Supplemental data

Methods.

C3bB and CVFB assembly in the presence and absence of lufaxin, analysis by SPR

Surface plasmon resonance measurements were performed using a Biacore T200 instrument. C3b and CVF were immobilized in pH 5.0 sodium acetate on CM5 chip surfaces at levels of 300-3000 RU using amine coupling methodology. FB and lufaxin, either individually or together, were injected over the surfaces in 10 mM HEPES, pH 7.4, 150 mM NaCl (HBS) buffer containing either 5 mM $MgCl_2$ or no divalent cation. For gel filtration and SDS-PAGE the C3bB-lufaxin-fXa complex was formed by mixing 0.56 nmol human C3b, 0.6 nmol FB, 1 nmol lufaxin and 1 nmol fXa in HBS containing 5 mM $MgCl_2$.

Assays of complement activation and thrombin generation

The mHam assay, conventional Ham assay, thrombinoscopy and thromboelastography were conducted as previously described[1-4]. All patients gave written informed consent. This study was approved by the institutional review board and conducted in accordance with the Declaration of Helsinki.

mHAM assay

TF1*PIGA*null cells were maintained at a density of 500,000 cells per milliliter daily. For the assay, the cells were seeded in a round-bottom 96-well plate at a density of 6700 cells per well in gelatin veronal buffer with Ca and Mg (GVB^{++}, Complement Technology, Inc). Serum either with or without inhibitors was diluted in gelatin veronal buffer and added to the wells. Cells were washed as described previously[5] and incubated with the cell proliferation reagent 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-

tetrazolio]-1.3-benzene disulfonate/WST-1 as previously described[5]. Absorbance was measured at 490 nm with a reference wavelength of 595 nm.

Ham assay

Acidified NHS was added to erythrocytes (5 x 10^7 cells/well) from a healthy or PNH individual as described previously[6] and 0.5mM EDTA was added to acidified NHS as a negative control. Samples were treated as described previously and the absorbance at 405 nm was measured in supernatants in a 96-well flat bottom plate using a plate reader[6]. The sample absorbance value was normalized by subtracting the absorbance of a blank sample containing erythrocytes. Hemolysis in each sample was compared to erythrocyte lysis in water (100 % value). Percent hemolysis was calculated as previously described.[6]

Crystallization and structure solution of lufaxin

Lufaxin was crystallized using the hanging drop vapor diffusion method from 100 mM HEPES, pH 7.0, 10 % PEG 6000. Crystals were soaked in the crystallization solution containing 15 % glycerol and flash cooled in liquid N_2 for X-ray diffraction. Crystals were prepared with 0.5 M potassium bromide added to the cryoprotectant solution of 100 mM HEPES, pH 7.0, 20 % PEG 6000. Diffraction data were collected at beamline 22-ID of the Southeast Regional Collaborative Access Team, APS, Argonne National Laboratory. Diffraction data were processed using XDS[7] and the structure of lufaxin was determined using single anomalous diffraction methods with the bromide-soaked crystals. Phasing using Phenix Autosol[8] initially produced a map that was not easily interpretable. Phase extension in Phenix Autobuild[8] using a native data set and incorporating non-crystallographic symmetry for two molecules in the asymmetric unit greatly improved the map and allowed for model building using Phenix Autobuild. The initial structure was then refined against the native data set and completed using iterative cycles of manual rebuilding using Coot [9] and refinement using Phenix Refine [8].

CryoEM sample preparation and data collection

The C3bB-lufaxin and C3bB-lufaxin-fXA complexes were prepared by mixing 0.57 nmol C3b, 0.57 nmol FB and 1.1 nmol of lufaxin in 1.0 mL HBS buffer containing 5 mM $MgCl_2$. For the C3bB-lufaxin-fXa complex 1.1 nmol of fXa was added to the above mixture. The sample was then concentrated to give a total protein concentration of 1.2-1.5 mg/mL using Amicon-Ultra centrifuge concentrators.

To prepare grids of the C3bB-lufaxin and C3bB-lufaxin-fXa complexes, 3 µL of protein sample were applied to freshly glow-discharged (easiGLow) C-flat grids (Protochips, CF1.2/1.3-3Au). Blotting was done at 6 °C and 100 % humidity using a Vitrobot Mark IV (Thermo-Fisher), with a 2.5 s blotting time and 4 pN blotting force for C3bB-lufaxin and a 4 s blotting time and 5 pN blotting force for C3bB-lufaxin-FXa. Grids were vitrified by plunging into liquid ethane and stored in liquid nitrogen before examination by cryo-EM. Images were recorded on a Glacios TEM (Thermo Fisher) at 200 kV and recorded at 36,000X magnification for C3bB-lufaxin and 45,000X magnification for C3bB-lufaxin-fXa, with a defocus range of -0.3 to -2.2 µm on K3 direct electron detector (Gatan) in super-resolution mode.

Cryo-EM Image processing

Movies of the C3bB-lufaxin and C3bB-lufaxin-fXa complexes were processed with MotionCor2[10], during which dose weighting was applied and the pixel size was binned to 1.12 Å/pixel for C3bB-lufaxin and0.92 Å/pixel for C3bB-lufaxin-FXa. The CTF (contrast transfer function) was estimated in Ctffind4[11]. Particle picking was conducted in Gautomatch (http://www.mrc-lmb.cam.ac.uk/kzhang/Gautomatch/) using references generated with EMAN2[12] resulting in 1,547,893 particles for C3bB-lufaxin and 713,201 for C3bB-lufaxin-fXa. These were extracted in RELION-3.0.8 or 3.2[13] with a box size of 220 x 220 pixels for C3bB-lufaxin and 300 x 300 pixels for C3bB-lufaxin-fXa. The picked-particles were subjected to 2D classification in RELION-3.0.8 to remove bad particles (Fig. S1). A previously calculated map was used as an initial reference for 3D classification. The best class was selected for subsequent gold-standard

refinement (FSC=0.143) in both RELION-3.0.8 and cisTEM [14] for C3bB-lufaxin and RELION 3.2 for C3bB-lufaxin-fXa (Fig. S1).

