FACTOR VIII COMPLEX WITH XTEN AND VON WILLEBRAND FACTOR PROTEIN, AND USES THEREOF

BACKGROUND OF THE INVENTION

[0001] Haemophilia A is a bleeding disorder caused by defects in the gene encoding coagulation factor VIII (FVIII) and affects 1-2 in 10,000 male births. Graw *et al., Nat. Rev. Genet.* 6(6): 488-501 (2005). Patients affected with hemophilia A can be treated with infusion of purified or recombinantly produced FVIII. All commercially available FVIII products, however, are known to have a half-life of about 8-12 hours, requiring frequent intravenous administration to the patients. *See* Weiner M.A. and Cairo, M.S., Pediatric Hematology Secrets, Lee, M.T., 12. Disorders of Coagulation, Elsevier Health Sciences, 2001; Lillicrap, D. Thromb. Res. 122 Suppl 4:S2-8 (2008). In addition, a number of approaches have been tried in order to extend the FVIII half-life. For example, the approaches in development to extend the half-life of clotting factors include pegylation, glycopegylation, and conjugation with albumin. See Dumont et al., Blood. 119(13): 3024-3030 (Published online Jan. 13, 2012). Regardless of the protein engineering used, however, the long acting FVIII products currently under development are reported to have limited half-lives – only to about 1.5 to 2 hours in preclinical animal models. *See id.* Consistent results have been demonstrated in humans, for example, rFVIIIFc was reported to improve half-life up to ~ 1.7 fold compared with ADVATE® in hemophilia A patients. *See Id.* Therefore, the half-life increases, despite minor improvements, may indicate the presence of other T1/2 limiting factors. *See* Liu, T. *et al.*, 2007 ISTH meeting, abstract #P-M-035; Henrik, A. *et al.*, 2011 ISTH meeting, abstract #P=MO-181; Liu, T. *et al.*, 2011 ISTH meeting abstract #P-WE-131.

[0002] Plasma von Willebrand Factor (VWF) has a half-life of approximately 12 hours (ranging from 9 to 15 hours). http://www.nhlbi.nih.gov/guidelines/vwd/2_scientificoverview.htm (last visited October 22, 2011). The VWF half-life may be affected by a number of factors: glycosylation pattern, ADAMTS-13 (a disintegrin and metalloprotease with thrombospondin motif-13), and various mutations in VWF.

[0003] In plasma, 95-98% of FVIII circulates in a tight non-covalent complex with full-length VWF. The formation of this complex is important for the

maintenance of appropriate plasma levels of FVIII in vivo. Lenting *et al., Blood.* 92(11): 3983-96 (1998); Lenting *et al., J. Thromb. Haemost.* 5(7): 1353-60 (2007). The full-length wild-type FVIII is mostly present as a heterodimer having a heavy chain (MW 200kD) and a light chain (MW 73kD). When FVIII is activated due to proteolysis at positions 372 and 740 in the heavy chain and at position 1689 in the light chain, the VWF bound to FVIII is removed from the activated FVIII. The activated FVIII, together with activated factor IX, calcium, and phospholipid ("tenase complex"), induces the activation of factor X, generating large amounts of thrombin. Thrombin, in turn, then cleaves fibrinogen to form soluble fibrin monomers, which then spontaneously polymerize to form the soluble fibrin polymer. Thrombin also activates factor XIII, which, together with calcium, serves to crosslink and stabilize the soluble fibrin polymer, forming crosslinked (insoluble) fibrin. The activated FVIII is cleared fast from the circulation by proteolysis.

[0004] Due to the frequent dosing and inconvenience caused by the dosing schedule, there is still a need to develop FVIII products requiring less frequent administration, *i.e.,* a FVIII product that has a half-life longer than the 1.5 to 2 fold half-life limitation.

BRIEF SUMMARY OF THE INVENTION

[0005] The present invention is directed to a chimeric protein comprising (i) a von Willebrand Factor (VWF) fragment comprising the D' domain and the D3 domain of VWF, (ii) an XTEN sequence, and (iii) a FVIII protein, wherein the VWF fragment and the XTEN sequence are linked by an optional linker and wherein the VWF fragment or the XTEN sequence is linked to or associated with the FVIII protein. The chimeric protein can comprise a single polypeptide chain comprising the VWF fragment, the XTEN sequence, and the FVIII protein, or two polypeptide chains, a first chain comprising the VWF fragment and the second chain comprising the FVIII protein, wherein the XTEN polypeptide is linked either to the VWF fragment or the FVIII protein.

[0006] In one embodiment, the chimeric protein of the invention comprises a formula comprising:

(a) V-X-FVIII,

(b) FVIII-X-V,

(c) V-X:FVIII,

(d) X-V:FVIII,

(e) FVIII:V-X, or

(f) FVIII:X-V,

wherein V comprises a VWF fragment,

X comprises one or more XTEN sequences, and

FVIII comprises a FVIII protein. The hyphen (-) can be a peptide bond or a linker, *e.g.,* a cleavable linker, while the colon (:) represents a chemical association or a physical association between the polypeptides, for example a covalent or non-covalent bond.

[0007] In another embodiment, the chimeric protein further comprises (iv) an immunoglobulin (Ig) constant region or a portion thereof (also indicated as F1 or a first Ig constant region or a portion thereof) linked to the VWF fragment, the XTEN sequence, the FVIII protein, or any combinations thereof. In other embodiments, the chimeric protein further comprises an additional Ig constant region or a portion thereof (also indicated as F2 or a second Ig constant region or a portion thereof). The first Ig constant region or a portion thereof can be linked to the VWF fragment or the XTEN sequence, and the second Ig constant region can be linked to the FVIII protein. The first Ig constant region, the second Ig constant region or a portion thereof, or both can extend the half-life of the FVIII protein.

[0008] In some embodiments, the second Ig constant region or a portion thereof (F2) is linked to the VWF fragment by a linker, *e.g.,* a processable linker. In other embodiments, the second Ig constant region or a portion thereof (F2) is associated with the (first) Ig constant region or a portion thereof (F1). The second Ig constant region or a portion thereof (F2) and the first Ig constant region or a portion thereof (F1) can be identical or different. The second Ig constant region or a portion thereof can be associated with the Ig constant region or a portion thereof by a covalent bond, *e.g.,* a disulfide bond. The VWF fragment linked to the first Ig constant region or a portion thereof may also be associated with the FVIII protein linked to the second Fc region by a non-covalent bond. In certain embodiments, the FVIII protein can further comprise one or more additional XTEN sequences which are linked to the C-terminus or N-terminus of the FVIII

protein or inserted immediately downstream of one or more amino acids in the FVIII protein (*e.g.,* one or more XTEN insertion sites). In some embodiments, the half-life of the FVIII protein is extended, compared to wild type FVIII or a FVIII protein without the VWF fragment.

[0009] In some embodiments, the chimeric protein comprises a formula comprising:

(g) V-L2-X-L1-F1:FVIII-L3-F2;

(h) V-L2-X-L1-F1:F2-L3-FVIII;

(i) F1-L1-X-L2-V:FVIII-L3-F2;

(j) F1-L1-X-L2-V:F2-L3-FVIII;

(k) V-L2-X-L1-F1-L4-FVIII-L3-F2;

(l) F2-L3-FVIII-L4-F1-L1-X-L2-V;

(m) FVIII-L3-F2-L4-V-L2-X-L1-F1; or

(n) F1-L1-X-L2-V-L4-F2-L3-FVIII,

wherein V comprises a VWF fragment,

each of L1, L2, and L3 comprises an optional linker, *e.g.,* a cleavable linker,

L4 is an optional linker, *e.g.,* a processable linker,

FVIII comprises a FVIII protein,

X comprises one or more XTEN sequences,

F1 comprises an optional first Ig constant region or a portion thereof,

F2 comprises an optional second Ig constant region or a portion thereof, and

(:) is a covalent bond or non-covalent bond.

[0010] The present invention is also directed to a chimeric protein comprising (i) a FVIII protein, (ii) an XTEN sequence, and (iii) an Ig constant region or a portion thereof, wherein the XTEN sequence is linked to the FVIII protein by an optional linker at the N-terminus or C terminus of the FVIII protein or inserted immediately downstream of one or more amino acids in the FVIII protein (*e.g.,* one or more insertion sites) and wherein the Ig constant region or a portion thereof is linked to or associated with the FVIII protein or the XTEN sequence. In one embodiment, the Ig constant region or a portion thereof useful for the chimeric protein comprises a first Fc region. In another embodiment, the chimeric protein further comprises an additional Ig constant region or a portion thereof. The additional Ig constant region or a portion thereof useful for the invention can

comprise a second Fc region, which is linked to or associated with the first Fc region, *e.g.,* by a covalent bond. In other embodiments, the first Fc region is linked to the second Fc region by a linker, *e.g.,* a processable linker.

[0011] In other aspects, a chimeric protein comprises (i) a FVIII protein, (ii) an XTEN sequence, (iii) a VWF fragment, and (iv) an Ig constant region or a portion thereof, which comprises the D' domain and the D3 domain of VWF, wherein the XTEN sequence is linked to the FVIII protein by an optional linker at the N-terminus or C terminus of the FVIII protein or inserted immediately downstream of one or more amino acids in the FVIII protein (*e.g.,* one or more insertion sites), the VWF fragment is linked to or associated with the FVIII protein or the XTEN sequence, and the Ig constant region or a portion thereof is linked to the FVIII protein, the XTEN sequence, the VWF fragment, or any combinations thereof. Non-limiting examples of the chimeric proteins may comprise a formula, which comprises:

(1) FVIII(X1)-L1-F1:V-L2-X2-L3-F2;

(2) FVIII(X1)-L1-F1:F2-L3-X2-L2-V;

(3) F1-L1-FVIII(X1):V-L2-X2-L3-F2;

(4) F1-L1-FVIII(X1):F2-L3-X2-L2-V;

(5) FVIII(X1)-L1-F1-L4-V-L2-X2-L3-F2;

(6) FVIII(X1)-L1-F1-L4-F2-L3-X2-L2-V;

(7) F1-L1-FVIII(X1)-L4-V-L2-X2-L3-F2, or

(8) F1-L1-FVIII(X1)-L4-F2-L3-X2-L2-V,

wherein FVIII(X1) comprises a FVIII protein and one or more XTEN sequences, wherein one or more of the XTEN sequences are linked to the N-terminus or C-terminus of the FVIII protein or inserted immediately downstream of one or more amino acids in the FVIII protein (*e.g.,* one or more XTEN insertion sites);

each of L1, L2, or L3 comprises an optional linker, *e.g.,* a cleavable linker;

L4 is a linker, a processable linker;

X2 comprises one or more XTEN sequences;

F1 comprises an Ig constant region or a portion thereof;

F2 comprises an optional additional Ig constant region or a portion thereof, and

V comprises a VWF fragment;

(-) is a peptide bond or one or more amino acids; and

(:) comprises a covalent bond or a non-covalent bond.

[0012] One aspect of the invention is that the VWF fragment useful for the chimeric protein does not bind to a VWF clearance receptor, which prevents or inhibits interaction of the FVIII protein with endogenous VWF. The chimeric protein comprising the VWF fragment thus has reduced clearance or is not cleared through a VWF clearance pathway. Another aspect of the invention is that the VWF fragment is capable of protecting the FVIII protein from one or more protease cleavages, protecting the FVIII protein from activation, stabilizing the heavy chain and/or the light chain of the FVIII protein, or preventing clearance of the FVIII protein by one or more scavenger receptors.

[0013] Because of the VWF fragment's ability to prevent or inhibit interaction between the FVIII protein and endogenous VWF, the half-life of the FVIII protein, is extended compared to a FVIII protein without the VWF fragment. In one embodiment, the half-life of the FVIII protein is extended at least about 1.5 times, at least about 2 times, at least about 2.5 times, at least about 3 times, at least about 4 times, at least about 5 times, at least about 6 times, at least about 7 times, at least about 8 times, at least about 9 times, at least about 10 times, at least about 11 times, or at least about 12 times longer than wild type FVIII. In another embodiment, the half-life of the FVIII protein is at least about 10 hours, at least about 11 hours, at least about 12 hours, at least about 13 hours, at least about 14 hours, at least about 15 hours, at least about 16 hours, at least about 17 hours, at least about 18 hours, at least about 19 hours, at least about 20 hours, at least about 21 hours, at least about 22 hours, at least about 23 hours, at least about 24 hours, at least about 36 hours, at least about 48 hours, at least about 60 hours, at least about 72 hours, at least about 84 hours, at least about 96 hours, or at least about 108 hours.

[0014] The Ig constant region or a portion thereof useful for the chimeric protein comprises a first Fc region, which is linked to the VWF fragment by an optional linker, *e.g.,* a cleavable linker. The chimeric protein can further comprise an additional Ig constant region or a portion thereof, which is linked to the FVIII protein or the XTEN sequence, the Ig constant region or a portion thereof, the VWF fragment, or any combinations thereof by an optional linker. In one embodiment, the additional Ig constant region or a portion thereof is linked to the

FVIII protein by an optional linker. The additional Ig constant region or a portion thereof can comprise a second Fc region.

[0015] The Ig constant region or a portion thereof useful in the present invention and the additional Ig constant region or a portion thereof useful in the present invention are identical or different.

[0016] In some aspects, the FVIII protein is linked to an XTEN sequence at the C-terminus or the N-terminus of the FVIII protein or inserted immediately downstream of one or more amino acids in mature native human FVIII (*e.g.,* one or more insertion sites) or any combinations thereof. One or more insertion sites in the FVIII protein can be located within one or more domains of the FVIII protein selected from the group consisting of the A1 domain, the a1 acidic region, the A2 domain, the a2 acidic region, the A3 domain, the B domain, the C1 domain, the C2 domain, and any combinations thereof or between one or more domains of the FVIII protein selected from the group consisting of the A1 domain and a1 acidic region, the a1 acidic region and A2 domain, the A2 domain and a2 acidic region, the a2 acidic region and B domain, the B domain and A3 domain, the A3 domain and C1 domain, the C1 domain and C2 domain, and any combinations thereof or between two domains of the FVIII protein selected from the group consisting of the A1 domain and a1 acidic region, the a1 acidic region and A2 domain, the A2 domain and a2 acidic region, the a2 acidic region and B domain, the B domain and A3 domain, the A3 domain and C1 domain, the C1 domain and C2 domain, and any combinations thereof.

