SUPPLEMENTARY APPENDIX

Factor VIII/V C-domain swaps reveal discrete C-domain roles in factor VIII function and intracellular trafficking

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Supplementary Methods

Materials

All chemicals used were of analytical grade. Fetal calf serum was from Hyclone (Logan, UT, USA). One shot *Stbl3* chemically competent cells, trypsin, and DMEM (4.5 g/L glucose) were obtained from Invitrogen (Breda, the Netherlands). DNA modifying enzymes were from NEB (Ipswich, MA, USA). EGM-2 bullet kit, penicillin and streptomycin were from Lonza (Walkersville, MD, USA). Collagen type 1 rat tail was from BD Biosciences (Uppsala, Sweden). Peroxidase-labeled polyclonal rabbit anti-human VWF antibody was supplied by DAKO (Glostrup, Denmark). Tissue culture flasks, multidishes and microtiterplates (Maxisorp) were from Nunc (Roskilde, Denmark). TMTs, hydroxylamine and chymotrypsin were from Thermo Scientific (Breda, the Netherlands). The chromogenic substrate S-2765 containing the thrombin inhibitor I-2581 was from the Coatest FVIII kit (Chromogenix-Instrumentation Laboratory, Milano, Italy).

Immunofluorescence microscopy of FVIII and FV

BOECs were grown on collagen-coated 1 cm-diameter glass coverslips. Confluent cells were fixed with 3.7% PFA in PBS for 15 minutes at room temperature. After fixation, the cells were labeled with monoclonal antibody CLB-RAg20[1] to detect VWF, followed by incubation with Alexa-633 conjugated secondary antibody and FITC-labeled EL-14[2] (directed against FVIII C2-domain), FITC-labeled KM33[3] (against FVIII C1-domain) or FITC-labeled CLB-FV-4 (directed against FV light chain)[4]. Cells were embedded in 4-88 Mowiol and stored at 4 °C until analysis. Z-stacks (0.4-µm intervals) were taken with confocal laser scanning microscopy using a Zeiss LSM510 equipped with Plan NeoFluar 63x/1.4 Oil objective (Carl Zeiss, Heidelberg, Germany). Images were processed with Zeiss LSM510 version 4.0 software and LSM image browser (Carl Zeiss, Heidelberg, Germany). Quantification of FVIII variants and FV antigen levels in the conditioned medium by enzyme-linked immunosorbent assay (ELISA) was essentially done as described elsewhere,[5,6] except that for determination

of FV antigen, the immobilized monoclonal antibody CLB-FV-4[4] was used and purified FV with known concentration served as reference.

Construction of FVIII-YFP/FV chimera pcDNA3.1(+) vectors

For construction of FVIII containing a FV C1- or C2-domain, FV C-domains were amplified from pcDNA3.1(+) encoding B-domain deleted FV 811-1491[6] using the following primers for the FV C1-domain:

5'-ATAACCGGTGGACTAAGCACTGGTATC-3' (sense) and
5'-TATTGGCGCGCCACCATTTACCTCACAACCTTG-3'(antisense).

For the FV C2-domain the following primers were used:

5'-ATAACGGTGGATGTTCCACACCCCTG-3' (sense) and
5'-TATTGGCGCGCCACCGTAAATATCACAGCCAAA-3' (antisense).

PCR products were ligated in pGEM-T Easy vector (Promega) and digested with *AgeI* and *AscI*. The FVIII C1- (ranging from Cys2021 to Asp2172) or C2-domain (ranging from Ser2173 to the C-terminal end) in pcDNA3.1(+) FVIIIYFP[7] were replaced for *AgeI-AscI* restriction sites by Quick Change mutagenesis using the following primers for the C1-domain (restriction sites are underlined):

5'-CTGGTGTACAGCAATAAG<u>ACCGGTGGCGCGCCA</u>AGTTGCAGCATGCCATTG-3'
(sense) and
5'-CAATGGCATGCTGCAACT<u>TGGCGCACCGGT</u>CTTATTGCTGTACACCAG-3'
(antisense).

Constructs were digested with *AgeI-AscI* and ligated with corresponding FV C-domain fragments. *AgeI* and *AscI* restriction sites bordering the C-domains were removed by Quick Change mutagenesis using appropriate primers. Coding sequences of the constructs were analyzed by sequencing. After sequencing we found that the first 5 amino acids of the swapped C1-domain were missing in the FVIII-YFP/FV-C1 variant. These were added using Quick Change mutagenesis with the following primers:

5'-CTGGTGTACAGCAATAAGTGTAGGATGCCAATGGGACTAAGCACTGGTATC-3' (sense) and

5'-GATACCAGTGCTTAGTCCCATTGGCATCCTACACTTATTGCTGTACACCAG-3' (antisense).