Model building and refinement of C3bB and C3bB-lufaxin complexes

Models of C3bB in the closed and open conformations were built from components of the C3bB complex crystallized in the presence of nickel ion (PDB accession code 2XWJ, [15]), the C3bB-FD complex crystallized in the presence of magnesium ion (PDB accession code 2XWB, [15]), the crystal structure of free, wild-type FB (PDB accession code 2OK5, [16]) and the structure of lufaxin described above. Positioning was assisted by the Phenix dock-in-map application [8]. For the C3bB-lufaxin-fXa complex, the structure of fXa was added to the model using coordinates from PDB depositions 1HCG[17] and 1FAX[18]. Models were refined using real-space methods in Phenix combined with manual rebuilding using Coot [9]. Model quality was evaluated using MolProbity [19].

Alphafold2 structure predictions.

For the prediction of C3bB-lufaxin and lufaxin-fXa complex structures, Alphafold 2.3.1 was run in multimer mode on the NIH Biowulf HPC cluster using sequences for the mature peptides of each component.

Analysis of CVFB and CVFB-lufaxin complexes.

CVFB complexes were formed by mixing 0.6 μM CVF and 1.1 μM FB in 10 mM Hepes pH 7.4, 0.15 M NaCl (HBS) containing 5 mM $MgCl_2$, $NiCl_2$ or no divalent cation. In experiments testing the role of lufaxin binding, the inhibitor was also added to a concentration of 1.2 μM. After 20 min incubation the samples were injected onto a Superdex 200 increase (10/30) column and eluted with HBS containing the appropriate divalent cation. Fractions (0.5 mL) were collected, separated by SDS-PAGE gels (NuPage) in MES running buffer and stained using silver (Thermo Scientific).

Lufaxin cleavage by FXa

Recombinant lufaxin containing a 6-His tag (1.9 μM) was incubated with fXa (3.8 μM) in the presence or absence of 5mM $CaCl_2$, in Tris-buffered saline pH 7.4, at 30°C for 3 hours. Immediately after incubation sample was mixed with NuPage LDS sample buffer and NuPAGE sample reducing agent, heated for 10 minutes at 94°C, and loaded in 4-12% BisTris gels. Two identical gels were run in parallel at 160 V using MES Buffer. After running, one of the gels was stained with Coomassie R-250, while the proteins from the other were transferred to a nitrocellulose membrane using an iBlot device.

The nitrocellulose membranes were blocked with 1% blotting grade milk in Tris Buffer Saline pH 8.0 with 0.05% Tween 20 (TBS-T) overnight at 4°C. Membrane was then incubated with 6x-His Tag monoclonal antibody (Invitrogen # MA-1-21315) diluted 1:3,000 in blocking buffer, for 1 hour at room temperature. After three washes with TBS-T, membrane was incubated with secondary antibody conjugated to alkaline phosphatase (anti-mouse IgG AP conjugated, SIGMA #A3562) diluted 1:10,000 in blocking buffer. Finally, after three washes with TBS-T membrane was incubated with Western Blue® stabilized substrate for alkaline phosphatase (Promega #S3841).