[0017] In one embodiment, the one or more insertion sites are located immediately downstream of one or more amino acids in mature native human FVIII (*e.g.,* SEQ ID NO: 4 [mature FVIII sequence-full length]) selected from the group consisting of the amino acid residues in Table 7, 8, 9, 10, 11, or any combinations thereof.

[0018] In another embodiment, the one or more insertion sites are located in one or more permissive loops of mature native human FVIII. In other embodiments, the one or more insertion sites are located in the a3 region of mature native human FVIII. For example, an XTEN sequence can be inserted immediately downstream of amino acid 1656 corresponding to SEQ ID NO: 4 (full length mature FVIII). In other embodiments, a FVIII protein is linked to at least two XTEN sequences, a

first XTEN sequence inserted within the a3 region, and a second XTEN sequence inserted within a permissive loop in the FVIII protein (*e.g.,* A1-1, A1-2, A2-1, A2-2, A3-1, or A3-2). In still other embodiments, a FVIII protein is linked to at least three XTEN sequences, a first XTEN sequence inserted within the a3 region and a second XTEN sequence and a third XTEN sequence inserted within one or two permissive loop in the FVIII protein (*e.g.,* A1-1, A1-2, A2-1, A2-2, A3-1, or A3-2).

[0019] In certain embodiments, the one or more insertion sites for one or more XTEN insertions are immediately downstream of one or more amino acids (numbered relative to mature FVIII sequence) selected from the group consisting of:

(1) amino acid 3, (2) amino acid 18, (3) amino acid 22,
(4) amino acid 26, (5) amino acid 32, (6) amino acid 40,
(7) amino acid 60, (8) amino acid 65, (9) amino acid 81,
(10) amino acid 116, (11) amino acid 119, (12) amino acid 130,
(13) amino acid 188, (14) amino acid 211, (15) amino acid 216,
(16) amino acid 220, (17) amino acid 224, (18) amino acid 230,
(19) amino acid 333, (20) amino acid 336, (21) amino acid 339,
(22) amino acid 375, (23) amino acid 399, (24) amino acid 403,
(25) amino acid 409, (26) amino acid 416, (26) amino acid 442,
(28) amino acid 487, (29) amino acid 490, (30) amino acid 494,
(31) amino acid 500, (32) amino acid 518, (33) amino acid 599,
(34) amino acid 603, (35) amino acid 713, (36) amino acid 745,
(37) amino acid 1656, (38) amino acid 1711, (39) amino acid 1720,
(40) amino acid 1725, (41) amino acid 1749, (42) amino acid 1796,
(43) amino acid 1802, (44) amino acid 1827, (45) amino acid 1861,
(46) amino acid 1896, (47) amino acid 1900, (48) amino acid 1904,
(49) amino acid 1905, (50) amino acid 1910, (51) amino acid 1937,

(52) amino acid 2019, (53) amino acid 2068, (54) amino acid 2111,
(55) amino acid 2120, (56) amino acid 2171, (57) amino acid 2188,
(58) amino acid 2227, (59) amino acid 2277, and
(60) two or more combinations thereof.

[0020] In some embodiments, one XTEN is inserted in the FVIII protein. In some embodiments, two XTENs are inserted in the FVIII protein. In some embodiments, 3 XTENs are inserted in the FVIII protein.

[0021] In a particular example, a first XTEN is inserted immediately downstream of amino acid 26 corresponding to SEQ ID NO: 4, and a second XTEN is inserted immediately downstream of amino acid 1720 corresponding to SEQ ID NO: 4 (full-length mature FVIII). In another example, a first XTEN is inserted immediately downstream of amino acid 403 corresponding to SEQ ID NO: 4, and a second XTEN is inserted immediately downstream of amino acid 1720 corresponding to SEQ ID NO: 4. In some examples, a first XTEN is inserted immediately downstream of amino acid 1656 corresponding to SEQ ID NO: 4, and a second XTEN is inserted immediately downstream of amino acid 1720 corresponding to SEQ ID NO: 4. In other examples, a first XTEN is inserted immediately downstream of amino acid 26 corresponding to SEQ ID NO: 4, a second XTEN is inserted immediately downstream of amino acid 1656 corresponding to SEQ ID NO: 4, and a third XTEN is inserted immediately downstream of amino acid 1720 corresponding to SEQ ID NO: 4. In yet other embodiments, a first XTEN is inserted immediately downstream of amino acid 403 corresponding to SEQ ID NO: 4, a second XTEN is inserted immediately downstream of amino acid 1656 corresponding to SEQ ID NO: 4, and a third XTEN is inserted immediately downstream of amino acid 1720 corresponding to SEQ ID NO: 4. In still other embodiments, a first XTEN is inserted between amino acids 403 and 404 corresponding to SEQ ID NO: 4, a second XTEN is inserted immediately downstream of amino acid 1656 corresponding to SEQ ID NO: 4, and a third XTEN is inserted immediately downstream of amino acid 1720 corresponding to SEQ ID NO: 4. In certain embodiments, a first XTEN is inserted immediately downstream of amino acid 26 corresponding to SEQ ID NO: 4 (full-

length mature FVIII), a second XTEN is inserted immediately downstream of amino acid 1720 corresponding to SEQ ID NO: 4, and a third XTEN is inserted immediately downstream of amino acid 1900 corresponding to SEQ ID NO: 4. In some embodiments, a first XTEN is inserted immediately downstream of amino acid 26 corresponding to SEQ ID NO: 4, a second XTEN is inserted immediately downstream of amino acid 1656 corresponding to SEQ ID NO: 2, a third XTEN is inserted immediately downstream of amino acid 1720 corresponding to SEQ ID NO: 4, and a fourth XTEN is inserted immediately downstream of amino acid 1900 corresponding to SEQ ID NO: 4. In another example, an XTEN is inserted immediately downstream of amino acid 745 corresponding to SEQ ID NO: 4. In an additional example, a first XTEN is inserted immediately downstream of amino acid 1656 corresponding to SEQ ID NO: 4 and a second XTEN is inserted immediately downstream of amino acid 1900 corresponding to SEQ ID NO: 4. In some embodiments, a first XTEN is inserted immediately downstream of amino acid 26 corresponding to SEQ ID NO: 4, a second XTEN is inserted immediately downstream of amino acid 1656 corresponding to SEQ ID NO: 4, and a third XTEN is inserted immediately downstream of amino acid 1900 corresponding to SEQ ID NO: 4. In another example, a first XTEN is immediately inserted downstream of amino acid 403 corresponding to SEQ ID NO: 4 and a second XTEN is inserted immediately downstream of amino acid 745 corresponding to SEQ ID NO: 4. In some embodiments, a first XTEN is inserted immediately downstream of amino acid 745 of corresponding to SEQ ID NO: 4, and a second XTEN is inserted immediately downstream of amino acid 1900 corresponding to SEQ ID NO: 4. In some embodiments, a first XTEN is inserted immediately downstream of amino acid 18 corresponding to SEQ ID NO: 4, and a second XTEN is inserted immediately downstream of amino acid 745 corresponding to SEQ ID NO: 4.

[0022] In some embodiments, the FVIII protein is a dual chain FVIII isoform. In some embodiments, the FVIII protein is a single chain FVIII isoform.

[0023] In some embodiments, the XTEN that is inserted is SEQ ID NO: 39 (AE288). In some examples, the XTENs that are inserted are SEQ ID NOs: 38 and 37 (AG144 and AE144). In some examples, the XTENs that are inserted are SEQ ID NOs: 37, 38 and 37 (AE144, AG144, and AE144). In some

embodiments. the XTENs that are inserted are SEQ ID NOs: 37 and 40 (AE144 and AE288). In some embodiments, the XTENs that are inserted are AE42 (SEQ ID NO: 36), AE72 (SEQ ID NO: 127), AE144_2A (SEQ IDNO: 128), AE144_3B (SEQ ID NO: 129), AE144_4A (SEQ ID NO: 130), AE144_5A (SEQ IDNO: 131), AE144_6B (SEQ IDNO: 132), AG144_A (SEQ ID NO: 133), AG144_B (SEQ IDNO: 134), AG144_C (SEQ ID NO: 135), AG144_F (SEQ IDNO: 136), AE864 (SEQ ID NO: 43), AE576 (SEQ ID NO: 41), AE288 (SEQ IDNO: 39), AE288_2 (SEQ ID NO: 137), AE144 (SEQ ID NO: 37), AG864 (SEQ ID NO: 44), AG576 (SEQ ID NO: 42), AG288 (SEQ ID NO: 40), AG144 (SEQ ID NO: 38), and any combinations thereof.

[0024] The FVIII protein useful in the invention can comprise B domain or a portion thereof, *e.g.,* SQ B domain deleted FVIII. In one embodiment, the FVIII protein comprises single chain FVIII. In another embodiment, the single chain FVIII contains at least one amino acid substitution at a residue corresponding to residue 1648, residue 1645, or both of full-length mature Factor VIII polypeptide (SEQ ID NO: 4) or residue 754, residue 751, or both of SQ BDD Factor VIII (SEQ ID NO: 6). In other embodiments, the amino acid substitution is an amino acid other than arginine. In some embodiments, the FVIII protein comprises a heavy chain of FVIII and a light chain of FVIII, wherein the heavy chain and the light chain are associated with each other by a metal bond.

[0025] The FVIII protein can have a low affinity to or does not bind to a low-density lipoprotein receptor-related protein (LRP), *e.g.,* by containing at least one amino acid substitution that lowers the affinity to or eliminates the binding to the LRP. Such at least one amino acid substitution can be at a residue corresponding to residue 471, residue 484, residue 487, residue 490, residue 497, residue 2092, residue 2093 or two or more combinations thereof of full-length mature FVIII. In a particular embodiment, the amino acid substitution at residue 471, 484, or 497 is an amino acid other than arginine, the amino acid substitution at residue 487 is an amino acid other than tyrosine, the amino acid substitution at residue 2092 is an amino acid other than lysine, or the amino acid substitution at residue 2093 is an amino acid other than phenylalanine.

[0026] In some embodiments, the FVIII protein contains at least one amino acid substitution, which induces the FVIII protein to be more stable than a FVIII

protein without the substitution. Such substitutions can be located in the A2 domain and the A3 domain of the FVIII protein, *e.g.,* at a residue corresponding to residue 664, residue 1826, residue 662, residue 1828, or two or more combinations thereof of full-length mature FVIII.

[0027] The VWF fragment useful for the present invention comprises a D' domain and D3 domain, which together are capable of binding to FVIII. The VWF fragment can comprise the amino acid sequence of the D' domain is at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 764 to 866 of SEQ ID NO: 2 and/or the amino acid sequence of the D3 domain is at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 867 to 1240 of SEQ ID NO: 2. In one embodiment, the VWF fragment is a monomer. In another embodiment, the VWF fragment comprises at least two VWF fragments, at least three VWF fragments, at least four VWF fragments, at least five VWF fragments, or at least six VWF fragments. In one embodiment, the two or more VWF fragments may be identical or they may be different. The VWF fragment can comprise an amino acid at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 764 to 1240 of SEQ ID NO: 2. The VWF fragment may consist essentially of or consist of amino acids 764 to 1240 of SEQ ID NO: 2. In certain embodiments, the VWF fragment can contain at least one amino acid substitution at a residue corresponding to residue 1099, residue 1142, or both residues 1099 and 1142 of SEQ ID NO: 2. In other embodiments, the VWF fragment further comprises the D1 domain, the D2 domain, or the D1 and D2 domains of VWF.

[0028] The VWF fragment may further comprise a VWF domain selected from the group consisting of the A1 domain, the A2 domain, the A3 domain, the D4 domain, the B1 domain, the B2 domain, the B3 domain, the C1 domain, the C2 domain, the CK domain, one or more fragments thereof, and any combinations thereof. For example, the VWF fragment can consist essentially of or consist of: (1) the D' and D3 domains of VWF or fragments thereof; (2) the D1, D', and D3 domains of VWF or fragments thereof; (3) the D2, D', and D3 domains of VWF or fragments thereof; (4) the D1, D2, D', and D3 domains of VWF or fragments thereof; or (5) the D1, D2, D', D3, and A1 domains of VWF or fragments thereof.

In some embodiments, the VWF fragment further comprises a signal peptide of VWF or FVIII which is operably linked to the VWF fragment.

[0029] One or more of the linkers useful in the invention have a length of at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1200, 1400, 1600, 1800, or 2000 amino acids. In some embodiments, one or more of the linkers have a length of about 1 to about 2000 amino acids. In one embodiment, one or more of the linkers have a length of at least about 20, 35, 42, 48, 73, 75, 95, 98, 144, 288, 324, 333, 576, or 864 amino acids. In another embodiment, one or more of the linkers comprise a gly/ser peptide, an XTEN sequence, or both. Examples of the gly/ser peptide include, but are not limited to, a formula of $(Gly_4Ser)_n$ (SEQ ID NO: 139) or $S(Gly_4Ser)_n$ (SEQ ID NO: 140), wherein n is a positive integer selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10. For example, the $(Gly_4Ser)_n$ linker can be $(Gly_4Ser)_3$ (SEQ ID NO: 63) or $(Gly_4Ser)_4$ (SEQ ID NO: 138). In one embodiment, the linker comprises at least one first cleavage site at the N-terminus of the linker, at least one second cleavage site at the C-terminus of the linker, or both. In another embodiment, the linker comprises 20 amino acids, 35 amino acids, 48 amino acids, 73 amino acids, or 95 amino acids thrombin cleavable linker. The cleavable linkers can comprise one or more of the cleavage sites by a protease selected from the group consisting of factor XIa, factor XIIa, kallikrein, factor VIIa, factor IXa, factor Xa, factor IIa (thrombin), Elastase-2, Granzyme-B, TEV, Enterokinase, Protease 3C, Sortase A, MMP-12, MMP-13, MMP-17, and MMP-20, *e.g.,* TLDPRSFLLRNPNDKYEPFWEDEEK (SEQ ID NO: 8). Non-limiting examples of one or more of the cleavage sites comprise an amino acid sequence selected from the group consisting of RRRR (SEQ ID NO: 9), RKRRKR (SEQ ID NO: 10), RRRRS (SEQ ID NO: 11), TQSFNDFTR (SEQ ID NO: 12), SVSQTSKLTR (SEQ ID NO: 13), DFLAEGGGVR (SEQ ID NO: 14), TTKIKPR (SEQ ID NO: 15), LVPRG (SEQ ID NO: 16), ALRPR (SEQ ID NO: 17), KLTRAET (SEQ ID NO: 18), DFTRVVG (SEQ ID NO: 19), TMTRIVGG (SEQ ID NO: 20), SPFRSTGG (SEQ ID NO: 21), LQVRIVGG (SEQ ID NO: 22), PLGRIVGG (SEQ ID NO:23), IEGRTVGG (SEQ ID NO: 24), LTPRSLLV (SEQ ID NO: 25), LGPVSGVP (SEQ ID NO: 26), VAGDSLEE (SEQ ID NO: 27),

GPAGLGGA (SEQ ID NO: 28), GPAGLRGA (SEQ ID NO: 29), APLGLRLR (SEQ ID NO: 30), PALPLVAQ (SEQ ID NO: 31), ENLYFQG (SEQ ID NO: 32), DDDKIVGG (SEQ ID NO: 33), LEVLFQGP (SEQ ID NO: 34), and LPKTGSES (SEQ ID NO: 35). In some embodiments, the first cleavage site and the second cleavage site are identical or different.