Construction of lentiviral vectors

Lentiviral plasmids encoding B-domain deleted FVIII (FVIII) and FVIII/FV chimeras were created by replacing fragment *NheI-XhoI* from pLV-CAG-FVIIIGFP vector[5] for the corresponding fragment of the pcDNA3.1(+) plasmids. To clone BDD-FV in lentiviral vector, pLV-CAG-FVIIIGFP vector was first digested with *NheI* followed by partial digestion with *NotI*. Briefly, *NheI*-digested construct was mixed on ice with serial dilutions of *NotI*, incubated for 15 minutes at 37 °C and heated to 65 °C for 20 minutes to inactivate the restriction enzyme. The fragment corresponding to lentiviral vector without insert was isolated from gel and ligated with the *NheI-NotI* fragment of FV in pcDNA3.1(+). Production of lentivirus, BOEC isolation and transduction with lentivirus have been described before.[5]

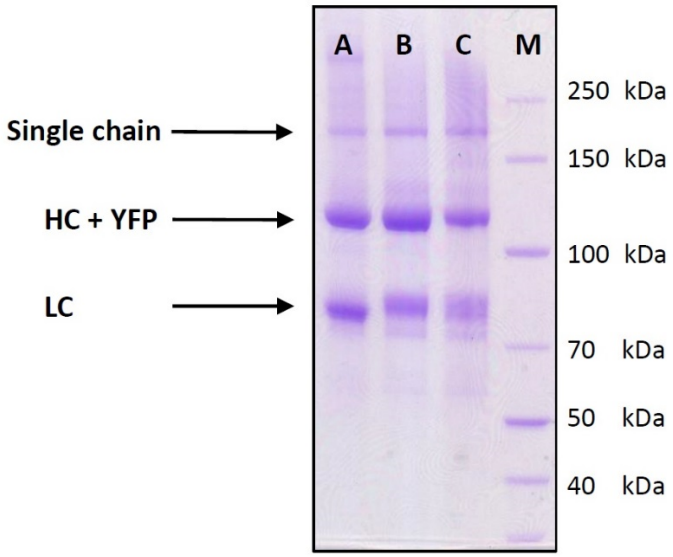
TMT-labeling of FVIII-YFP variants and mass spectrometry analysis

TMT-labeling was done as essentially described elsewhere.[8] Briefly, labeling was initiated by addition of 2.5 mM of TMT labels to 140 nM of the chimeric FVIII-YFP/FV protein or 140 nM of the normal FVIII-YFP protein in buffer containing 20 mM HEPES, 5 mM $CaCl_2$ and 175 mM NaCl and incubated at 25 °C (Eppendorf Thermomixer comfort, 600 rpm). In our

experiments, TMT-127 was used for the chimeric protein and TMT-126 was used for the FVIII-YFP or FV. The labeling reaction was quenched after 15 minutes by addition of 500 mM of hydroxylamine. TMT-labeled wild-type protein was pooled in a 1:1 molar ratio with either TMT-labeled FVIII-YFP/FV-C1 or FVIII-YFP/FV-C2. The pooled protein mixture was reduced and acetylated as described previously.[8] Peptides were obtained after digestion by chymotrypsin with subsequent desalting using a C18 Ziptip (Millipore, Amsterdam, the Netherlands), both performed according to the manufacturer's instructions. Peptides were separated by reverse phase chromatography and sprayed into a Thermo Scientific LTQ OrbitrapXL mass spectrometer. Peptides from the chimeras and FVIII-YFP were separated on a C18 column with 0.5% (v/v) acetic acid using two different gradients from 0% up to 60% (v/v) of acetonitrile: a (short) 60 minute gradient and a (long) 160 minute gradient both with a wash peak of 98% (v/v) acetonitrile for 5 minutes. Peptides acquired from the chimeras and FV wild-type were separated using the long 160 minute gradient. Peptide identification and TMT quantification by mass spectrometric analysis was done as described elsewhere.[8]