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Supplemental Figures

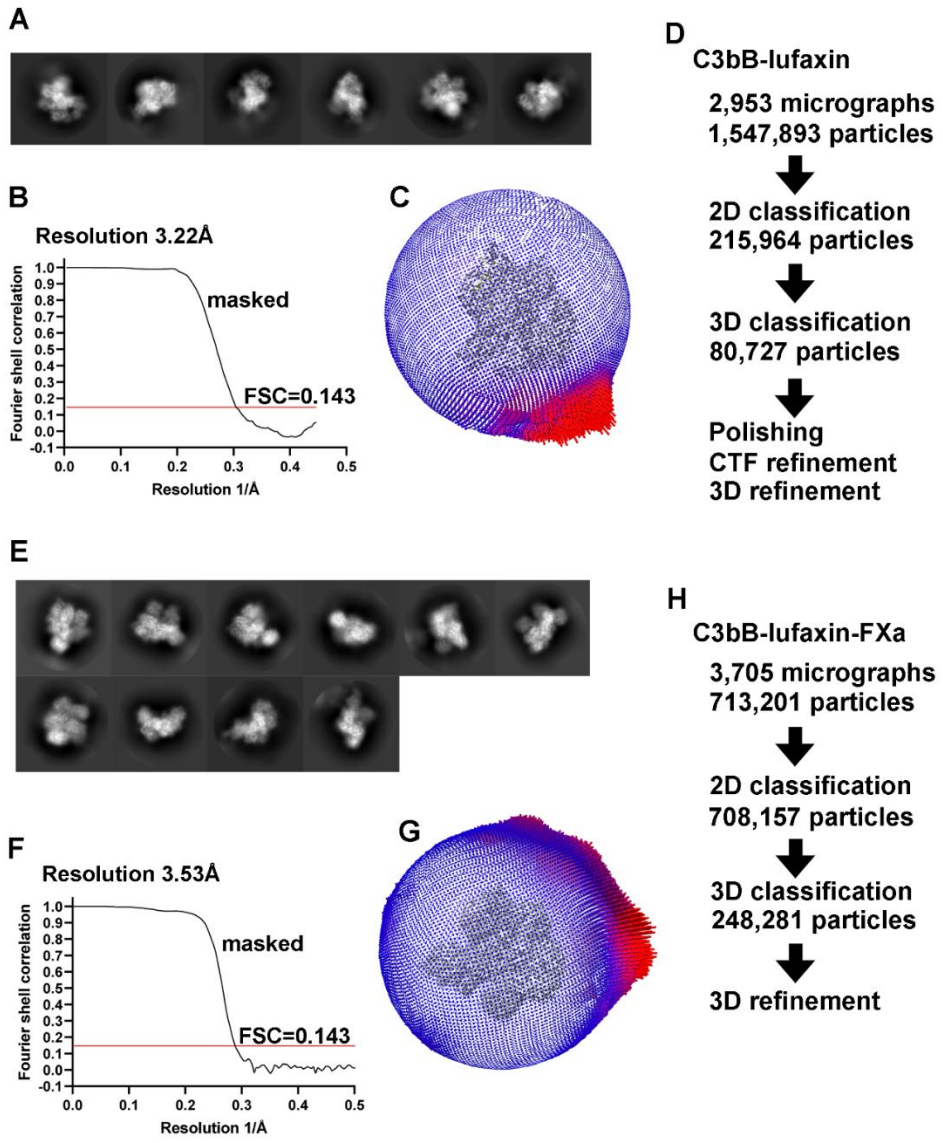

Figure S1. Cryo-EM of C3bB-lufaxin and C3bB-lufaxin-fXa in the presence of Mg^{2+} ion. **A** - 2D class averages from processing of C3bB-lufaxin images. **B** – FSC plot for C3bB-lufaxin after masking. **C** – Orientation plot for C3bB-lufaxin particle distribution. **D** – Workflow for processing of the C3bB-lufaxin data. **E** - 2D class averages from processing of C3bB-lufaxin-fXa images. **F** - FSC plot for C3bB-lufaxin-fXa after masking. **G** – Orientation plot for C3bB-lufaxin-fXa particle distribution. **H** – Workflow for processing of C3bB-lufaxin-fXa data.

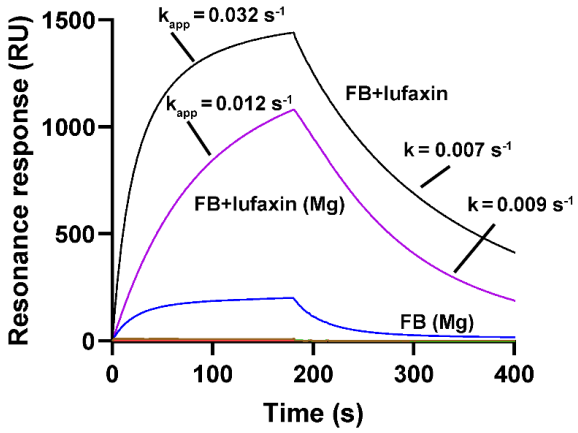

Figure S2. Assembly of C3bB complex components and lufaxin on a C3bB SPR surface (3000 RU). SPR analysis of alternative C3 convertase assembly in the presence and absence of lufaxin on an immobilized C3b surface. Black- 57 nM FB, 500 nM lufaxin injected in the absence of divalent cation, purple- FB-lufaxin mixture injected in the presence of 5 mM Mg^{2+}, blue-FB with 5 mM Mg^{2+}, green- lufaxin with Mg^{2+}, red – FB without divalent cation, brown – lufaxin without divalent cation. The green, red and brown lines run near zero on the Y-axis making them difficult to distinguish from one another. Apparent first order rate constants (k_{app}) for the association phase are shown on the left side of the corresponding curve, and first order rate constants (k) for the dissociation phase are shown on the right side of the corresponding curve.