[0030] The XTEN sequence useful for the invention can be selected from the group consisting of AE42 (SEQ ID NO: 36), AE144 (SEQ ID NO: 37), AG144 (SEQ ID NO: 38), AE288 (SEQ ID NO: 39), AG288 (SEQ ID NO: 40), AE576 (SEQ ID NO: 41). AG576 (SEQ ID NO: 42), AE864 (SEQ ID NO: 43), AE72 (SEQ ID NO: 127), AE144_2A (SEQ ID NO: 128), AE144_3B (SEQ ID NO: 129), AE144_4A (SEQ ID NO: 130), AE144_5A (SEQ ID NO: 131), AE144_6B (SEQ ID NO: 132), AG144_A (SEQ ID NO: 133), AG144_B (SEQ ID NO: 134), AG144_C (SEQ ID NO:135), AG144_F (SEQ ID NO: 136), AE288_2 (SEQ ID NO: 137), or AG864 (SEQ ID NO: 44). In a particular embodiment, the XTEN sequence comprises AE288 or AG288.

[0031] The chimeric protein of the invention can be polysialylated, pegylated, or hesylated.

[0032] The present invention is also directed to a polynucleotide or a set of polynucleotides encoding the chimeric protein. The polynucleotide can further comprise a polynucleotide chain, which encodes PC5 or PC7. The invention is also directed to a vector comprising the polynucleotide or the set of polynucleotides and one or more promoter operably linked to the polynucleotide or the set of polynucleotides. The vector can further comprise an additional vector, which comprises a polynucleotide chain encoding PC5 or PC7. The invention is also drawn to a host cell comprising the polynucleotide or the vector. The host cell can be a mammalian cell, *e.g.,* HEK293 cell, CHO cell, or BHK cell. In some embodiments, the PC5 or PC7 of the host cell cleaves the D1D2 domains of VWF.

[0033] The invention is also directed to a pharmaceutical composition comprising the chimeric protein, the polynucleotide, the vector, or the host cell, and a pharmaceutically acceptable carrier. The composition of the invention thus has an extended half-life compared to wild type FVIII protein. The half-life of the FVIII protein is extended at least about 1.5 times, at least about 2 times, at least about

2.5 times, at least about 3 times, at least about 4 times, at least about 5 times, at least about 6 times, at least about 7 times, at least about 8 times, at least about 9 times, at least about 10 times, at least about 11 times, or at least about 12 times longer than wild type FVIII. The half-life of Factor VIII is at least about 17 hours, at least about 18 hours, at least about 19 hours, at least about 20 hours, at least about 21 hours, at least about 22 hours, at least about 23 hours, at least about 24 hours, at least about 25 hours, at least about 26 hours, at least about 27 hours, at least about 28 hours, at least about 29 hours, at least about 30 hours, at least about 31 hours, at least about 32 hours, at least about 33 hours, at least about 34 hours, at least about 35 hours, at least about 36 hours, at least about 48 hours, at least about 60 hours, at least about 72 hours, at least about 84 hours, at least about 96 hours, or at least about 108 hours.

[0034] The composition of the present invention can be administered by a route selected from the group consisting of topical administration, intraocular administration, parenteral administration, intrathecal administration, subdural administration and oral administration. In one embodiment, the composition is administered via parenteral administration, *e.g.,* intravenous or subcutaneous administration. The composition of the invention is useful to treat a bleeding disease or condition in a subject in need thereof. The bleeding disease or condition is selected from the group consisting of a bleeding coagulation disorder, hemarthrosis, muscle bleed, oral bleed, hemorrhage, hemorrhage into muscles, oral hemorrhage, trauma, trauma capitis, gastrointestinal bleeding, intracranial hemorrhage, intra-abdominal hemorrhage, intrathoracic hemorrhage, bone fracture, central nervous system bleeding, bleeding in the retropharyngeal space, bleeding in the retroperitoneal space, bleeding in the illiopsoas sheath and any combinations thereof. In one embodiment, the subject treated with the chimeric protein is scheduled to undergo a surgery. In another embodiment, the treatment is prophylactic or on-demand.

[0035] The invention is also directed to a method of preventing or inhibiting binding of a FVIII protein with endogenous VWF comprising adding an effective amount of the chimeric protein, the polynucleotide vector, the host cell, or the composition to a subject in need thereof, wherein the VWF fragment binds to the FVIII protein and thus prevents or inhibits binding of endogenous VWF. The

present invention is further directed to a method of extending or increasing the half-life of the FVIII protein, wherein the method comprises administering an effective amount of the chimeric protein, the polynucleotide, the vector, the host cell, or the composition to a subject in need thereof, wherein the VWF fragment binds to the FVIII protein and thus extends or increases the half-life of the FVIII protein. Also provided is a method of preventing or inhibiting clearance of a FVIII protein from a cell, wherein the method comprises administering an effective amount of the chimeric protein, the polynucleotide, the vector, the host cell, or the composition to a cell comprising a FVIII protein or a polynucleotide encoding the FVIII protein, wherein the protein having VWF activity binds to the FVIII protein. The subject useful for the present methods is an animal, *e.g.,* a human, *e.g.,* a patient suffering from hemophilia A.

[0036] The present invention also provides a method of treating a bleeding disease or disorder in a subject in need thereof comprising administering an effective amount of the chimeric protein, the polynucleotide, the vector, the host cell, or the composition, wherein the bleeding disease or disorder is selected from the group consisting of a bleeding coagulation disorder, hemarthrosis, muscle bleed, oral bleed, hemorrhage, hemorrhage into muscles, oral hemorrhage, trauma, trauma capitis, gastrointestinal bleeding, intracranial hemorrhage, intra-abdominal hemorrhage, intrathoracic hemorrhage, bone fracture, central nervous system bleeding, bleeding in the retropharyngeal space, bleeding in the retroperitoneal space, and bleeding in the illiopsoas sheath. The treatment can be prophylactic or on-demand. In one embodiment, the effective amount is 0.1 μg/kg to 500 mg/kg.

[0037] The invention also includes a method of making a chimeric protein, comprising transfecting one or more host cell with the polynucleotide or the vector and expressing the chimeric protein in the host cell.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0038] Figure 1A-D. Schematic diagrams of VWF fragments. Fig. 1A shows three exemplary VWF fragments useful for the invention, *e.g.,* VWF-002, VWF-010, and VWF-013. VWF-002 contains amino acids 1 to 477 of SEQ ID NO: 124 (amino acids 764 to 1240 of SEQ ID NO: 2) and is synthesized without the pre/propeptide sequences. VWF-010 contains the D1D2 domains in addition to the D'D3 domains. VWF-013 contains the D1D2D'D3 domains in addition to

alanine residues substituting cysteines at residues 336 and 379 of SEQ ID NO: 123 Fig. 1B shows VWF-031, which contains the D1D2D'D3 domains fused to an Ig constant region or a portion thereof, *e.g.,* an Fc region, by a cleavable linker, *e.g.,* a 48 amino acids thrombin cleavable linker. Fig. 1C shows VWF-025, which is a nucleotide sequence encoding D1D2D'D3 domains contained in pLIVE vector, and VWF-029, which is a nucleotide sequence encoding D1D2D'D3 domains with two amino acid substitutions, C336A and C379A, in pLIVE vector. Fig. 1D shows full-length VWF fragment comprising propeptide (the D1 and D2 domains) and mature subunits (the D', D3, A1, A2, A3, D4, B1-3, C1-2 domains). The VWF fragment is about 250 kDa protein and forms multimers (> 20 MDa) by disulfide bonding. The VWF fragment associates with FVIII (95-98%) in non-covalent complex and then extends half-life of FVIII by protecting FVIII from protease cleavage/activation, stabilizing heavy & light chain, and preventing clearance of FVIII by scavenger receptors. The VWF fragment also can limit half-life of FVIII by clearance of FVIII-VWF complex through VWF receptors and preventing pinocytosis and recycling of rFVIIIFc.

[0039] Figure 2. Pharmacokinetic profile of rFVIII-XTEN (rFVIII-AE288 or rFVIII-288AE) in VWF D'D3 expression mice or in FVIII and VWF double knockout (DKO) mice. Figure 2A shows the timeline of hydrodynamic injection (HDI) of the D'D3 domain encoding plasmid DNA (VWF-025) (day -5), intravenous dosing of rFVIII-XTEN AE288 (day 0), and PK sample collection (day 5). Figure 2B shows FVIII activity measured by a FVIII chromogenic assay after IV dosing of rFVIII-XTEN288 in D1D2D'D3 mice (inverted triangle) and rFVIII-XTEN288 in DKO mice (diamond). Fig. 2C shows the D'D3 plasma level (ng/mL) after administration of VWF-025. The X axis represents time in hours.

[0040] Figure 3. Schematic diagram of exemplary VWF:FVIII heterodimer constructs. The constructs have the common structure represented as formula FVIII-F1-L1-V-X-L2-F2, but contain examples of different variable linkers. The construct (FVIII-161) shown contains a heterodimeric FVIII (the heavy chain and the light chain are associated by a metal bond) linked to a first Fc region and a VWF fragment, which is the D' and D3 domains of VWF (*i.e.,* amino acids 1 to 477 of SEQ ID NO: 2 with amino acid substitutions C336A and C379A) linked to an XTEN sequence, which is further linked to a cleavable linker and a second Fc

region. The XTEN sequence contained in FVIII-161 is an XTEN AE288 sequence, and the linker is a thrombin cleavable linker, which has 35 amino acids. In FVIII-161, the FVIII protein linked to the first Fc region is linked to the VWF fragment by a processable linker. Upon expression, the processable linker can be cleaved by an intracellular processing enzyme, thus making the construct three polypeptide chains associated with each other.

[0041] Figure 4 is schematic diagrams of FVIII-VWF heterodimer or monomer examples. FVIII-168, FVIII-175, FVIII-172, FVIII-174, and FVIII170. Construct FVIII-168 comprises a single chain FVIII sequence (having an alanine residue substitute the arginine residues at residues 1645 and 1648) linked to a first Fc region, which is then fused to a VWF fragment linked to a second Fc region by a thrombin cleavable linker, which has 48 amino acids. AE288 XTEN is inserted in the B domain of the single chain FVIII sequence. The linkage between the first Fc region and the VWF fragment comprises a linker that is capable of being cleaved by an intracellular processing enzyme, *i.e.,* processable linker. Construct FVIII-175 comprises a single chain FVIII (having an alanine residue substitute the arginine residues at residues 1645 and 1648) linked to AE288 XTEN and a first Fc region, which is linked to a second Fc region by a linker, *e.g.,* a processable linker. AE288 XTEN is inserted in the B domain of the single chain FVIII sequence. Construct FVIII-172 comprises two polypeptide chains, a first chain comprising a heavy chain FVIII sequence fused to AE288 XTEN, a second chain comprising a light chain FVIII sequence, a first Fc region, a linker (*e.g.,* a processable linker), a VWF fragment, a thrombin cleavable linker (*e.g.,* 48 amino acids), and a second Fc region. Construct FVIII-174 comprises two polypeptide chains, a first chain comprising a heavy chain FVIII sequence fused to AE288 XTEN and a second chain comprises a light chain FVIII, a first Fc region, a linker (*e.g.,* a processable linker), and a second Fc region. Construct FVIII-170 comprises a VWF fragment, AE288 XTEN, a linker (*e.g.,* a thrombin cleavable linker, which is 35 amino acids in length), and a single chain FVIII sequence.

[0042] Figure 5. Pharmacokinetic profile of FVIII/VWF heterodimers containing an XTEN sequence in combination with an Fc region. Constructs FVIII-161, FVIII-168, and FVIII-172 were administered to FVIII:VWF double knockout (DKO) mice by Hydrodynamic injection (HDI) at 100ug/mouse dose. Construct

FVIII-170 was administered to FVIII:VWF DKO mice by HDI at 50 μg/mouse dose. The post-HDI plasma FVIII activity was analyzed by FVIII chromogenic assay for 24 hr post-HDI. The FVIII activity of the FVIII:VWF heterodimers containing an XTEN sequence and Fc domains was compared with the FVIII activity of BDD-FVIII without the VWF fragment, XTEN sequence, and Fc domains.

[0043] Figure 6. Schematic diagrams of FVIII-VWF heterodimer examples co-transfection system. Fig. 6A. Construct FVIII-169 contains the full-length FVIII sequence (with an alanine residue substituting the arginine residues at 1645 and 1648 and with an XTEN sequence inserted in the single chain FVIII sequence), which is linked to an Fc region. VWF-031 contains the D1D2D'D3 fragment (with an alanine residue substituting the cysteine residues at 336 and 379) which is linked to another Fc region with a 48 thrombin cleavable linker. After intracellular processing, construct FVIII-169 produces a full length single chain FVIII (SCFVIII) fused to one Fc fragment and an XTEN sequence, and construct VWF-031 produces a 477 amino acid D'D3 fragment linked to another Fc fragment. Two covalent bonds can be formed between the Fc fragments that are linked to the SC FVIII or the D'D3 fragment, this in turn allows a non-covalent association of FVIII and D'D3. Fig. 6B. Construct FVIII-173 contains a heterodimeric FVIII sequence, a heavy chain FVIII sequence linked to an XTEN sequence and a light chain FVIII sequence linked to an Fc region. VWF-031 is described above. After intracellular processing, construct FVIII-173 produces a heterodimeric protein, a heavy chain FVIII fused to an XTEN sequence, a light chain FVIII fused to one Fc fragment, and construct VWF-031 produces a 477 amino acid D'D3 fragment linked to another Fc fragment. Two covalent bonds can be formed between the Fc fragments that are linked to the light chain FVIII or the D'D3 fragment, this in turn allows a non-covalent association of FVIII and D'D3.