Supplementary Figures

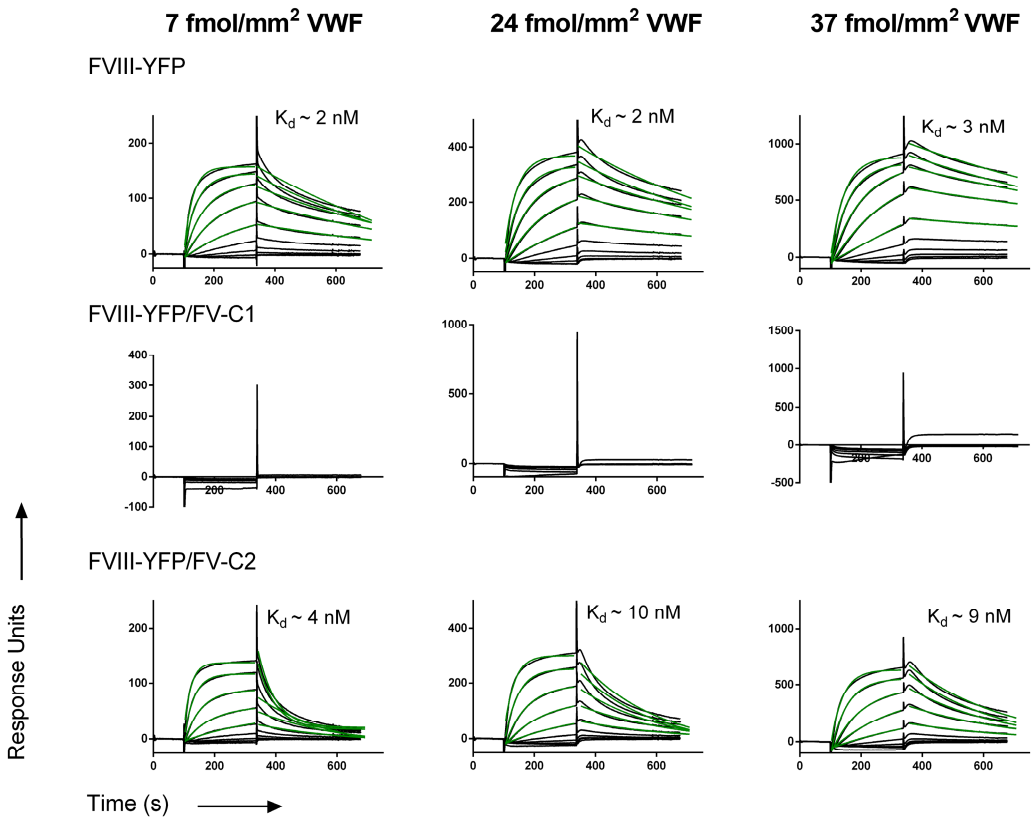
Figure S1



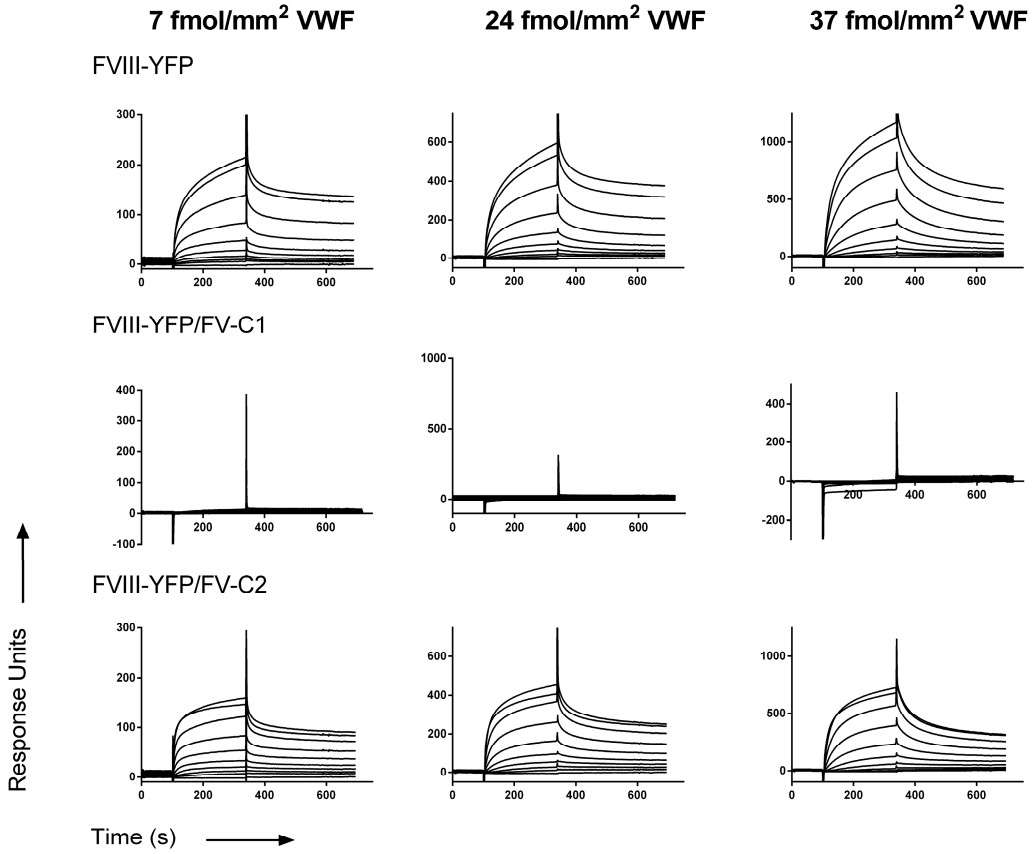
Supplementary Figure S1. SDS PAGE analysis of purified chimeras. (A) Purified FVIII-YFP, (B) FVIII-YFP/FV-C1 and (C) FVIII-YFP/FV-C2 were run on a 4-12% gradient SDS PAGE (Invitrogen). Indicated are the YFP-carrying heavy chain domain (HC+YFP) of approx. 110 kDa and light chain (LC) of approx. 80 kDa as well as a minor single chain component. Masses were inferred using a PageRuler Broad Range (Thermo Scientific) marker (M).