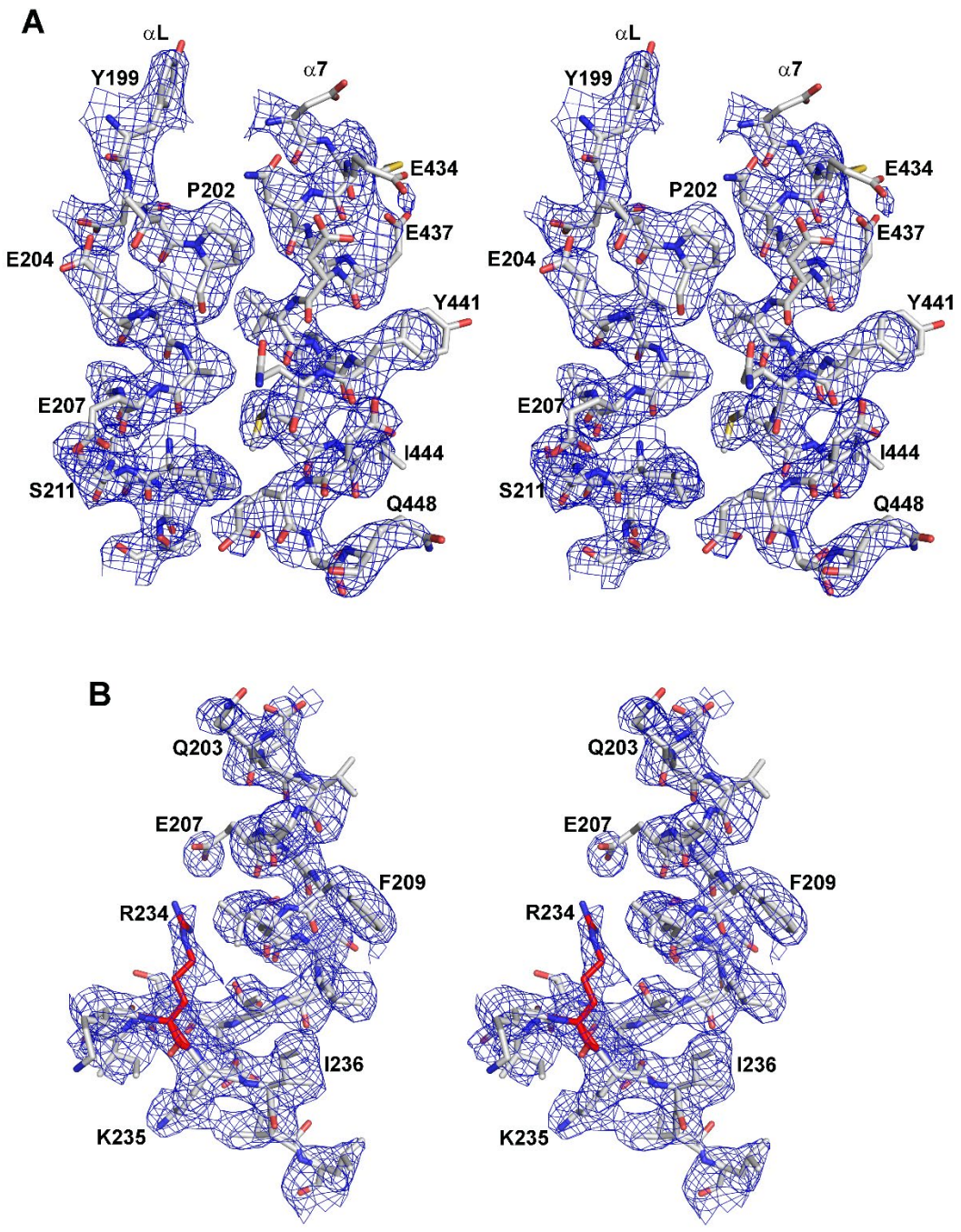

Figure S3. Structure of αL/α7 and the scissile bond region in the C3bB-lufaxin structure. **A** - Stereoview of map density covering helices αL and α7 of the vWA domain of FB contoured at 4.0 r.m.s.d. **B** - Stereoview of map density covering the scissile bond loop region of FB contoured at 4.0 r.m.s.d. Arg 234, the residue at the "P1" cleavage position of the SP domain is colored in red.

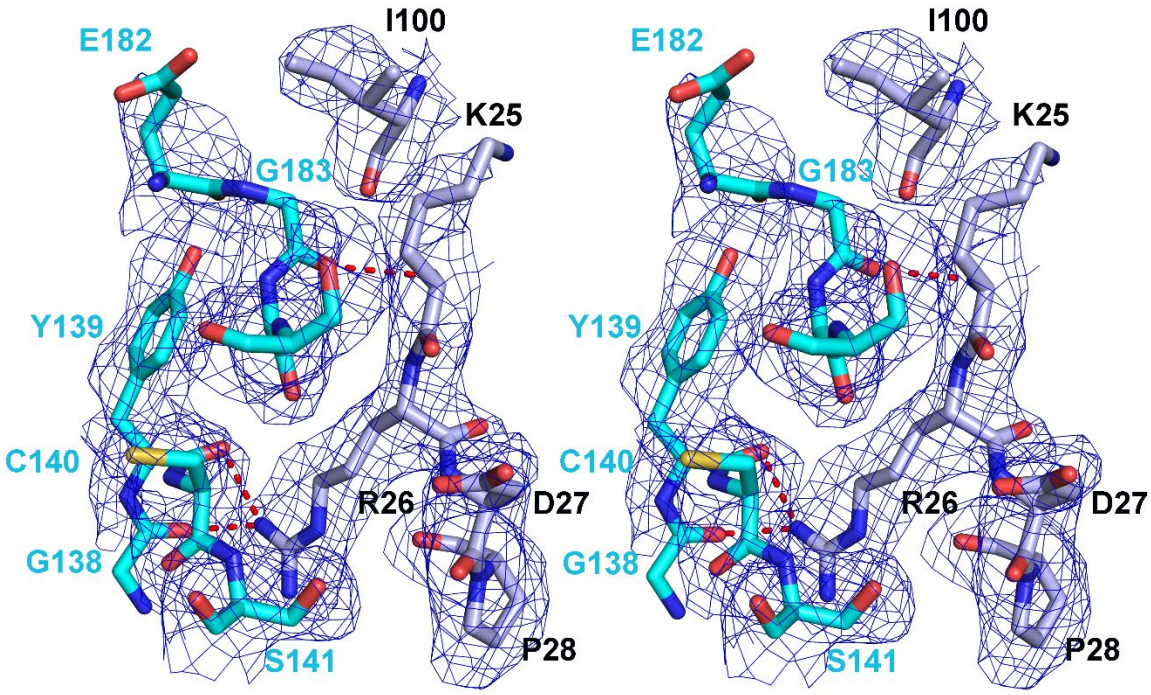

Figure S4. Stereoview of map density covering the interaction interface between lufaxin and the CCP3 domain of FB contoured at 2.5 r.m.s.d. Lufaxin residues are colored light blue and labeled in black, while FB residues are colored in cyan and labeled in cyan. Hydrogen bonds are shown as red dashed lines.

