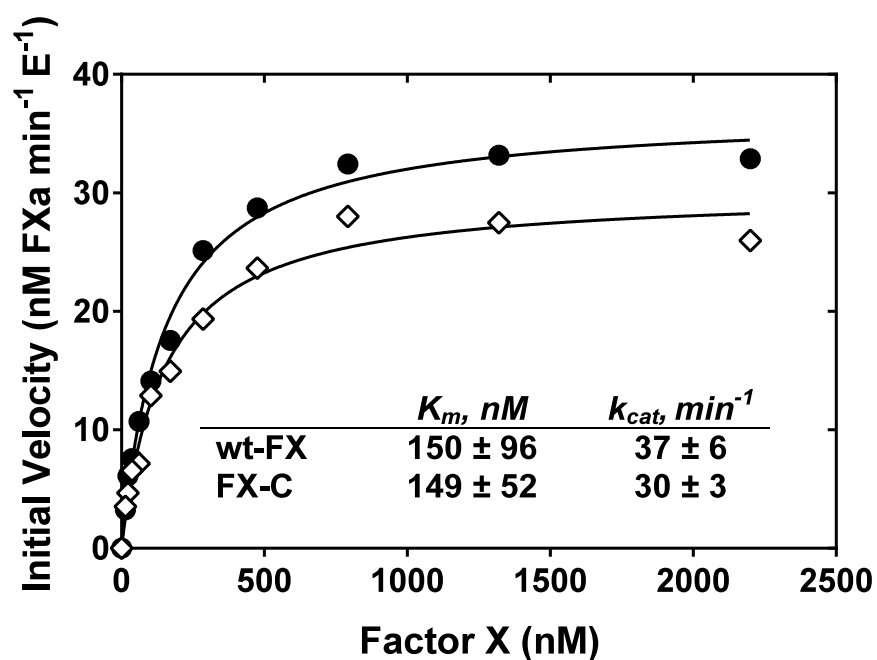
147 samples of pd-PC (66 nM final). No significant activation of pd-PC was observed

148 given that the chromogenic activity in samples 2 – 8 corresponded to the conversion

149 of S2366 by 5 nM of either wt-FXa or FXa-C. The data represents one experiment.

150

150



151

152 **Supplementary Fig. 10. Activation of factor X variants via the intrinsic**
153 **activation pathway.** The rate of FX (13 – 2200 nM) activation for wild-type (wt-FX;
154 closed circles) or variant C (FX-C; open diamonds) by the intrinsic FVIIIa/FIXa
155 tenase complex (PCPS, 20 μ M; FIXa, 0.5 nM; FVIIIa, 5 nM) was determined as
156 described in ‘Materials’. Individual data points represent the average of two
157 independent experiments. The lines were drawn by fitting the data to the Michaelis-
158 Menten equation using non-linear regression, and the obtained values for K_m and k_{cat}
159 ± 1 standard deviation of the induced fit are shown in the inset.

160

160 **Supplementary Table 1. Factor Xa-initiated thrombin generation in factor X-**
 161 **depleted plasma in the absence or presence of apixaban.**

	pd-FXa	wt-FXa	FXa-A	FXa-B	FXa-C
<i>No inhibitor</i>					
Lag time (min)	0.14 ± 0.05	0.33 ± 0.06	2.28 ± 0.77	1.85 ± 0.44	0.42 ± 0.08
Time to peak (min)	0.74 ± 0.05	1.03 ± 0.10	3.75 ± 1.15	3.57 ± 0.99	1.30 ± 0.09
Peak height (nM)	938 ± 121	836 ± 11	625 ± 144	512 ± 170	827 ± 29
Velocity index (nM min⁻¹)	1565 ± 201	1203 ± 97	473 ± 228	323 ± 156	937 ± 25
ETP (nM min)	2573 ± 264	2667 ± 175	2313 ± 396	1979 ± 717	2792 ± 86
<i>2 μM apixaban</i>					
Lag time (min)	4.43 ± 1.71	5.22 ± 1.43	3.29 ± 2.08	2.02 ± 0.94	0.47 ± 0.12
Time to peak (min)	8.14 ± 2.63	8.82 ± 1.81	5.75 ± 3.41	4.07 ± 1.78	1.42 ± 0.20
Peak height (nM)	278 ± 128	272 ± 54	490 ± 214	435 ± 86	818 ± 40
Velocity index (nM min⁻¹)	87 ± 62	77 ± 22	289 ± 228	243 ± 113	868 ± 102
ETP (nM min)	1845 ± 296	2031 ± 187	2059 ± 375	1824 ± 442	2823 ± 95

162

163 Thrombin generation was measured for 60 minutes at 37°C in FX-depleted plasma
 164 supplemented with 20 μM PCPS. Thrombin generation was initiated by the addition
 165 of 5 nM plasma-derived human FXa (pd-FXa), recombinant wild-type human FXa
 166 (wt-FXa), or chimeric FXa-A, FXa-B, or FXa-C premixed with HBS (no inhibitor) or
 167 apixaban (2 μM) and supplemented with CaCl₂ and a thrombin fluorogenic substrate
 168 as detailed in 'Materials'. All values represent averages ± 1 standard deviation
 169 obtained from at least three independent experiments.