[0044] Figure 7. Binding Affinity of Exemplary FVIII:VWF containing an XTEN sequence and Fc domains to immobilized hVWF in Octet assay. The binding affinity for FVIII-169/VWF-031 and FVIII-057 (rFVIIIFc) fused to immobilized hVWF was tested using biolayer interferometry based measurements (Octet assay). Figure 7A shows binding response in nanomoles of FVIII169 and FVIIIFc

drug substance (a positive control) to immobilized hVWF. Figure 7B shows binding response of human IgG1 (a negative control) to immobilized human VWF.

[0045] Figure 8. Pharmacokinetic (PK) profile of FVIII-169 in HemA and FVIII:VWF double knockout (DKO) mice. Figure 8A shows the PK profile of FVIII-169/VWF-031 and FVIIIFc in HemA mice. HemA mice were treated with a single intravenous dose of FVIII-169/VWF-031 at 200 IU/kg. Plasma samples collected from the mice were tested by FVIII chromogenic assay. Half-life of FVIII-169/VWF-031 was calculated using WinNonlin program. Figure 8B shows the PK profile of FVIII-169/VWF-031, FVIII-169/Fc, and FVIIIFc in FVIII/VWF DKO mice.

[0046] Figure 9. PK profile of FVIII-XTEN variants in D'D3 expressing FVIII/VWF DKO mice. Figure 9A shows comparison of the PK profile of the FVIII-XTEN variants, FVIII with one XTEN, FVIII with two XTENs, and FVIII with three XTENs. One, two, or three XTENs were inserted in various portions of FVIII including C-terminus and B-domain. CT indicates that an XTEN is linked to the C-terminus of FVIII. Insertion site B/CT indicates that one XTEN is inserted between amino acid residue 745 and amino acid residue 746 of the FVIII protein and another XTEN is linked to the C-terminus of the FVIII protein. The amino acid residue numbering corresponds to the SQ BDD FVIII protein sequence. Insertion site 1900/B/CT indicates that a first XTEN is inserted between amino acid residue 1900 and amino acid residue 1901 of FVIII, a second XTEN is inserted between amino acid residue 745 and amino acid residue 746 of FVIII, and a third XTEN is linked to the C-terminus of FVIII. The mouse strain used to administer the FVIII-XTEN variants is a DKO mouse strain expressing D'D3 domains. Figure 9B shows the PK profile of FVIII-XTEN with three XTEN insertions. The FVIII-XTEN (1900/B/CT) variant was administered to either the FVIII/VWF DKO mice or HemA mice. The half-life of FVIII-XTEN (1900/B/CT) is compared.

[0047] Figure 10. FVIII activity of FVIIIFc (hollow triangle), FVIII169:Fc (filled circle), and FVIII169:VWF31 (hollow triangle) in mouse DKO plasma measured by chromogenic assay. FVIII:Fc contains a dual-chain FVIII (Heavy chain and Light chain) fused to an Fc dimer (*i.e.,* monomer-dimer hybrid). FVIII169 is

described above (containing AE288 in the B domain, immediately downstream of amino acid 745 corresponding to mature FVIII sequence). FVIII169:Fc contains FVIII169 fused to an Fc dimer. FVIII169:VWF31 contains VWF31 in addition to the Fc dimer, FVIII169 fused to the first Fc region and VWF31 fused to the second Fc region, wherein the first Fc region and the second Fc region form a covalent bond, *e.g.,* one or more disulfide bonds.

[0048] Figure 11. Effects of Fc, XTEN, and VWF-D'D3 fragments on FVIII half-life extension. BDD-FVIII (REFACTO®) (square), FVIIFc (circle), FVIII169/Fc (triangle), and FVIII169/VWF031 (inverted triangle) were administered to FVIII and VWF double knockout (DKO) mice. The FVIII activity was measured by chromogenic assay, and the half-life was calculated using the WinNonlin-Phoenix program. X-axis shows time, and the Y-axis shows the FVIII plasma activity in mU/mL.

[0049] Figure 12A-C. Effects of different XTENs in rFVIII-XTEN/VWF heterodimer in HemA mice. Figure 12A shows the FVIII plasma activity normalized to 5 min value (%) of two XTENs inserted immediately downstream of residues 1900 and 1656 corresponding to mature FVIII sequence (*i.e.,* FVIII-195 (dual chain FVIII isoform) and FVIII-199 (single chain FVIII isoform)), compared to FVIII-169 containing an XTEN immediately downstream of residue 745 corresponding to mature FVIII sequence. FVIII-169/VWF-031 (filled circle), FVIII-199/VWF-031 (filled square), and FVIII-195/VWF031 (hollow square) were administered in HemA mice to measure the FVIII plasma activity. Figure 12B shows the half-life extension effect of the second XTEN insertion immediately downstream of residues 403 (A2 domain) and 745 (B domain) (*i.e.,* FVIII-203) and residues 745 (B domain) and 1900 (A3 domain) (FVIII-204) corresponding to mature FVIII sequence compared to FVIII-169 (an XTEN insertion in B domain only). FVIII-204/VWF031 (filled triangle), FVIII-169/VWF-031 (filled circle), FVIII-203/VWF-031 (filled square), and scBDD-FVIII (hollow diamond) were administered to HemA mice. The X-axis shows FVIII plasma activity normalized to 5 min value (%), and the y-axis shows time in hours. Figure 12C shows the half-life extension effect of the two XTEN insertions immediately downstream of residues 18 (A1 domain) and 745 (B domain) (*i.e.,* FVIII-205) compared to FVIII-169 (a single XTEN insertion in the B domain) and

single chain FVIII without any Fc regions or any XTENs (*i.e.,* FVIII-207). Figure 12C additionally shows the half-life extension effect of three XTEN insertions incorporated immediately downstream of residues 26 (A1 domain), 1656 (A3 domain), and 1900 (A3 domain) (*i.e.,* FVIII-201) compared to FVIII-169 (a single XTEN insertion immediately downstream of residue 745). . FVIII-205/VWF-031 (filled square), FVIII-201/VWF-031 (inverted triangle), FVIII-169/VWF-031 (filled circle), and FVIII-207 (hollow diamond) were administered to HemA mice. The FVIII plasma activity normalized to 5 min value (%) (X-axis) was measured over time in hours (Y-axis).

[0050] Figure 13. FVIII activity of rFVIII-XTEN/VWF-XTEN heterodimer in FVIII/VWF DKO mice. FVIII activity of plasma samples was analyzed by FVIII chromogenic assay, and the regression curve of plasma FVIII activity (X-axis) as a function of time (Y-axis) was plotted. FVIII-155 (scFVIIIFc without any XTENs) was co-expressed with VWF-034 (VWF-Fc with AE 288 XTEN plus a 35 residue thrombin cleavable linker). The half-life of FVIII-155/VWF-034 was compared with that of FVIII-169/VWF-031, which has a AE 288 XTEN inserted into the B domain junction (immediately downstream of residue 745 corresponding to mature FVIII polypeptide) of FVIII.

[0051] Figure 14A-H. Schematic diagrams of various rFVIII-XTEN/VWF constructs. These constructs are also described in other sections herein. Fig. 14A shows single chain B domain deleted FVIII protein (sometimes indicated herein as scBDD-FVIII). The scBDD-FVIII constructs contain two substitutions at residues 1645 and 1648 from Arg to Ala. Figure 14B shows two polypeptide chain construct (FVIII155/VWF031), the first chain comprising single chain FVIII linked to an Fc region without any XTENS and the second chain comprising the VWF D'D3 fragment linked to an Fc region. This construct is used as a control. Fig. 14C shows two polypeptide chain construct (FVIII199/VWF031), the first chain comprising single chain FVIII linked to an Fc region, in which a first XTEN is inserted immediately downstream of residue 1900 corresponding to mature FVIII sequence and a second XTEN is inserted immediately downstream of residue 1656 corresponding to mature FVIII sequence, and the second chain comprising the VWF D'D3 fragment linked to an Fc region. Fig. 14D shows two polypeptide chain construct (FVIII201/VWF031), the first chain comprising single

chain FVIII protein linked to an Fc region, in which a first XTEN is inserted immediately downstream of residue 26 corresponding to mature FVIII sequence, a second XTEN is inserted immediately downstream of residue 1656 corresponding to mature FVIII sequence, and a third XTEN is inserted immediately downstream of residue 1900 corresponding to mature FVIII sequence ,and the second chain comprising the VWF D'D3 fragment linked to an Fc region. Fig. 14E shows two polypeptide chain constructs (FVIII169/VWF031), the first chain comprising single chain FVIII protein linked to an Fc region, in which an XTEN is inserted immediately downstream of residue 745 (indicated as "B") corresponding to mature FVIII sequence, and the second chain comprising the VWF D'D3 fragment linked to an Fc region. Fig. 14F shows two polypeptide chain construct (FVIII203/VWF031), the first chain comprising single chain FVIII protein, in which a first XTEN is inserted at residue 745 ("B") corresponding to mature FVIII sequence and a second XTEN is inserted at residue 1900 corresponding to mature FVIII sequence, and the second chain comprising the VWF D'D3 fragment linked to an Fc region. Fig. 14G shows two polypeptide chain construct (FVIII204/VWF031), the first chain comprising single chain FVIII protein linked to an Fc region, in which a first XTEN is inserted immediately downstream of residue 403 corresponding to mature FVIII sequence and a second XTEN is inserted immediately downstream of residue 745 ("B") corresponding to mature FVIII sequence, and a second chain comprising the VWF D'D3 fragment linked to an Fc region. Fig. 14H shows two polypeptide chain construct (FVIII205/VWF031), the first chain comprising single chain FVIII, in which a first XTEN is inserted immediately downstream of residue 18 corresponding to mature FVIII sequence and a second XTEN is inserted immediately downstream of residue 745 ("B") corresponding to mature FVIII sequence, and the second chain comprising the VWF D'D3 fragment linked to an Fc region.

[0052] Figure 15. FVIII activity of rFVIII-XTEN/VWF and BDD-FVIII in FVIII/VWF DKO mice. FVIII activity of plasma samples was analyzed by FVIII chromogenic assay, and the regression curve of plasma FVIII activity (X-axis) as a function of time (Y-axis) was plotted. The half-life of rFVIII-XTEN/VWF (FVIII-205/VWF-031) was compared with that of BDD-FVIII and rFVIIIFc.

[0053] Figure 16. Efficacy of FVIII-XTEN-Fc:VWF-Fc heterodimers in HemA mice using tail clip bleeding model. The HemA mice tail clip bleeding model was used to compare the efficacy of FVIII169/VWF034, FVIII205/VWF031, and BDD-FVIII. The median blood loss in ml for 200 IU/kg of FVIII169/VWF034 and FVIII205/VWF031 is compared with 200 IU/kg of BDD-FVIII, 65 IU/kg of BDD-FVIII, 20 IU/kg of BDD-FVIII, and vehicle.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

[0054] It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, "a nucleotide sequence," is understood to represent one or more nucleotide sequences. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

[0055] The term "polynucleotide" or "nucleotide" is intended to encompass a singular nucleic acid as well as plural nucleic acids, and refers to an isolated nucleic acid molecule or construct, *e.g.*, messenger RNA (mRNA) or plasmid DNA (pDNA). In certain embodiments, a polynucleotide comprises a conventional phosphodiester bond or a non-conventional bond (*e.g.*, an amide bond, such as found in peptide nucleic acids (PNA)). The term "nucleic acid" refers to any one or more nucleic acid segments, *e.g.*, DNA or RNA fragments, present in a polynucleotide. By "isolated" nucleic acid or polynucleotide is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, a recombinant polynucleotide encoding a Factor VIII polypeptide contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) from other polynucleotides in a solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of polynucleotides of the present invention. Isolated polynucleotides or nucleic acids according to the present invention further include such molecules produced synthetically. In addition, a polynucleotide or a nucleic acid can include regulatory elements such as promoters, enhancers, ribosome binding sites, or transcription termination signals.

[0056] As used herein, a "coding region" or "coding sequence" is a portion of polynucleotide which consists of codons translatable into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is typically not translated into an amino acid, it may be considered to be part of a coding region, but any flanking sequences, for example promoters, ribosome binding sites, transcriptional terminators, introns, and the like, are not part of a coding region. The boundaries of a coding region are typically determined by a start codon at the 5' terminus, encoding the amino terminus of the resultant polypeptide, and a translation stop codon at the 3' terminus, encoding the carboxyl terminus of the resulting polypeptide. Two or more coding regions of the present invention can be present in a single polynucleotide construct, *e.g.*, on a single vector, or in separate polynucleotide constructs, *e.g.*, on separate (different) vectors. It follows, then, that a single vector can contain just a single coding region, or comprise two or more coding regions, *e.g.*, a single vector can separately encode a binding domain-A and a binding domain-B as described below. In addition, a vector, polynucleotide, or nucleic acid of the invention can encode heterologous coding regions, either fused or unfused to a nucleic acid encoding a binding domain of the invention. Heterologous coding regions include without limitation specialized elements or motifs, such as a secretory signal peptide or a heterologous functional domain.

[0057] Certain proteins secreted by mammalian cells are associated with a secretory signal peptide which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Those of ordinary skill in the art are aware that signal peptides are generally fused to the N-terminus of the polypeptide, and are cleaved from the complete or "full-length" polypeptide to produce a secreted or "mature" form of the polypeptide. In certain embodiments, a native signal peptide or a functional derivative of that sequence that retains the ability to direct the secretion of the polypeptide that is operably associated with it. Alternatively, a heterologous mammalian signal peptide, *e.g.*, a human tissue plasminogen activator (TPA) or mouse ß-glucuronidase signal peptide, or a functional derivative thereof, can be used.

[0058] The term "downstream" refers to a nucleotide sequence that is located 3' to a reference nucleotide sequence. In certain embodiments, downstream nucleotide

sequences relate to sequences that follow the starting point of transcription. For example, the translation initiation codon of a gene is located downstream of the start site of transcription.

[0059] The term "upstream" refers to a nucleotide sequence that is located 5' to a reference nucleotide sequence. In certain embodiments, upstream nucleotide sequences relate to sequences that are located on the 5' side of a coding region or starting point of transcription. For example, most promoters are located upstream of the start site of transcription.