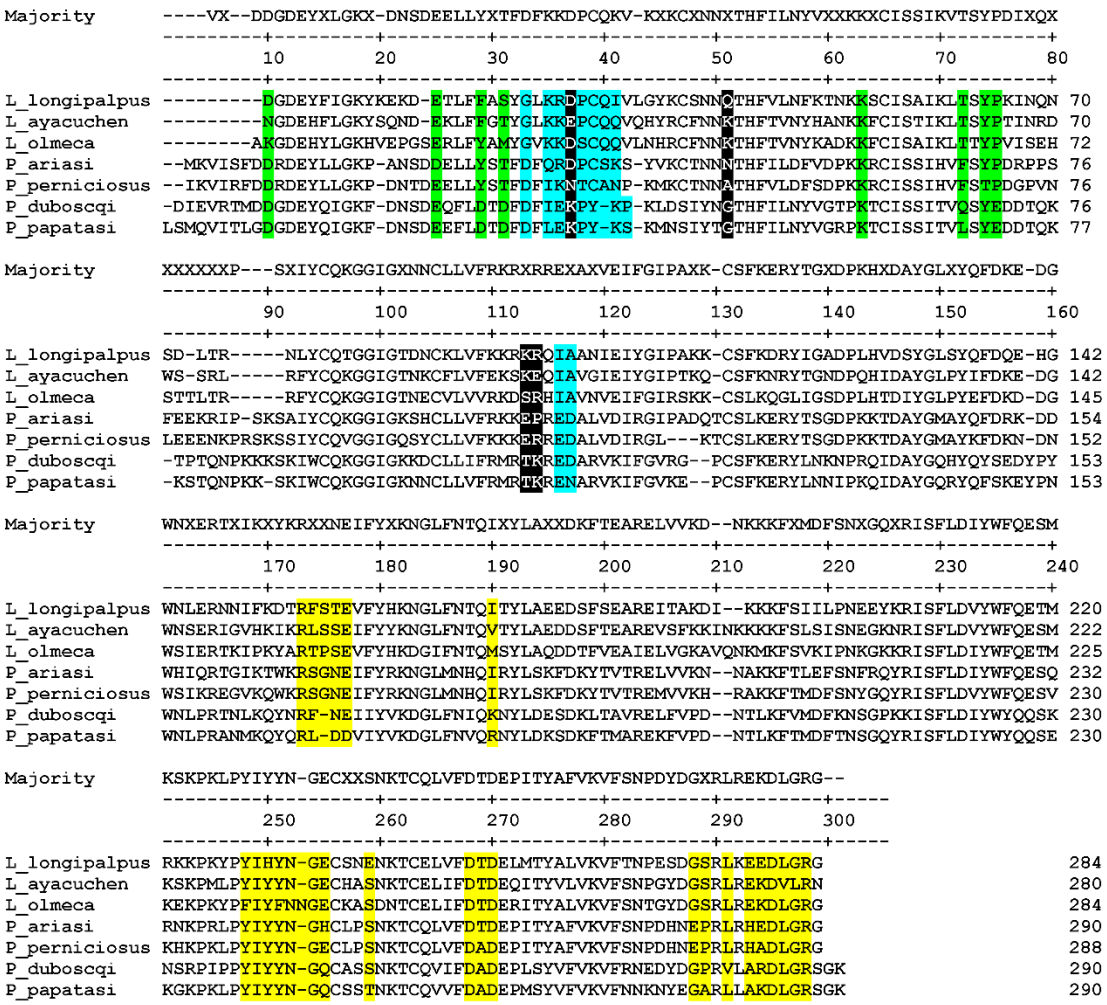

Figure S5. Sequence alignment of lufaxin and orthologs from new- and old-world sand fly species. L_longipalp: *Lutzomyia longipalpis* (lufaxin); L_ayacuchen: *Lutzomyia ayacuchensis*; L_olmeca: *Lutzomyia olmeca*; P_ariasi: *Phlebotomus ariasi*; P_pernicios: *Phlebotomus perniciosus*; P_duboscqi: *Phlebotomus duboscqi*; P_papatasi: *Phlebotomus papatasi*. N-terminal domain residues interacting (as indicated by PISA) with the Ba fragment are highlighted in cyan, those interacting with the vWA domain are highlighted in black and those interacting with the CUB domain are highlighted in green. In the C-terminal domain residues interacting with the SP domain of fXa are highlighted in yellow.

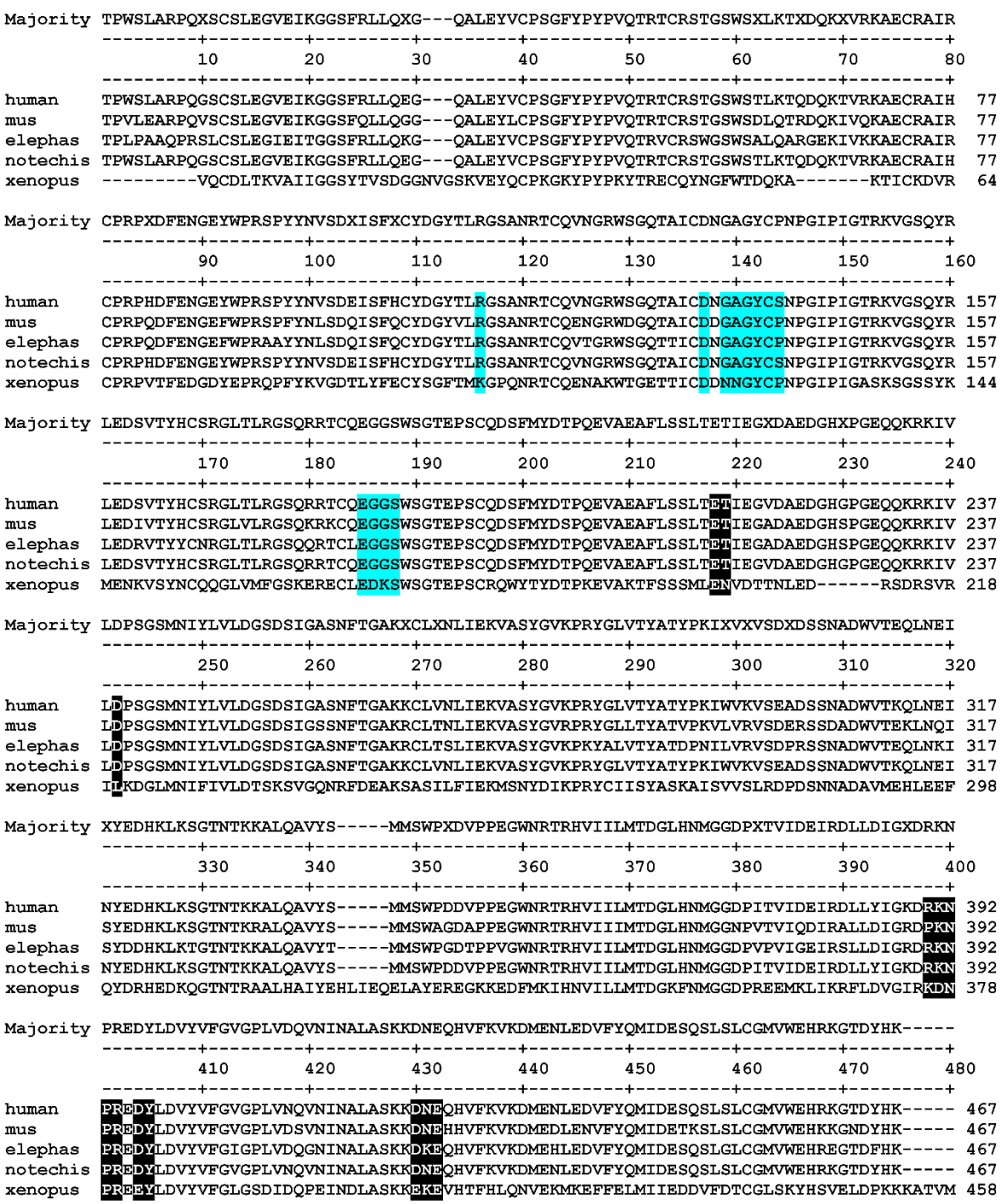

Figure S6. Amino alignment of FB (minus the SP domain) from vertebrate species representing mammals (human, *Mus* and *Elephas*), reptiles (*Notechis*) and amphibians (*Xenopus*). Residues in human FB from the Ba fragment (cyan) vWA domain (black) that interact with lufaxin are highlighted along with corresponding residues in other species.