170

170 **Supplementary Table 2. Tissue factor-initiated thrombin generation in factor X-**
 171 **depleted plasma spiked with factor X variants.**

	no inhibitor	2.0 μ M APX	6.0 μ M APX	0.6 μ M EDX	2.0 μ M EDX
<i>wt-FX</i>					
Lag time (min)	2.23 \pm 0.27	10.59 \pm 1.51	17.77 \pm 4.05	11.95 \pm 1.27	22.86 \pm 5.54
Time to peak (min)	5.12 \pm 0.65	49.57 \pm 1.81	51.82 \pm 0.52	37.14 \pm 8.60	45.70 \pm 9.59
Peak height (nM)	86 \pm 25	8 \pm 2	4 \pm 1	13 \pm 3	7 \pm 2
Velocity index (nM min⁻¹)	31 \pm 13	<1	<1	<1	<1
ETP (nM min)	646 \pm 88	NA	NA	NA	NA
<i>FX-C</i>					
Lag time (min)	2.98 \pm 0.30	3.00 \pm 0.22	3.25 \pm 0.22	3.43 \pm 0.30	4.16 \pm 0.54
Time to peak (min)	7.32 \pm 0.73	7.67 \pm 0.50	8.17 \pm 0.50	8.53 \pm 0.86	10.34 \pm 1.25
Peak height (nM)	93 \pm 18	83 \pm 12	86 \pm 14	85 \pm 22	62 \pm 18
Velocity index (nM min⁻¹)	22 \pm 6	18 \pm 4	18 \pm 4	17 \pm 6	10 \pm 4
ETP (nM min)	806 \pm 99	776 \pm 30	789 \pm 43	846 \pm 131	722 \pm 70

172
 173 Thrombin generation (TG) was measured for 60 minutes at 37°C in FX-depleted
 174 plasma supplemented with 2 pM tissue factor (TF), 20 μ M PCPS, and either 1 U ml⁻¹
 175 of wt-FX (7 μ g ml⁻¹) or FX-C (15 μ g ml⁻¹). Thrombin generation was initiated with
 176 CaCl₂ and a thrombin fluorogenic substrate as detailed in 'Materials'. Experimental
 177 data was obtained in the absence or presence of either 2 μ M or 6 μ M apixaban
 178 (APX), or 0.6 μ M or 2 μ M edoxaban (EDX). All values represent averages \pm 1
 179 standard deviation obtained from at least three independent experiments. NA, not
 180 applicable: for these experiments thrombin generation was insufficient, precluding an
 181 accurate assessment of the ETP.

182

182 **Supplementary Table 3. Tissue factor-initiated thrombin generation parameters**
 183 **in normal pooled plasma spiked with factor X-C and apixaban**

	NPP	APX + 5 µg ml⁻¹	APX + 10 µg ml⁻¹	APX + 20 µg ml⁻¹	APX + 40 µg ml⁻¹	+ 40 µg ml⁻¹
2 pM TF						
Lag time (min)	3.27 ± 0.16	4.45 ± 0.08	3.98 ± 0.06	3.69 ± 0.19	3.89 ± 0.20	3.25 ± 0.25
Time to peak (min)	6.65 ± 0.28	12.88 ± 0.38	11.44 ± 0.31	10.35 ± 0.63	10.35 ± 0.51	8.58 ± 0.63
Peak height (nM)	96 ± 22	50 ± 20	68 ± 17	77 ± 11	84 ± 11	87 ± 2
Velocity index (nM min⁻¹)	29 ± 6	6 ± 2	9 ± 2	12 ± 2	13 ± 3	16 ± 1
ETP (nM min)	731 ± 127	719 ± 246	798 ± 191	740 ± 96	722 ± 45	696 ± 27
6 pM TF						
Lag time (min)	1.94 ± 0.15	3.04 ± 0.06	2.56 ± 0.10	2.35 ± 0.12	2.27 ± 0.04	2.08 ± 0.14
Time to peak (min)	4.10 ± 0.23	7.54 ± 0.56	6.10 ± 0.42	5.39 ± 0.30	5.35 ± 0.29	4.67 ± 0.14
Peak height (nM)	171 ± 23	120 ± 36	143 ± 26	168 ± 22	176 ± 16	190 ± 12
Velocity index (nM min⁻¹)	79 ± 9	27 ± 8	40 ± 8	55 ± 6	57 ± 6	74 ± 5
ETP (nM min)	792 ± 100	864 ± 268	839 ± 204	849 ± 118	883 ± 86	868 ± 25

184

185 Thrombin generation (TG) was measured for 60 minutes at 37°C in normal pooled
 186 plasma supplemented with 2 or 6 pM tissue factor (TF), 20 µM PCPS, and 5 – 40 µg
 187 ml⁻¹ FX-C in the presence of 2 µM apixaban (APX). Thrombin generation was
 188 initiated with CaCl₂ and a thrombin fluorogenic substrate as detailed in ‘Materials’. All
 189 values represent averages ± 1 standard deviation obtained from at least three
 190 independent experiments.

191

191 **Supplementary References**

- 192 1 Bos, M. H. & Camire, R. M. Procoagulant adaptation of a blood coagulation
193 prothrombinase-like enzyme complex in australian elapid venom. *Toxins* **2**, 1554-
194 1567, doi:10.3390/toxins2061554 (2010).

195