[0060] As used herein, the term "regulatory region" refers to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding region, and which influence the transcription, RNA processing, stability, or translation of the associated coding region. Regulatory regions may include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures. If a coding region is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

[0061] A polynucleotide which encodes a gene product, *e.g.*, a polypeptide, can include a promoter and/or other transcription or translation control elements operably associated with one or more coding regions. In an operable association a coding region for a gene product, *e.g.*, a polypeptide, is associated with one or more regulatory regions in such a way as to place expression of the gene product under the influence or control of the regulatory region(s). For example, a coding region and a promoter are "operably associated" if induction of promoter function results in the transcription of mRNA encoding the gene product encoded by the coding region, and if the nature of the linkage between the promoter and the coding region does not interfere with the ability of the promoter to direct the expression of the gene product or interfere with the ability of the DNA template to be transcribed. Other transcription control elements, besides a promoter, for example enhancers, operators, repressors, and transcription termination signals, can also be operably associated with a coding region to direct gene product expression.

[0062] A variety of transcription control regions are known to those skilled in the art. These include, without limitation, transcription control regions which function in vertebrate cells, such as, but not limited to, promoter and enhancer segments from cytomegaloviruses (the immediate early promoter, in conjunction with intron-A), simian virus 40 (the early promoter), and retroviruses (such as Rous sarcoma virus). Other transcription control regions include those derived from vertebrate genes such as actin, heat shock protein, bovine growth hormone and rabbit ß-globin, as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (*e.g.*, promoters inducible by interferons or interleukins).

[0063] Similarly, a variety of translation control elements are known to those of ordinary skill in the art. These include, but are not limited to ribosome binding sites, translation initiation and termination codons, and elements derived from picornaviruses (particularly an internal ribosome entry site, or IRES, also referred to as a CITE sequence).

[0064] The term "expression" as used herein refers to a process by which a polynucleotide produces a gene product, for example, an RNA or a polypeptide. It includes without limitation transcription of the polynucleotide into messenger RNA (mRNA), transfer RNA (tRNA), small hairpin RNA (shRNA), small interfering RNA (siRNA) or any other RNA product, and the translation of an mRNA into a polypeptide. Expression produces a "gene product." As used herein, a gene product can be either a nucleic acid, *e.g.*, a messenger RNA produced by transcription of a gene, or a polypeptide which is translated from a transcript. Gene products described herein further include nucleic acids with post transcriptional modifications, *e.g.*, polyadenylation or splicing, or polypeptides with post translational modifications, *e.g.*, methylation, glycosylation, the addition of lipids, association with other protein subunits, or proteolytic cleavage.

[0065] A "vector" refers to any vehicle for the cloning of and/or transfer of a nucleic acid into a host cell. A vector may be a replicon to which another nucleic acid segment may be attached so as to bring about the replication of the attached segment. A "replicon" refers to any genetic element (*e.g.*, plasmid, phage, cosmid, chromosome, virus) that functions as an autonomous unit of replication *in vivo*,

i.e., capable of replication under its own control. The term "vector" includes both viral and nonviral vehicles for introducing the nucleic acid into a cell *in vitro*, *ex vivo* or *in vivo*. A large number of vectors are known and used in the art including, for example, plasmids, modified eukaryotic viruses, or modified bacterial viruses. Insertion of a polynucleotide into a suitable vector can be accomplished by ligating the appropriate polynucleotide fragments into a chosen vector that has complementary cohesive termini.

[0066] Vectors may be engineered to encode selectable markers or reporters that provide for the selection or identification of cells that have incorporated the vector. Expression of selectable markers or reporters allows identification and/or selection of host cells that incorporate and express other coding regions contained on the vector. Examples of selectable marker genes known and used in the art include: genes providing resistance to ampicillin, streptomycin, gentamycin, kanamycin, hygromycin, bialaphos herbicide, sulfonamide, and the like; and genes that are used as phenotypic markers, *i.e.*, anthocyanin regulatory genes, isopentanyl transferase gene, and the like. Examples of reporters known and used in the art include: luciferase (Luc), green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), -galactosidase (LacZ), -glucuronidase (Gus), and the like. Selectable markers may also be considered to be reporters.

[0067] The term "plasmid" refers to an extra-chromosomal element often carrying a gene that is not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear, circular, or supercoiled, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

[0068] Eukaryotic viral vectors that can be used include, but are not limited to, adenovirus vectors, retrovirus vectors, adeno-associated virus vectors, and poxvirus, *e.g.*, vaccinia virus vectors, baculovirus vectors, or herpesvirus vectors. Non-viral vectors include plasmids, liposomes, electrically charged lipids (cytofectins), DNA-protein complexes, and biopolymers.

[0069] A "cloning vector" refers to a "replicon," which is a unit length of a nucleic acid that replicates sequentially and which comprises an origin of replication, such as a plasmid, phage or cosmid, to which another nucleic acid segment may be attached so as to bring about the replication of the attached segment. Certain cloning vectors are capable of replication in one cell type, *e.g.*, bacteria and expression in another, *e.g.*, eukaryotic cells. Cloning vectors typically comprise one or more sequences that can be used for selection of cells comprising the vector and/or one or more multiple cloning sites for insertion of nucleic acid sequences of interest.

[0070] The term "expression vector" refers to a vehicle designed to enable the expression of an inserted nucleic acid sequence following insertion into a host cell. The inserted nucleic acid sequence is placed in operable association with regulatory regions as described above.

[0071] Vectors are introduced into host cells by methods well known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter.

[0072] "Culture," "to culture" and "culturing," as used herein, means to incubate cells under *in vitro* conditions that allow for cell growth or division or to maintain cells in a living state. "Cultured cells," as used herein, means cells that are propagated *in vitro*.

[0073] As used herein, the term "polypeptide" is intended to encompass a singular "polypeptide" as well as plural "polypeptides," and refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term "polypeptide" refers to any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, "protein," "amino acid chain," or any other term used to refer to a chain or chains of two or more amino acids, are included within the definition of "polypeptide," and the term "polypeptide" can be used instead of, or interchangeably with any of these terms. The term "polypeptide" is also intended to refer to the products of post-expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups,

proteolytic cleavage, or modification by non-naturally occurring amino acids. A polypeptide can be derived from a natural biological source or produced recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It can be generated in any manner, including by chemical synthesis.

[0074] An "isolated" polypeptide or a fragment, variant, or derivative thereof refers to a polypeptide that is not in its natural milieu. No particular level of purification is required. For example, an isolated polypeptide can simply be removed from its native or natural environment. Recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for the purpose of the invention, as are native or recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique.

[0075] Also included in the present invention are fragments or variants of polypeptides, and any combination thereof. The term "fragment" or "variant" when referring to polypeptide binding domains or binding molecules of the present invention include any polypeptides which retain at least some of the properties (*e.g.*, FcRn binding affinity for an FcRn binding domain or Fc variant, coagulation activity for an FVIII variant, or FVIII binding activity for the VWF fragment) of the reference polypeptide. Fragments of polypeptides include proteolytic fragments, as well as deletion fragments, in addition to specific antibody fragments discussed elsewhere herein, but do not include the naturally occurring full-length polypeptide (or mature polypeptide). Variants of polypeptide binding domains or binding molecules of the present invention include fragments as described above, and also polypeptides with altered amino acid sequences due to amino acid substitutions, deletions, or insertions. Variants can be naturally or non-naturally occurring. Non-naturally occurring variants can be produced using art-known mutagenesis techniques. Variant polypeptides can comprise conservative or non-conservative amino acid substitutions, deletions or additions.

[0076] The term "VWF fragment" or "VWF fragments" used herein means any VWF fragments that interact with FVIII and retain at least one or more properties that are normally provided to FVIII by full-length VWF, *e.g.,* preventing

premature activation to FVIIIa, preventing premature proteolysis, preventing association with phospholipid membranes that could lead to premature clearance, preventing binding to FVIII clearance receptors that can bind naked FVIII but not VWF-bound FVIII, and/or stabilizing the FVIII heavy chain and light chain interactions.

[0077] A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, if an amino acid in a polypeptide is replaced with another amino acid from the same side chain family, the substitution is considered to be conservative. In another embodiment, a string of amino acids can be conservatively replaced with a structurally similar string that differs in order and/or composition of side chain family members.

[0078] As known in the art, "sequence identity" between two polypeptides is determined by comparing the amino acid sequence of one polypeptide to the sequence of a second polypeptide. When discussed herein, whether any particular polypeptide is at least about 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% identical to another polypeptide can be determined using methods and computer programs/software known in the art such as, but not limited to, the BESTFIT program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). BESTFIT uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981), to find the best segment of homology between two sequences. When using BESTFIT or any other sequence alignment program to determine whether a particular sequence is, for example, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is

calculated over the full-length of the reference polypeptide sequence and that gaps in homology of up to 5% of the total number of amino acids in the reference sequence are allowed.

[0079] As used herein, an "amino acid corresponding to" or an "equivalent amino acid" in a VWF sequence or a FVIII protein sequence is identified by alignment to maximize the identity or similarity between a first VWF or FVIII sequence and a second VWF or FVIII sequence. The number used to identify an equivalent amino acid in a second VWF or FVIII sequence is based on the number used to identify the corresponding amino acid in the first VWF or FVIII sequence.

[0080] As used herein, the term "insertion site" refers to a position in a FVIII polypeptide, or fragment, variant, or derivative thereof, which is immediately upstream of the position at which a heterologous moiety can be inserted. An "insertion site" is specified as a number, the number being the number of the amino acid in mature native FVIII (SEQ ID NO:4) to which the insertion site corresponds, which is immediately N-terminal to the position of the insertion. For example, the phrase "a3 comprises an XTEN at an insertion site which corresponds to amino acid 1656 of SEQ ID NO: 4" indicates that the heterologous moiety is located between two amino acids corresponding to amino acid 1656 and amino acid 1657 of SEQ ID NO: 4.

[0081] The phrase "immediately downstream of an amino acid" as used herein refers to position right next to the terminal carboxyl group of the amino acid. Similarly, the phrase "immediately upstream of an amino acid" refers to the position right next to the terminal amine group of the amino acid. Therefore, the phrase "between two amino acids of an insertion site" as used herein refers to a position in which an XTEN or any other polypeptide is inserted between two adjacent amino acids. Thus, the phrases "inserted immediately downstream of an amino acid" and "inserted between two amino acids of an insertion site" are used synonymously with "inserted at an insertion site."

[0082] The terms "inserted," "is inserted," "inserted into" or grammatically related terms, as used herein refers to the position of an XTEN in a chimeric polypeptide relative to the analogous position in native mature human FVIII. As used herein the terms refer to the characteristics of the recombinant FVIII polypeptide relative to native mature human FVIII, and do not indicate, imply or infer any methods or

process by which the chimeric polypeptide was made. For example, in reference to a chimeric polypeptide provided herein, the phrase "an XTEN is inserted into immediately downstream of residue 745 of the FVIII polypeptide" means that the chimeric polypeptide comprises an XTEN immediately downstream of an amino acid which corresponds to amino acid 745 in native mature human FVIII, *e.g.,* bounded by amino acids corresponding to amino acids 745 and 746 of native mature human FVIII.

[0083] A "fusion" or "chimeric" protein comprises a first amino acid sequence linked to a second amino acid sequence with which it is not naturally linked in nature. The amino acid sequences which normally exist in separate proteins can be brought together in the fusion polypeptide, or the amino acid sequences which normally exist in the same protein can be placed in a new arrangement in the fusion polypeptide, *e.g.*, fusion of a Factor VIII domain of the invention with an Ig Fc domain. A fusion protein is created, for example, by chemical synthesis, or by creating and translating a polynucleotide in which the peptide regions are encoded in the desired relationship. A chimeric protein can further comprises a second amino acid sequence associated with the first amino acid sequence by a covalent, non-peptide bond or a non-covalent bond.

[0084] As used herein, the term "half-life" refers to a biological half-life of a particular polypeptide *in vivo*. Half-life may be represented by the time required for half the quantity administered to a subject to be cleared from the circulation and/or other tissues in the animal. When a clearance curve of a given polypeptide is constructed as a function of time, the curve is usually biphasic with a rapid α-phase and longer β-phase. The α-phase typically represents an equilibration of the administered Fc polypeptide between the intra- and extra-vascular space and is, in part, determined by the size of the polypeptide. The β-phase typically represents the catabolism of the polypeptide in the intravascular space. In some embodiments, FVIII and chimeric proteins comprising FVIII are monophasic, and thus do not have an alpha phase, but just the single beta phase. Therefore, in certain embodiments, the term half-life as used herein refers to the half-life of the polypeptide in the β-phase. The typical β-phase half-life of a human antibody in humans is 21 days.

[0085] The term "linked" as used herein refers to a first amino acid sequence or nucleotide sequence covalently or non-covalently joined to a second amino acid sequence or nucleotide sequence, respectively. The first amino acid or nucleotide sequence can be directly joined or juxtaposed to the second amino acid or nucleotide sequence or alternatively an intervening sequence can covalently join the first sequence to the second sequence. The term "linked" means not only a fusion of a first amino acid sequence to a second amino acid sequence at the C-terminus or the N-terminus, but also includes insertion of the whole first amino acid sequence (or the second amino acid sequence) into any two amino acids in the second amino acid sequence (or the first amino acid sequence, respectively). In one embodiment, the first amino acid sequence can be linked to a second amino acid sequence by a peptide bond or a linker. The first nucleotide sequence can be linked to a second nucleotide sequence by a phosphodiester bond or a linker. The linker can be a peptide or a polypeptide (for polypeptide chains) or a nucleotide or a nucleotide chain (for nucleotide chains) or any chemical moiety (for both polypeptide and polynucleotide chains). The term "linked" is also indicated by a hyphen (-).

[0086] As used herein the term "associated with" refers to a covalent or non-covalent bond formed between a first amino acid chain and a second amino acid chain. In one embodiment, the term "associated with" means a covalent, non-peptide bond or a non-covalent bond. This association can be indicated by a colon, *i.e.,* (:). In another embodiment, it means a covalent bond except a peptide bond. For example, the amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a thiol group on a second cysteine residue. In most naturally occurring IgG molecules, the CH1 and CL regions are associated by a disulfide bond and the two heavy chains are associated by two disulfide bonds at positions corresponding to 239 and 242 using the Kabat numbering system (position 226 or 229, EU numbering system). Examples of covalent bonds include, but are not limited to, a peptide bond, a metal bond, a hydrogen bond, a disulfide bond, a sigma bond, a pi bond, a delta bond, a glycosidic bond, an agnostic bond, a bent bond, a dipolar bond, a Pi backbond, a double bond, a triple bond, a quadruple bond, a quintuple bond, a sextuple bond, conjugation, hyperconjugation, aromaticity, hapticity, or antibonding. Non-limiting examples

of non-covalent bond include an ionic bond (*e.g.,* cation-pi bond or salt bond), a metal bond, an hydrogen bond (*e.g.,* dihydrogen bond, dihydrogen complex, low-barrier hydrogen bond, or symmetric hydrogen bond), van der Walls force, London dispersion force, a mechanical bond, a halogen bond, aurophilicity, intercalation, stacking, entropic force, or chemical polarity.