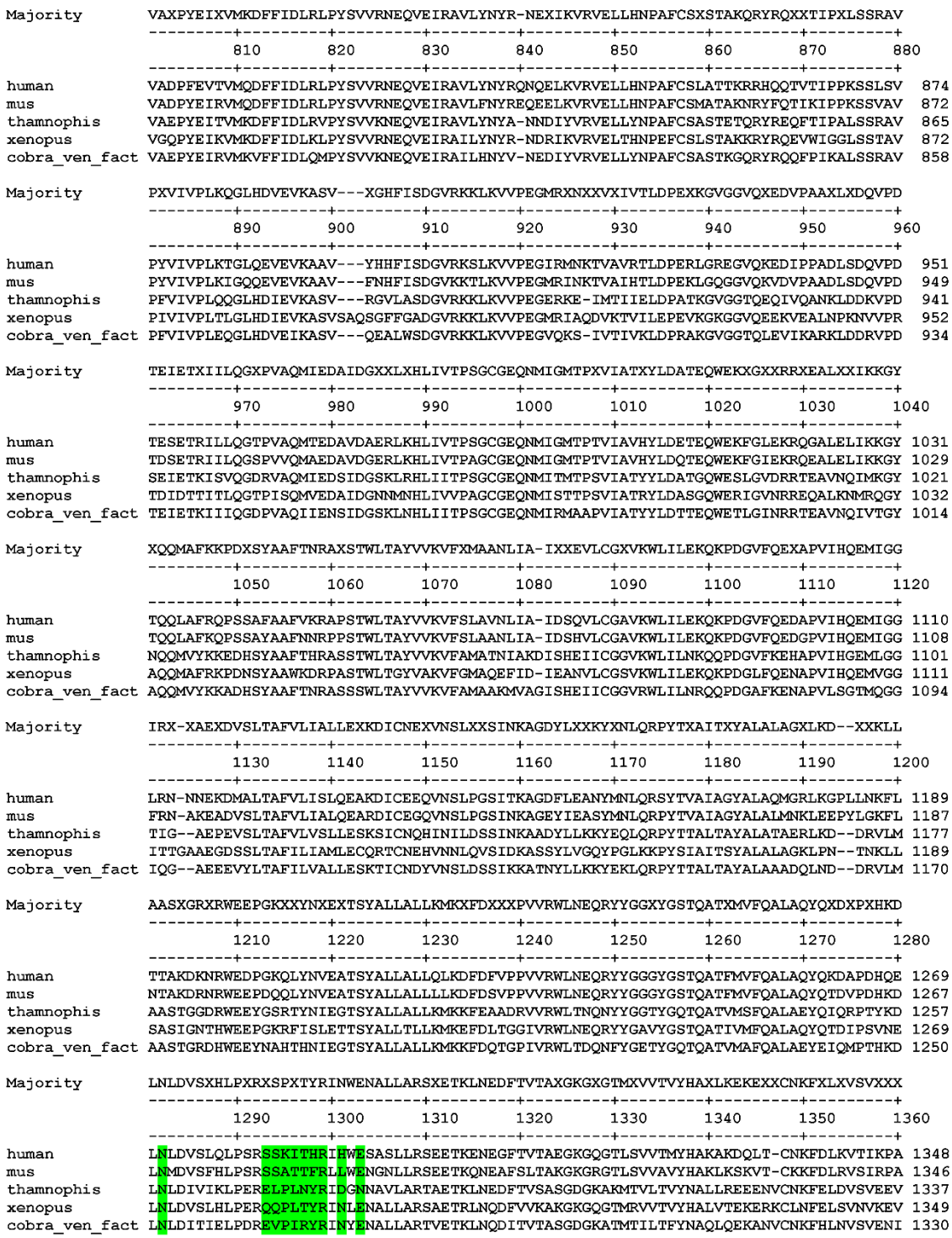

Figure S7. Amino acid alignment of region of C3 corresponding to the CUB and TED domains from various vertebrate species including mammals (human, *Mus*), reptiles (*Thamnophis*), and amphibians (*Xenopus*) as well as the snake venom component, cobra venom factor. Residues highlighted in green correspond to those of human C3b interacting with lufaxin.