Figure S2

A

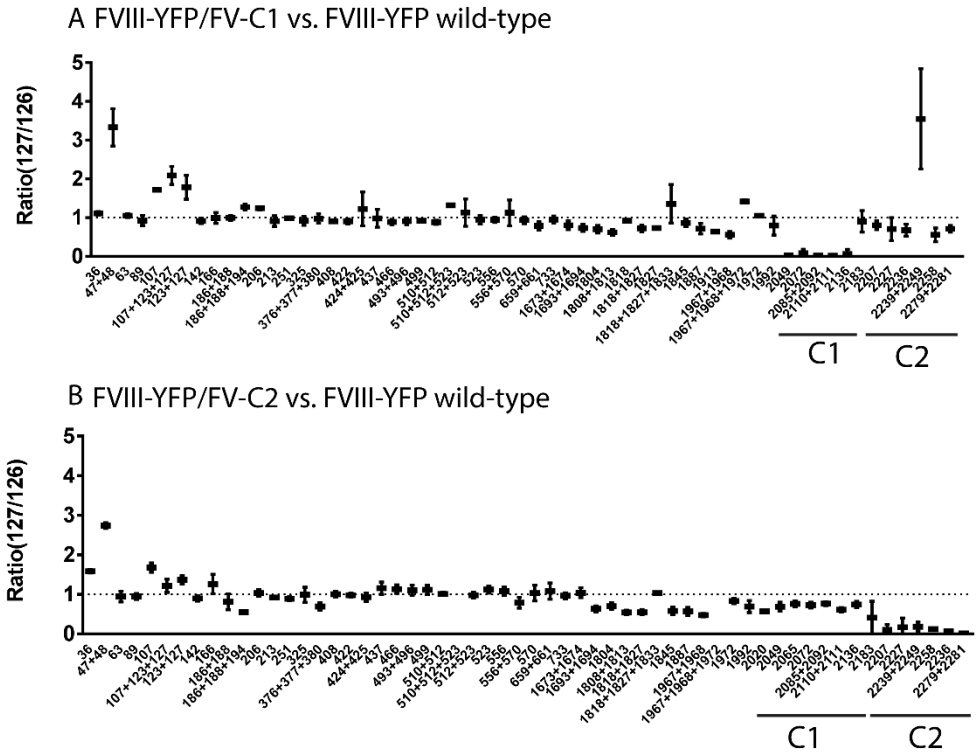

7 fmol/mm2 VWF
24 fmol/mm2 VWF
37 fmol/mm2 VWF
FVIII-YFP
Kd ~ 2 nM
Kd ~ 2 nM
Kd ~ 3 nM
FVIII-YFP/FV-C1
FVIII-YFP/FV-C2
Kd ~ 4 nM
Kd ~ 10 nM
Kd ~ 9 nM
Response Units
Time (s)