[0087] The term "monomer-dimer hybrid" used herein refers to a chimeric protein comprising a first polypeptide chain and a second polypeptide chain, which are associated with each other by a disulfide bond, wherein the first chain comprises a clotting factor, *e.g.,* Factor VIII, and a first Fc region and the second chain comprises, consists essentially of, or consists of a second Fc region without the clotting factor. The monomer-dimer hybrid construct thus is a hybrid comprising a monomer aspect having only one clotting factor and a dimer aspect having two Fc regions.

[0088] As used herein, the term "cleavage site" or "enzymatic cleavage site" refers to a site recognized by an enzyme. Certain enzymatic cleavage sites comprise an intracellular processing site. In one embodiment, a polypeptide has an enzymatic cleavage site cleaved by an enzyme that is activated during the clotting cascade, such that cleavage of such sites occurs at the site of clot formation. Exemplary such sites include, *e.g.,* those recognized by thrombin, Factor XIa or Factor Xa. Exemplary FXIa cleavage sites include, *e.g.,* TQSFNDFTR (SEQ ID NO: 45) and SVSQTSKLTR (SEQ ID NO: 46). Exemplary thrombin cleavage sites include, *e.g.,* DFLAEGGGVR (SEQ ID NO: 47), TTKIKPR (SEQ ID NO: 48), LVPRG (SEQ ID NO: 49) and ALRPR (amino acids 1 to 5 of SEQ ID NO: 50). Other enzymatic cleavage sites are known in the art.

[0089] As used herein, the term "processing site" or "intracellular processing site" refers to a type of enzymatic cleavage site in a polypeptide which is a target for enzymes that function after translation of the polypeptide. In one embodiment, such enzymes function during transport from the Golgi lumen to the trans-Golgi compartment. Intracellular processing enzymes cleave polypeptides prior to secretion of the protein from the cell. Examples of such processing sites include, *e.g.,* those targeted by the PACE/furin (where PACE is an acronym for Paired basic Amino acid Cleaving Enzyme) family of endopeptidases. These enzymes are localized to the Golgi membrane and cleave proteins on the carboxyterminal

side of the sequence motif Arg-[any residue]-(Lys or Arg)-Arg. As used herein the "furin" family of enzymes includes, *e.g.,* PCSK1 (also known as PC1/Pc3), PCSK2 (also known as PC2), PCSK3 (also known as furin or PACE), PCSK4 (also known as PC4), PCSK5 (also known as PC5 or PC6), PCSK6 (also known as PACE4), or PCSK7 (also known as PC7/LPC, PC8, or SPC7). Other processing sites are known in the art.

[0090] In constructs that include more than one processing or cleavage site, it will be understood that such sites may be the same or different.

[0091] The term "Furin" refers to the enzymes corresponding to EC No. 3.4.21.75. Furin is subtilisin-like proprotein convertase, which is also known as PACE (Paired basic Amino acid Cleaving Enzyme). Furin deletes sections of inactive precursor proteins to convert them into biologically active proteins. During its intracellular transport, pro-peptide of VWF can be cleaved from mature VWF molecule by a Furin enzyme. In some embodiments, Furin cleaves the D1D2 from the D'D3 of VWF. In other embodiments, a nucleotide sequence encoding Furin can be expressed together with the nucleotide sequence encoding a VWF fragment so that D1D2 domains can be cleaved off intracellularly by Furin.

[0092] In constructs that include more than one processing or cleavage site, it will be understood that such sites may be the same or different.

[0093] A "processable linker" as used herein refers to a linker comprising at least one intracellular processing site, which is described elsewhere herein.

[0094] Hemostatic disorder, as used herein, means a genetically inherited or acquired condition characterized by a tendency to hemorrhage, either spontaneously or as a result of trauma, due to an impaired ability or inability to form a fibrin clot. Examples of such disorders include the hemophilias. The three main forms are hemophilia A (factor VIII deficiency), hemophilia B (factor IX deficiency or "Christmas disease") and hemophilia C (factor XI deficiency, mild bleeding tendency). Other hemostatic disorders include, *e.g.,* Von Willebrand disease, Factor XI deficiency (PTA deficiency), Factor XII deficiency, deficiencies or structural abnormalities in fibrinogen, prothrombin, Factor V, Factor VII, Factor X or factor XIII, Bernard-Soulier syndrome, which is a defect or deficiency in GPIb. GPIb, the receptor for VWF, can be defective and lead to lack of primary clot formation (primary hemostasis) and increased bleeding

tendency), and thrombasthenia of Glanzman and Naegeli (Glanzmann thrombasthenia). In liver failure (acute and chronic forms), there is insufficient production of coagulation factors by the liver; this may increase bleeding risk.

[0095] The chimeric molecules of the invention can be used prophylactically. As used herein the term "prophylactic treatment" refers to the administration of a molecule prior to a bleeding episode. In one embodiment, the subject in need of a general hemostatic agent is undergoing, or is about to undergo, surgery. The chimeric protein of the invention can be administered prior to or after surgery as a prophylactic. The chimeric protein of the invention can be administered during or after surgery to control an acute bleeding episode. The surgery can include, but is not limited to, liver transplantation, liver resection, dental procedures, or stem cell transplantation.

[0096] The chimeric protein of the invention is also used for on-demand treatment. The term "on-demand treatment" refers to the administration of a chimeric molecule in response to symptoms of a bleeding episode or before an activity that may cause bleeding. In one aspect, the on-demand treatment can be given to a subject when bleeding starts, such as after an injury, or when bleeding is expected, such as before surgery. In another aspect, the on-demand treatment can be given prior to activities that increase the risk of bleeding, such as contact sports.

[0097] As used herein the term "acute bleeding" refers to a bleeding episode regardless of the underlying cause. For example, a subject may have trauma, uremia, a hereditary bleeding disorder (*e.g.,* factor VII deficiency) a platelet disorder, or resistance owing to the development of antibodies to clotting factors.

[0098] Treat, treatment, treating, as used herein refers to, *e.g.,* the reduction in severity of a disease or condition; the reduction in the duration of a disease course; the amelioration of one or more symptoms associated with a disease or condition; the provision of beneficial effects to a subject with a disease or condition, without necessarily curing the disease or condition, or the prophylaxis of one or more symptoms associated with a disease or condition. In one embodiment, the term "treating" or "treatment" means maintaining a FVIII trough level at least about 1 IU/dL, 2 IU/dL, 3 IU/dL, 4 IU/dL, 5 IU/dL, 6 IU/dL, 7 IU/dL, 8 IU/dL, 9 IU/dL, 10 IU/dL, 11 IU/dL, 12 IU/dL, 13 IU/dL, 14 IU/dL, 15 IU/dL, 16 IU/dL, 17

IU/dL, 18 IU/dL, 19 IU/dL, or 20 IU/dL in a subject by administering a chimeric protein or a VWF fragment of the invention. In another embodiment, treating or treatment means maintaining a FVIII trough level between about 1 and about 20 IU/dL, about 2 and about 20 IU/dL, about 3 and about 20 IU/dL, about 4 and about 20 IU/dL, about 5 and about 20 IU/dL, about 6 and about 20 IU/dL, about 7 and about 20 IU/dL, about 8 and about 20 IU/dL, about 9 and about 20 IU/dL, or about 10 and about 20 IU/dL. Treatment or treating of a disease or condition can also include maintaining FVIII activity in a subject at a level comparable to at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20% of the FVIII activity in a non-hemophiliac subject. The minimum trough level required for treatment can be measured by one or more known methods and can be adjusted (increased or decreased) for each person.

CHIMERIC PROTEINS

[0099] The present invention is directed to extending the half-life of a Factor VIII protein using a VWF fragment and an XTEN sequence by preventing or inhibiting a FVIII half-life limiting factor, *i.e.,* endogenous VWF, from associating with the FVIII protein. Endogenous VWF associates with about 95% to about 98% of FVIII in non-covalent complexes. While endogenous VWF is a FVIII half-life limiting factor, endogenous VWF bound to a FVIII protein is also known to protect FVIII in various ways. For example, full length VWF (as a multimer having about 250 kDa) can protect FVIII from protease cleavage and FVIII activation, stabilize the FVIII heavy chain and/or light chain, and prevent clearance of FVIII by scavenger receptors. But, at the same time, endogenous VWF limits the FVIII half-life by preventing pinocytosis and by clearing FVIII-VWF complex from the system through the VWF clearance pathway. It is believed, while not bound by a theory, that endogenous VWF is a half-life limiting factor that prevents the half-life of a FVIII protein fused to a half-life extender from being longer than about two-fold that of wild-type FVIII. Therefore, the present invention is directed to preventing or inhibiting interaction between endogenous VWF and a FVIII protein using a VWF fragment, thereby increasing a half-life of the FVIII protein by using an XTEN sequence alone or an XTEN sequence in combination with an Ig constant region or a portion thereof. The

XTEN sequence can be linked to the FVIII protein or the VWF fragment. The FVIII protein associated with the VWF fragment is thus cleared from the circulation more slowly by one or more VWF clearance receptors and then can have the full half-life extension of the XTEN sequence or the XTEN sequence in combination of the Ig constant region, as compared to wild type FVIII or a FVIII protein without the VWF fragment.

[0100] In one embodiment, a VWF fragment is associated (or linked) with the FVIII protein by a covalent or a non-covalent bond. In some instances, however, the physical blockage or chemical association (*e.g.,* non-covalent bonding) between the VWF fragment and the FVIII protein may not be strong enough to provide a stable complex comprising the FVIII protein and the VWF fragment in the presence of endogenous VWF. For example, a VWF fragment forming a non-covalent bond with a FVIII protein without any other connections may readily be dissociated *in vivo* from the FVIII protein in the presence of endogenous VWF, replacing the VWF fragment (*e.g.,* recombinant VWF, *i.e.,* rVWF) with endogenous VWF. Therefore, the FVIII protein non-covalently bound to endogenous VWF would undergo the VWF clearance pathway and be readily cleared from the system. In order to prevent the dissociation of the VWF fragment with the FVIII protein, in some embodiments, the association or linkage between the FVIII protein and the VWF fragment is a covalent bond, *e.g.,* a peptide bond, one or more amino acids, or a disulfide bond. In certain embodiments, the association (*i.e.,* linkage) between the adjunct moiety and the FVIII protein is a peptide bond or a linker between the FVIII protein and the VWF fragment ("FVIII/VWF linker"). Non-limiting examples of the linker are described elsewhere herein. In some embodiments, the VWF fragment is a polypeptide comprising, consisting essentially of, or consisting of at least about 10, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2500, 3000, or 4000 amino acids. Non-limiting examples of the VWF fragment are described elsewhere herein.

[0101] In certain embodiments, the VWF fragment chemically (*e.g.,* non-covalently) binds to or physically blocks one or more VWF binding sites on a FVIII protein. The VWF binding site on a FVIII protein is located within the A3 domain or the C2 domain of the FVIII protein. In still other embodiments, the

VWF binding site on a FVIII protein is located within the A3 domain and C2 domain. For example, the VWF binding site on a FVIII protein can correspond to amino acids 1669 to 1689 and/or 2303 to 2332 of SEQ ID NO: 4 [full-length mature FVIII].

[0102] The invention also provides a chimeric protein (comprising a FVIII protein and a VWF fragment) further comprising one or more XTEN sequences, which provide additional half-life extension properties. The one or more XTEN sequences can be inserted within the FVIII protein or the VWF fragment or linked to the N-terminus or the C-terminus of the FVIII protein or the VWF fragment. The invention also includes a FVIII protein linked to an XTEN sequence (a first half-life extending moiety) and an Ig constant region or a portion thereof (a second half-life extending moiety) so that the two half-life extending moieties extend the half-life of the FVIII protein through two different mechanisms.

[0103] In some embodiments, a chimeric protein comprises a FVIII protein linked to a first Ig constant region or a portion thereof (*e.g.,* a first FcRn binding partner), a VWF fragment linked to a second Ig constant region or a portion thereof (*e.g.,* a second FcRn binding partner), and one or more XTEN sequences inserted or linked to the FVIII protein or the VWF fragment, wherein the VWF fragment prevents the FVIII half-life limiting factor (*e.g.,* endogenous VWF) from binding to the FVIII protein, wherein the first and second Ig constant regions or portions thereof forms a covalent bond, *e.g.,* a disulfide bond, and the one or more XTEN sequences extends the half-life of the FVIII protein.

[0104] In certain embodiments, a chimeric protein of the invention comprises a FVIII protein linked to a VWF fragment by an optional linker (*i.e.,* FVIII/VWF linker) and one or more XTEN sequences inserted or linked to the FVIII protein or the VWF fragment, wherein the VWF fragment prevents the FVIII half-life limiting factor (*e.g.,* endogenous VWF) from binding to the FVIII protein and the one or more XTEN sequences extends the half-life of the FVIII protein. In one aspect, the optional linker (FVIII/VWF linker) comprises a sortase recognition motif. In another aspect, the optional linker (FVIII/VWF linker) comprises a cleavable site. Examples of the cleavage linker (*i.e.,* linker containing one or more cleavage site) are described elsewhere herein.

[0105] The chimeric protein of the present invention includes, but is not limited to:

(1) a VWF fragment comprising a D' domain and a D3 domain, an XTEN sequence, and FVIII, wherein the XTEN sequence is linked to the VWF fragment;

(2) a FVIII protein, an XTEN sequence, and an Ig constant region or a portion thereof, wherein the FVIII protein is linked to an XTEN sequence and the Ig constant region or a portion thereof, or

(3) a FVIII protein, an XTEN sequence, and a VWF fragment, wherein the XTEN sequence is linked to the FVIII protein at the C-terminus or N-terminus or inserted immediately downstream of one or more amino acids (*e.g.,* one or more XTEN insertion sites) of FVIII, and the VWF fragment and the FVIII protein are associated with each other.