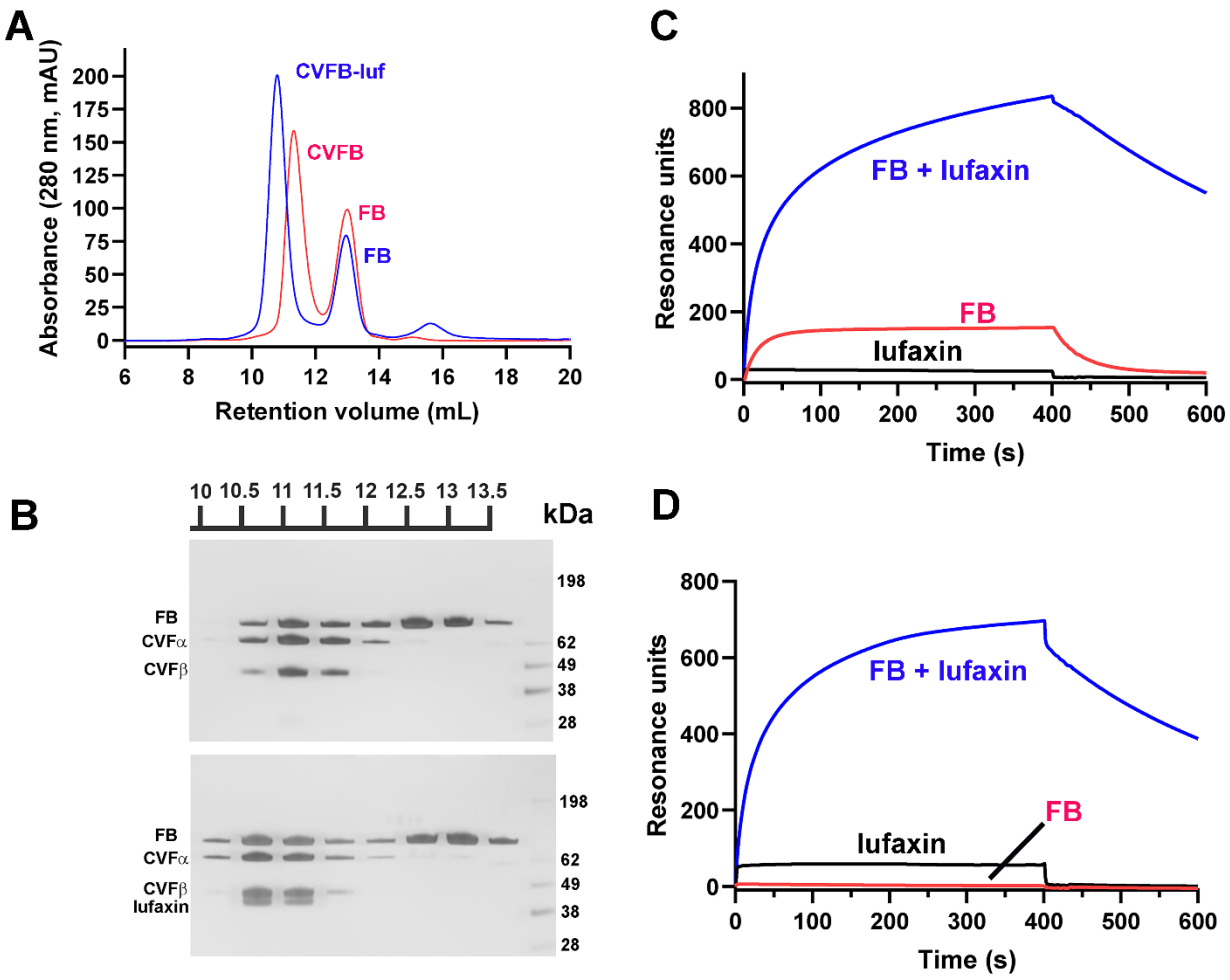

Figure S8. Lufaxin binding with CVFB. **A, B** – Formation of a CVF complex with FB and lufaxin in the absence and presence of Mg^{2+}. **A** - Analysis by size exclusion chromatography of Superdex-200 shows formation of a CVFB in the presence of 5 mM Mg^{2+} (red trace). Addition of lufaxin increases the size of the complex (blue trace). **B** - SDS-PAGE (10% gel) of fractions (with retention volumes on the scale above the gels) shows the formation of CVFB in the absence of lufaxin (upper gel) and the incorporation of lufaxin into the complex when mixed with CVF and FB (lower gel). The gels are stained with silver. **C** - Surface plasmon resonance analysis of FB and lufaxin binding to an immobilized CVF surface in the presence of 5 mM Mg^{2+}. Blue trace FB (57 nM) + lufaxin (0.5 μM), Red trace FB alone, black trace lufaxin alone. **D** – The experiment shown in panel C performed in the absence of Mg^{2+}. Chromatographic and SPR analyses indicate that lufaxin stabilizes the binding of FB with CVF much like it does with C3b.

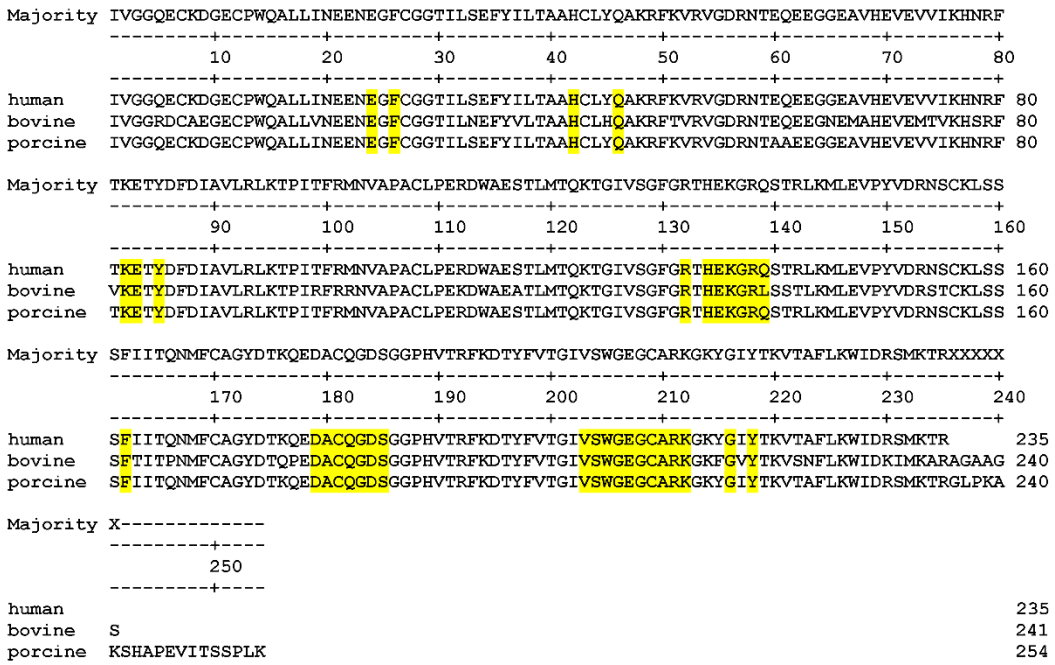

Figure S9. Amino acid alignment of the heavy chain of FXa from mammal species with regions of the human protein that interact with lufaxin (along with corresponding residues from other species) highlighted in yellow.