B



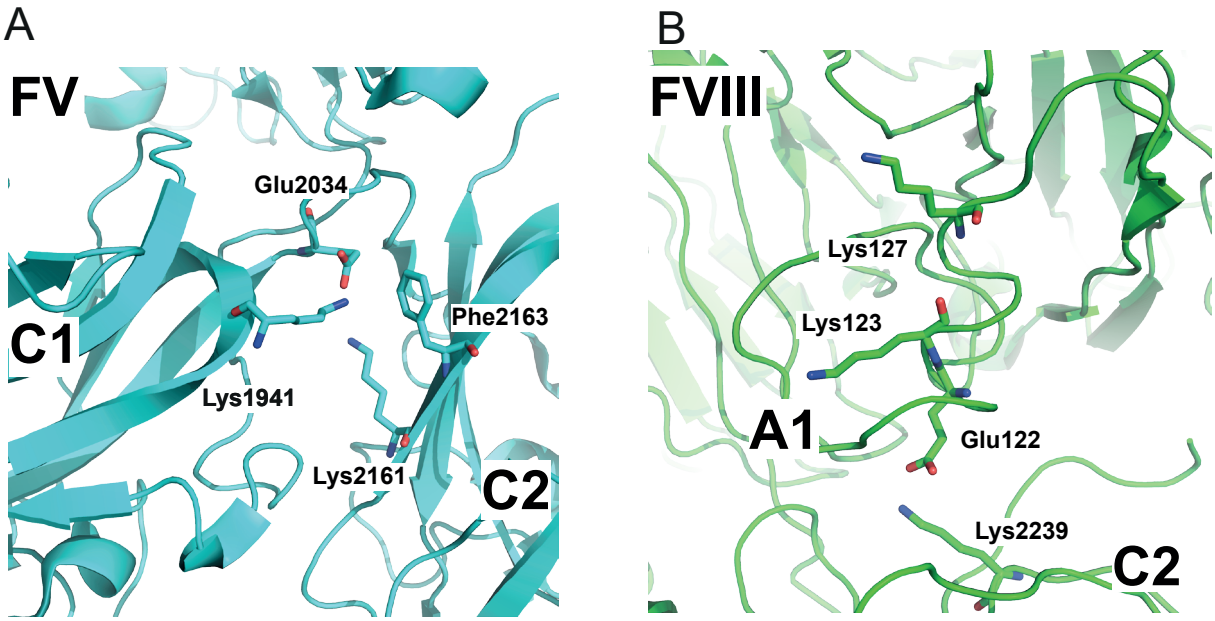
Supplementary Figure S2. Interaction of FVIII-YFP variants with immobilized VWF. Displayed are the sensorgrams acquired during the SPR experiments done at (A) pH 7.4 and (B) pH 5.5. FVIII-YFP variants with different concentrations ranging from 0.23 to 60 nM were passed over a chip with different VWF densities (7 fmol/mm^2, 24 fmol/mm^2 and 37 fmol/mm^2). Spikes are due to minor differences in buffer composition between samples and running buffer. Ignoring these imperfections, sensorgrams of FVIII-YFP and FVIII-YFP/FV-C2 were fitted using a Langmuir 1:1 binding model.[9] The association phase was fitted using a k_{off} value obtained from a separate dissociation phase fitting (also using a Langmuir 1:1 binding model).[9] The obtained K_d values of the binding curves at pH 7.4 are an average of the K_d values obtained from the fitted traces displayed in green solid curves. At pH 5.5 the dissociation phase displays partially irreversible binding which precluded calculation of K_d values.

Figure S3



Supplementary Figure S3. Overview of TMT-labeled FVIII-YFP/FV-C1 and FVIII-YFP/FV-C2 in comparison with FVIII-YFP. The mean TMT-127/TMT-126 ratio with ± SD (black lines) is given for peptides containing the same lysine residues in (A) FVIII-YFP/FV-C1 and (B) FVIII-YFP/FV-C2 compared to FVIII-YFP. Most of the TMT-127/TMT-126 ratios are around 1, indicating unchanged lysine accessibility. The TMT-127/TMT126 ratios for lysine residues in the C1-domain or C2-domain are near zero for the respective experiments were either the FVIII-YFP/FV-C1 chimera or the FVIII-YFP/FV-C2 chimera was compared to FVIII-YFP. This could be explained because peptides containing lysines of FVIII-YFP are not present in the chimeras' swapped C-domains. Therefore, only FVIII-YFP TMT-126 labeled lysine residues could be detected.

Figure S4



Supplementary Figure S4. Potentially disrupted C1/C2 domain and A1/C2 domain interfaces. (A) Within FV, two lysine residues Lys1941 and Lys2161 on the opposite C-domain could interact with Glu2034 (salt-bridge) or Phe2163 (cation-π). Swapping of a C-domain would leave half the interacting residues disengaged. (B) Within FVIII, Lys2239 appears to interact with Glu122 on the A1-domain. Disruption of this interaction could also have its effect on the nearby located lysine residues Lys123 and Lys127. Images were created with PyMol (Schrödinger, Cambridge, MA, USA).

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