(1) Von Willebrand Factor (VWF) fragment linked to XTEN, and FVIII

[0106] The present invention is directed to a chimeric protein comprising (i) a VWF fragment comprising a D' domain and a D3 domain of VWF, (ii) an XTEN sequence, and (iii) a FVIII protein, wherein (i), (ii), and (iii) are linked to or associated with each other. The VWF fragment linked to the XTEN sequence, as a part of a chimeric protein in the present invention, associates with the FVIII protein, thus preventing or inhibiting interaction between endogenous VWF and the FVIII protein. In certain embodiments, the VWF fragment, which is capable of preventing or inhibiting binding of the FVIII protein with endogenous VWF, can at the same time have at least one VWF-like FVIII protecting property. Examples of the VWF-like FVIII protecting properties include, but are not limited to, protecting FVIII from protease cleavage and FVIII activation, stabilizing the FVIII heavy chain and/or light chain, and preventing clearance of FVIII by scavenger receptors. As a result, the VWF fragment can prevent clearance of the FVIII protein through the VWF clearance pathway, thus reducing clearance of FVIII from the circulatory system. In some embodiments, the VWF fragments of the present invention bind to or are associated with a FVIII protein and/or physically or chemically block the VWF binding site on the FVIII protein. The FVIII protein associated with the VWF fragment is thus cleared from the circulation more slowly, as compared to wild type FVIII or FVIII not associated with the VWF fragment.

[0107] In one embodiment, the invention is directed to a chimeric protein comprising (i) a VWF fragment comprising the D' domain and the D3 domain of VWF, (ii) an XTEN sequence, and (iii) a FVIII protein, wherein the XTEN sequence is linked to the VWF fragment (*e.g.,* (a1) V-X or (a2) X-V, wherein V comprises a VWF fragment and X comprises an XTEN sequence), and the VWF fragment is linked to or associated with the FVIII protein. In another embodiment, the VWF fragment and the XTEN sequence can be linked by a linker (*e.g.,* (a3) V-L-X or (a4) X-L-V) or a peptide bond. The linker can be a cleavable linker, *e.g.,* a thrombin cleavable linker, which can be cleaved at the site of coagulation. In other embodiments, the VWF fragment, the XTEN sequence, and the FVIII protein are placed in a single polypeptide chain. In still other embodiments, the chimeric protein comprises two polypeptide chains, a first chain comprising the VWF fragment and the XTEN sequence and a second chain comprising the FVIII protein. In yet other embodiments, the chimeric protein comprises three polypeptide chains, a first chain comprising the VWF fragment and the XTEN sequence, a second chain comprising a light chain of FVIII and a third chain comprising a heavy chain of FVIII, wherein the first chain and the second chain are associated with each other (*e.g.,* covalent bond, *e.g.*, disulfide bond), and the second chain and the third chain are associated with each other (*e.g.*, metal bond). In still other embodiments, the XTEN sequence can be linked to the N-terminus or the C-terminus of the VWF fragment or inserted immediately downstream of one or more amino acids in the VWF fragment.

[0108] In certain embodiments, a chimeric protein of the invention comprises a formula comprising:

(a) V-X-FVIII,

(b) FVIII-X-V,

(c) V-X:FVIII,

(d) X-V:FVIII,

(e) FVIII:V-X,

(f) FVIII:X-V, or

(a5) X-V-FVIII,

wherein V comprises a VWF fragment,

X comprises one or more XTEN sequences,

FVIII comprises a FVIII protein;

(-) represents a peptide bond or one or more amino acids; and

(:) is a chemical association or a physical association. In one embodiment, (:) represents a chemical association, *e.g.*, at least one non-peptide bond. In another embodiment, the chemical association, *i.e.,* (:) is a covalent bond. In other embodiments, the chemical association, *i.e.,* (:) is a non-covalent interaction, *e.g.,* an ionic interaction, a hydrophobic interaction, a hydrophilic interaction, a Van der Waals interaction, or a hydrogen bond. In other embodiments, (:) is a non-peptide covalent bond. In still other embodiments, (:) is a peptide bond. In yet other embodiments, (:) represents a physical association between two sequences, wherein a portion of a first sequence is in close proximity to a second sequence such that the first sequence shields or blocks a portion of the second sequence from interacting with another moiety, and further that this physical association is maintained without allowing the second sequence to interact with other moieties. The orientation of the polypeptide formulas herein is listed from N-terminus (left) to C-terminus (right). For example, formula V-X-FVIII means formula NH2-V-X-FVIII -COOH. In one embodiment, the formulas described herein can comprise any additional sequences between the two moieties. For example, formula V-X-FVIII can further comprise any sequences at the N-terminus of V between V and X, between X and FVIII, or at the C-terminus of FVIII unless otherwise specified. In another embodiment, the hyphen (-) indicates a peptide bond.

[0109] In other embodiments, a chimeric protein of the invention comprises a formula comprising:

(a) V(X1)-X2-FVIII,

(b) FVIII-X2-V(X1),

(c) V(X1):FVIII,

(d) FVIII:V(X1), or

(a5) X2-V(X1)-FVIII,

wherein V(X1) comprises a VWF fragment and a first XTEN sequence (X1), wherein the XTEN sequence is inserted immediately downstream of one or more amino acids in the VWF fragment,

X2 comprises one or more optional XTEN sequences,

FVIII comprises a FVIII protein;

(-) is a peptide bond or one or more amino acids; and

(:) is a chemical association or a physical association.

[0110] In some embodiments, a chimeric protein comprises (i) a VWF fragment comprising a D' domain and a D3 domain of VWF, (ii) an XTEN sequence, (iii) a FVIII protein, (iv) a first optional linker, and (v) a second optional linker, wherein the XTEN sequence is linked to the VWF fragment and/or to the FVIII protein by the linker. In certain embodiments, a chimeric protein comprises a formula comprising:

(b1) V-L1-X-L2-FVIII,

(b2) FVIII-L2-X-L1-V,

(b3) V-L1-X:FVIII,

(b4) X-L1-V:FVIII,

(b5) FVIII:V-L1-X,

(b6) FVIII:X-L1-V,

(b7) X-L1-V-L2-FVIII, or

(b8) FVIII-L2-V-L1-X,

wherein V comprises a VWF fragment,

X comprises one or more XTEN sequences,

FVIII comprises a FVIII protein,

L1 comprises a first optional linker, *e.g.,* a first cleavable linker,

L2 comprises a second optional linker, *e.g.,* a second cleavable linker or an optional processable linker;

(-) is a peptide bond or one or amino acids; and

(:) is a chemical association or a physical association. In one embodiment, (:) represents a chemical association, *e.g.*, at least one non-peptide bond. In another embodiment, the chemical association, *i.e.,* (:) is a covalent bond. In other embodiments, the chemical association, *i.e.,* (:) is a non-covalent interaction, *e.g.,* an ionic interaction, a hydrophobic interaction, a hydrophilic interaction, a Van der Waals interaction, or a hydrogen bond. In other embodiments, (:) is a non-peptide covalent bond. In still other embodiments, (:) is a peptide bond. In yet other embodiments, (:) represents a physical association between two sequences, wherein a portion of a first sequence is in close proximity to a second sequence such that the first sequence shields or blocks a portion of the second sequence

from interacting with another moiety, and further that this physical association is maintained without allowing the second sequence to interact with other moieties. The orientation of the polypeptide formulas herein is listed from N-terminus (left) to C-terminus (right). For example, formula (b1) V-L1-X-L2-FVIII means formula NH2-V-L1-X-L2-FVIII-COOH. In one embodiment, the formulas described herein can comprise any additional sequences between the two moieties. In another embodiment, the hyphen (-) indicates a peptide bond.

[0111] Another aspect of the present invention is to provide a FVIII chimeric protein having reduced or no interactions with a FVIII half-life limiting factor, *e.g.,* endogenous VWF, and at the same time maximizing the half-life of the FVIII protein using an XTEN sequence (a first half-life extender) in combination with a second half-life extender or a moiety providing a covalent bond between the FVIII protein and the VWF fragment, *e.g.,* an Ig constant region or a portion thereof. In one embodiment, a chimeric protein of the invention comprises (i) a VWF fragment comprising a D' domain and a D3 domain of VWF, (ii) an XTEN sequence, (iii) a FVIII protein, and (iv) an Ig constant region or a portion thereof (also referred to herein as F), wherein (1) the VWF fragment is linked to the XTEN sequence by an optional linker, *e.g.,* a cleavable linker, (2) the VWF fragment is associated with or linked to the FVIII protein by an additional optional linker, *e.g.,* a cleavable linker, and (3) the Ig constant region or a portion thereof is linked to the VWF fragment, the XTEN sequence, or the FVIII protein. In another embodiment, a chimeric protein of the invention comprises (i) a VWF fragment comprising a D' domain and a D3 domain of VWF, (ii) an XTEN sequence, (iii) a FVIII protein, (iv) an Ig constant region or a portion thereof (F1 or a first Ig constant region or a portion thereof), and (v) an additional Ig constant region or a portion thereof (F2 or a second Ig constant region or a portion thereof), wherein (1) the VWF fragment is linked to the XTEN sequence by an optional linker, *e.g.,* a cleavable linker, (2) the XTEN sequence or the VWF fragment is linked to the Ig constant region or a portion thereof, (3) the FVIII is linked to the additional Ig constant region or a portion thereof, and (4) the Ig constant region or a portion thereof is associated with or linked to the additional Ig constant region or a portion thereof. In one embodiment, the association or linkage between the two Ig constant regions or a portion thereof is a covalent bond, *e.g.,* a disulfide bond. In

another embodiment, the association or linkage between the two Ig constant regions or a portion thereof is a processable linker, wherein the processable linker is intracellularly processed by a protease. For example, the chimeric protein comprises a formula comprising:

(g) V-L2-X-L1-F1: FVIII-L3-F2;

(h) V-L2-X-L1-F1:F2-L3-FVIII;

(i) F-L1-X-L2-V: FVIII-L3-F2;

(j) F-L1-X-L2-V:F2-L3-FVIII;

(k) V-L2-X-L1-F1-L4-FVIII-L3-F2;

(l) F2-L3-FVIII-L4-F1-L1-X-L2-V;

(m) FVIII-L2-F2-L4-V-L2-X-L1-F1; or

(n) F1-L1-X-L2-V-L4-F2-L2-FVIII,

wherein V comprises a VWF fragment,

each of L1 and L3 comprises an optional linker,

L2 comprises an optional linker, *e.g.,* a cleavable linker,

L4 is an optional linker, *e.g.,* a processable linker,

FVIII comprises a FVIII protein,

X comprises one or more XTEN sequences,

F1 comprises an optional Ig constant region or a portion thereof,

F2 comprises an optional additional Ig constant region or a portion thereof;

(-) is a peptide bond or one or more amino acids; and

(:) is a chemical association or a physical association.

[0112] In some embodiments, the FVIII protein in any constructs or formulas disclosed herein can further comprises at least one, at least two, at least three, at least four, at least five, or at least six XTEN sequences, each of the XTEN sequences inserted immediately downstream of one or more amino acids in the FVIII protein or linked to the N-terminus or the C-terminus of the FVIII protein. Non-limiting examples of the XTEN insertion sites are disclosed elsewhere herein.

[0113] In one embodiment, (:) represents a chemical association, *e.g.,* at least one non-peptide bond. In another embodiment, the chemical association, *i.e.,* (:) is a covalent bond. In other embodiments, the chemical association, *i.e.,* (:) is a non-covalent interaction, *e.g.,* an ionic interaction, a hydrophobic interaction, a

hydrophilic interaction, a Van der Waals interaction, or a hydrogen bond. In other embodiments, (:) is a non-peptide covalent bond. In still other embodiments, (:) is a peptide bond. In yet other embodiments, (:) represents a physical association between two sequences, wherein a portion of a first sequence is in close proximity to a second sequence such that the first sequence shields or blocks a portion of the second sequence from interacting with another moiety, and further that this physical association is maintained without allowing the second sequence to interact with other moieties. The orientation of the polypeptide formulas herein is listed from N-terminus (left) to C-terminus (right). For example, formula (n) F1-L1-X-L2-V-L4-F2-L2-FVIII means formula NH2-F1-L1-X-L2-V-L4-F2-L2-FVIII -COOH. In one embodiment, the formulas described herein can comprise any additional sequences between the two moieties. In another embodiment, the hyphen (-) indicates a peptide bond.

[0114] In one embodiment, either or both of the Ig constant region or a portion thereof (sometimes indicated herein by "F" or "F1") and the additional Ig constant region or a portion thereof (sometimes indicated herein by "F2") linked to the VWF fragment or the FVIII protein can extend the half-life of the VWF fragment, the FVIII protein, or both. In another embodiment, a pair of the Ig constant region or a portion thereof (sometimes indicated herein by "F" or "F1") and the additional Ig constant region or a portion thereof (sometimes indicated herein by "F2"), each of which are linked to the VWF fragment and the FVIII protein, provides a bond stronger than the non-covalent bond between the FVIII protein and the VWF fragment, *i.e.,* a covalent bond, *e.g.,* a disulfide bond, thereby preventing endogenous VWF from replacing the VWF fragment *in vivo*. F1 or F2 can comprise an Fc region or an FcRn binding partner. In other embodiments, either or both of F1 and F2 linked to the VWF fragment and/or the FVIII protein form a covalent bond (*e.g.,* a disulfide bond) between F1 and F2, thereby placing the VWF fragment and the FVIII protein in close proximity to prevent interaction of the FVIII protein with the VWF fragment. In some embodiments, F1 and F2 are identical or different. Non-limiting examples of F1 and F2 can be selected from the group consisting of a CH1 domain, a CH2 domain, a CH3 domain, a CH4 domain, a hinge domain, any functional fragments, derivatives, or analogs thereof, and two or more combinations thereof. In one embodiment, F1, F2, or both

comprise at least one CH1 domain, at least one CH2 domain, at least one CH3 domain, at least one CH4 domain, or the functional fragments, derivatives, or analogs thereof. In another embodiment, F1, F2, or both comprise at least one hinge domain or portion thereof and at least one CH2 domain or portion thereof (*e.g.,* in the hinge-CH2 orientation). In other embodiments, F1, F2, or both comprise at least one CH2 domain or portion thereof and at least one CH3 domain or portion thereof (*e.g.,* in the CH2-CH3 orientation.) Examples of the combination include, but are not limited to, a CH2 domain, a CH3 domain, and a hinge domain, which are also known as an Fc region (or Fc domain), *e.g.,* a first Fc region or a first FcRn binding partner for F1 and a second Fc region or a second FcRn binding partner for F2. In other embodiments, F1 is linked to the VWF fragment by a linker, and/or F2 is linked to the FVIII protein by a linker. In some embodiments, F1 and/or F2 comprises, consisting essentially of, or consisting of a hinge region. Additional non-limiting examples of the Fc regions or the FcRn binding partners are described elsewhere herein.