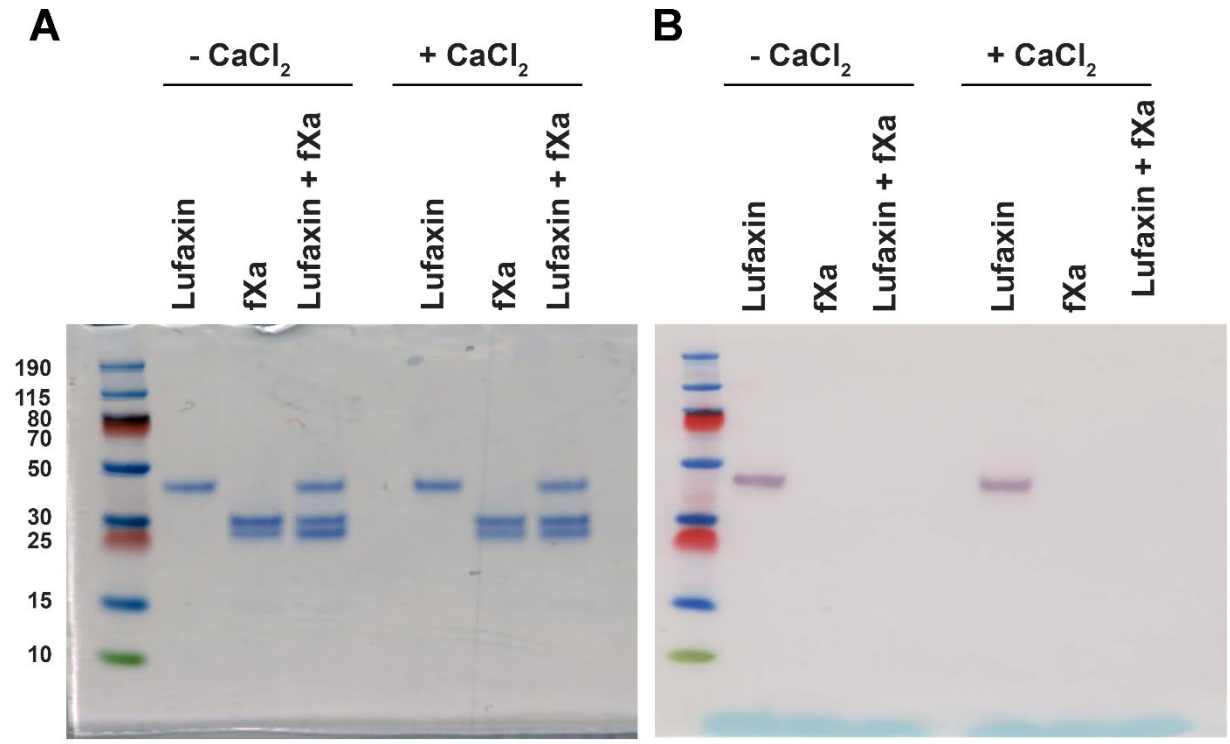

Figure S10. Factor Xa cleaves lufaxin. Recombinant lufaxin containing a 6-His tag (1.9 μM) was incubated with fXa (3.8 μM) in the presence or absence of 5mM $CaCl_2$, in Tris-buffered saline pH 7.4, at 30°C for 3 hours. Same concentrations of lufaxin or FXa alone were used as controls. Immediately after incubation samples (indicated in the figure) were prepared and loaded on 4-12% Bis-Tris gels run in parallel. After running, one of the gels was stained with Coomassie blue (left panel) and the other one was transferred to a nitrocellulose membrane used in western blot assay to detect 6x-his tag (right panel). Lufaxin cleavage by fXa is almost imperceptible if evaluated by its apparent molecular weight in the Coomassie stained gel (left panel), nevertheless it is evident by the loss of the fragment containing the His-tag (right panel), in a reaction that occurs regardless of the presence of $CaCl_2$. Pre-stained molecular weight markers (sizes indicated in kDa) were run in the first lane of each gel.

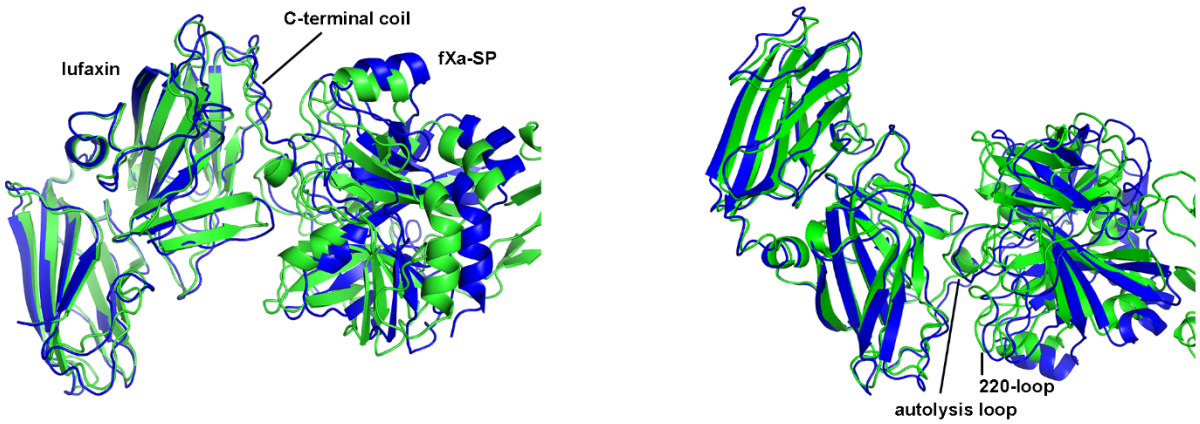

Figure S11. Superposition of cryo-EM model (blue) showing lufaxin and fXa (heavy chain) superposed with a prediction produced with alphafold 2.3.1 (green). After its recent release, alphafold 2.3.1 was used to produce a model for the fXa-lufaxin structure that closely resembles the cryo-EM model. Two views of the model are show related by rotation of approximately 180° around the vertical axis. Arg 277 of the C-terminal coil of lufaxin is bound at the S1 subsite of fXa and very similar interactions occur at the autolysis loop and the 220-loop of fXa. The alphafold model is "tighter" than the cryo EM model with the two components lying generally closer to one another at the interface of lufaxin and fXa. Inputs for the prediction were one chain of the lufaxin sequence (mature), one heavy chain of fXa and one light chain of fXa (both mature).