[0115] In certain embodiments, a chimeric protein of the invention comprises two polypeptide chains, a first polypeptide chain comprising, consisting essentially of, or consisting of a VWF fragment comprising a D' domain and a D3 domain, an XTEN sequence, a first Ig constant region or a portion thereof (*e.g.,* a first Fc region), and an optional linker between the VWF fragment and the XTEN sequence or the XTEN sequence or the first Ig constant region or a portion thereof and a second polypeptide chain comprising, consisting essentially of, or consisting of a FVIII protein and a second Ig constant region or a portion thereof (*e.g.,* a second Fc region). The linker between the VWF fragment and the first Ig constant region or a portion thereof can be a cleavable linker, *e.g.,* a thrombin cleavable linker, which can be cleaved at the site of coagulation. In some embodiments, the first polypeptide chain and the second polypeptide chain are associated with each other. The association between the first chain and the second chain prevents replacement of the first chain comprising the VWF fragment with endogenous VWF *in vivo*. In one embodiment, the association between the first chain and the second chain can be a covalent bond. In a particular embodiment, the covalent bond is a disulfide bond. In some embodiments, the FVIII protein in the second chain further comprises one or more XTEN sequences linked to the C-terminus or

N-terminus of the FVIII protein or inserted immediately downstream of one or more amino acids (*e.g.,* at least one insertion site disclosed herein) in the FVIII protein. Non-limiting examples of the insertion sites are described elsewhere herein.

[0116] In other embodiments, a chimeric protein of the invention comprises three polypeptide chains, wherein a first polypeptide chain comprises, consists essentially of, or consists of a heavy chain of a FVIII protein, a second polypeptide chain comprises, consists essentially of, or consists of a light chain of a FVIII protein fused to a first Ig constant region or a portion thereof (*e.g.,* a first Fc region), and a third polypeptide chain comprises, consists essentially of, or consists of a VWF fragment comprising a D' domain and a D3 domain, an XTEN sequence, a second Ig constant region or a portion thereof (*e.g.*, a second Fc region), and an optional linker between the XTEN sequence and the second Ig constant region or a portion thereof or the VWF fragment and the XTEN sequence. The linker in the third chain can be a cleavable linker, which is cleaved at the site of coagulation, *e.g.,* a thrombin cleavage site. In some embodiments, the heavy chain FVIII or the light chain FVIII is linked to one or more XTEN sequences, which can be linked to the N-terminus, the C-terminus, or inserted within one or more insertion sites within the FVIII sequence. Non-limiting examples of the insertion sites are disclosed elsewhere herein.

[0117] In yet other embodiments, a chimeric protein of the invention comprises two polypeptide chains, a first polypeptide chain comprising, consisting essentially of, or consisting of a heavy chain of a FVIII protein and a second polypeptide chain comprising, consisting essentially of, or consisting of a light chain of a FVIII protein, a first Ig constant region or a portion thereof (*e.g.,* a first Fc region), a first linker (*e.g.,* a processable linker, which contains one or more protease cleavage sites comprising one or more intracellular processing sites), a VWF fragment, a second linker (*e.g.,* a thrombin cleavable linker), an XTEN sequence, and a second Ig constant region or a portion thereof (*e.g.,* a second Fc region), wherein the light chain of the FVIII protein is linked to the first Ig constant region or a portion thereof (*e.g.,* the first Fc region), which is further linked to the VWF fragment by the first linker, and wherein the VWF fragment is linked to the XTEN sequence, which is further linked to the second Ig constant

region or a portion thereof by the second linker. In certain embodiments, the first linker is a processable linker, and the second linker is a cleavable linker. Upon expression, the chimeric protein can be processed by an intracellular processing enzyme, which cleaves the processable linker, and thus the chimeric protein can comprise, consists essentially of, or consists of three polypeptide chains. In addition, the VWF fragment can be cleaved off at the site of coagulation due to the cleavable linker.

[0118] In certain embodiments, a chimeric protein of the invention comprises one polypeptide chain, which comprises a single chain FVIII protein, a first Ig constant region or a portion thereof (*e.g.,* a first Fc region), a first linker (*e.g.,* a processable linker), a VWF fragment, an XTEN sequence, a second linker (*e.g.,* a thrombin cleavable linker), and a second Ig constant region or a portion thereof (*e.g.,* a second Fc region), wherein the single chain FVIII protein is linked to the first Ig constant region or a portion thereof, which is also linked to the VWF fragment by the first linker, and the VWF fragment is linked to the XTEN sequence, which is further linked to the second Ig constant region or a portion thereof. In one embodiment, the VWF fragment and the XTEN sequence are linked by the second linker. In another embodiment, the XTEN sequence and the second Ig constant region or a portion thereof are linked by the second linker. In other embodiments, the second chain further comprises a third linker. The single polypeptide chain can thus comprise the VWF fragment linked to the XTEN sequence by the second linker and the XTEN linked to the second Ig constant region or a portion thereof by the third linker. The second linker and the third linker can be identical or different. In one embodiment, the first linker is a processable linker. In another embodiment, the second linker or the third linker is a cleavable linker comprising one or two cleavable sites. In a specific embodiment, the second linker is a thrombin cleavable linker. The linkers useful in the invention are described elsewhere herein.

(2) FVIII, XTEN, and Fc

[0119] A chimeric protein of the invention also comprises (i) a FVIII protein, (ii) an XTEN sequence (a first half-life extender), and (iii) an Ig constant region or a portion thereof (a second half-life extender), in which the XTEN sequence is linked to the FVIII protein by an optional linker and the Ig constant region or a

portion thereof by an additional optional linker. The XTEN sequence and the Ig constant region or a portion thereof can be used together to extend half-life of the FVIII protein. In one embodiment, the chimeric protein is a monomer. In another embodiment, the chimeric protein is a dimer (a homodimer or a heterodimer).

[0120] The present invention is also directed to a chimeric protein comprising (i) a FVIII protein, (ii) an XTEN sequence, (iii) an Ig constant region or a portion thereof (*i.e.,* a first Ig constant region or a portion thereof, "F," or "F1"), and (iv) an additional Ig constant region or a portion thereof (*i.e.,* a second Ig constant region or a portion thereof or "F2"). In one embodiment, the XTEN sequence is linked to the FVIII protein at the C-terminus or the N-terminus or inserted immediately downstream of one or more amino acids in the FVIII protein (*e.g.,* one or more XTEN insertion sites), the FVIII protein is linked to the first Ig constant region or a portion thereof, and the first Ig constant region or a portion thereof and the second Ig constant region or a portion thereof are associated with or linked to each other by an optional linker. In certain aspects, the chimeric protein is a monomer-dimer hybrid, which comprises a first polypeptide chain and a second polypeptide chain, wherein the first polypeptide chain comprises a FVIII protein, an XTEN sequence, and a first Ig constant region or a portion thereof, and the second polypeptide chain comprises, consists essentially of, or consists of a second Ig constant region or a portion thereof without the FVIII protein and wherein the first chain and the second chain are associated with each other. The association between the Ig constant region or a portion thereof (*e.g.,* the first Fc region) and the additional Ig constant region or a portion thereof (*e.g.,* a second Fc region) is a chemical association or a physical association. In certain embodiments, the chemical association is a covalent bond. In other embodiments, the chemical association is a non-covalent interaction, *e.g.,* an ionic interaction, a hydrophobic interaction, a hydrophilic interaction, a Van der Waals interaction, or a hydrogen bond. In other embodiments, the association is a non-peptide covalent bond. In still other embodiments, the association is a peptide bond.

[0121] In other aspects, the chimeric protein is a single polypeptide chain comprising a FVIII protein, an XTEN sequence, a first Ig constant region or a portion thereof, a linker, *e.g.,* a processable linker, and a second Ig constant region

or a portion thereof, wherein the single polypeptide chain is processed after expression by an intracellular enzyme and becomes two polypeptide chains.

[0122] In one embodiment, the Ig constant region or a portion thereof (sometimes indicated herein by "F" or "F1") linked to the FVIII protein can extend the half-life of the FVIII protein together with the XTEN sequence. In another embodiment, the Ig constant region or a portion thereof ("F" or "F1") is an Fc region or an FcRn binding partner described elsewhere herein.

[0123] In other embodiments, the additional Ig constant region or a portion thereof (sometimes indicated herein by "F2" or a second Ig constant region or a portion thereof) associated with or linked to the first Ig constant region or a portion thereof can also extend the half-life of the FVIII protein. In other embodiments, the second Ig constant region or a portion thereof ("F2") together with the first Ig constant region or a portion thereof and the XTEN sequence can extend the half-life of the FVIII protein. The additional Ig constant region or a portion thereof can be an Fc region or an FcRn binding partner described elsewhere herein.

[0124] In certain embodiments, the second Ig constant region or a portion thereof associated with the first Ig constant region or a portion thereof is further linked to a VWF fragment described elsewhere herein and an optional XTEN sequence.

[0125] In some embodiments, either or both of the Ig constant region or a portion thereof ("F" or "F1" or a first Ig constant region or a portion thereof) and an additional Ig constant region or a portion thereof (*i.e.,* a second Ig constant region or a portion thereof or "F2") (indicated in this paragraph as "the Ig constant regions or portion thereof") can include, but not limited to, a CH1 domain, a CH2 domain, a CH3 domain, a CH4 domain, a hinge domain, any functional fragments, derivatives, or analogs thereof or two or more combinations thereof. In one embodiment, the Ig constant region or a portion thereof comprises at least one CH1 domain, at least one CH2 domain, at least one CH3 domain, at least one CH4 domain, or the functional fragments, derivatives, or analogues thereof. In another embodiment, the Ig constant region or a portion thereof comprises at least one hinge domain or portion thereof and at least one CH2 domain or portion thereof (*e.g.,* in the hinge-CH2 orientation). In other embodiments, the Ig constant domain or portion thereof comprises at least one CH2 domain or portion thereof

and at least one CH3 domain or portion thereof (*e.g.,* in the CF2-CH3 orientation). Examples of the combination include, but are not limited to, a CH2 domain, a CH3 domain, and a hinge domain, which are also known as an Fc region (or Fc domain), *e.g.,* first Fc region. Additional examples of the Ig constant regions or portion thereof are described elsewhere herein.

[0126] The chimeric protein of the invention can have an extended half-life of the FVIII protein compared to wild-type FVIII. In one embodiment, the half-life of the FVIII protein is extended at least about 1.5 times, at least about 2 times, at least about 2.5 times, at least about 3 times, at least about 4 times, at least about 5 times, at least about 6 times, at least about 7 times, at least about 8 times, at least about 9 times, at least about 10 times, at least about 11 times, or at least about 12 times longer than the half-life of wild type FVIII. In another embodiment, the half-life of the FVIII protein is at least about 10 hours, at least about 11 hours, at least about 12 hours, at least about 13 hours, at least about 14 hours, at least about 15 hours, at least about 16 hours, at least about 17 hours, at least about 18 hours, at least about 19 hours, at least about 20 hours, at least about 21 hours, at least about 22 hours, at least about 23 hours, at least about 24 hours, at least about 36 hours, at least about 48 hours, at least about 60 hours, at least about 72 hours, at least about 84 hours, at least about 96 hours, or at least about 108 hours.

(3) FVIII, XTEN, and VWF

[0127] In one aspect, a chimeric protein of the present invention comprises (i) a FVIII protein, (ii) an XTEN sequence, and (iii) a VWF fragment comprising a D' domain and a D3 domain of VWF, wherein the FVIII protein is linked to the XTEN sequence and wherein the FVIII protein is associated with or linked to the VWF fragment. In one embodiment, the VWF fragment of the chimeric protein described herein is not capable of binding to a VWF clearance receptor. In another embodiment, the VWF fragment is capable of protecting the FVIII protein from one or more protease cleavages, protecting the FVIII protein from activation, stabilizing the heavy chain and/or the light chain of the FVIII protein, or preventing clearance of the FVIII protein by one or more scavenger receptors. In other embodiments, the VWF fragment prevents or inhibits binding of endogenous VWF to the VWF binding site in the FVIII protein. The VWF binding site can be located in the A3 domain or the C2 domain of the FVIII protein or both the A3

domain and the C2 domain. In a specific embodiment, the VWF binding site comprises the amino acid sequence corresponding to amino acids 1669 to 1689 and/or amino acids 2303 to 2332 of SEQ ID NO: 2.

[0128] In another aspect, a chimeric protein comprises (i) a FVIII protein, (ii) an XTEN sequence, (iii) a VWF fragment, which comprises a D' domain and a D3 domain of VWF, and (iv) an Ig constant region or a portion thereof, wherein the XTEN sequence is linked to the FVIII protein at the C-terminus or the N-terminus or inserted immediately downstream of one or more amino acids (*e.g.,* one or more XTEN insertion sites disclosed herein) in the FVIII protein, the VWF fragment is linked to or associated with the FVIII protein or the XTEN sequence, and the Ig constant region or a portion thereof is linked to the FVIII protein, the XTEN sequence, the VWF fragment, or any combinations thereof. The Ig constant region or a portion thereof useful for chimeric proteins of the invention is described elsewhere herein. In one embodiment, the Ig constant region or a portion thereof is capable of extending the half-life of a FVIII protein. In another embodiment, the Ig constant region or a portion thereof comprises a first Fc region or a first FcRn binding partner. In yet other embodiments, the Ig constant region or a portion thereof is linked to the FVIII protein by an optional linker. In still other embodiments, the linker comprises a cleavable linker. The chimeric protein can be a single polypeptide chain, *i.e.,* a monomer (*i.e.,* a single chain), containing (i), (ii), (iii), and (iv) or two chains containing a first chain comprising (i) and (ii) and a second chain comprising (iii) and (iv). In other aspects, the chimeric protein is a dimer (*e.g.,* a homodimer or a heterodimer). In one embodiment, the chimeric protein comprises two chains, each comprising (i), (ii), (iii), and (iv).

[0129] In certain embodiments, a chimeric protein comprises (i) a FVIII protein, (ii) an XTEN sequence, (iii) a VWF fragment, which comprises a D' domain and a D3 domain of VWF, (iv) an Ig constant region or a portion thereof (sometimes also indicated as "F," "a first Ig constant region or a portion thereof", or "F2"), and (v) an additional Ig constant region or a portion thereof (sometimes also indicated as "F2" or "a second Ig constant region or a portion thereof"), wherein (1) the FVIII protein is linked to the XTEN sequence at the C-terminus or N-terminus of the FVIII protein or inserted immediately downstream of one or more amino acids (*e.g.,* one or more XTEN insertion sites disclosed herein) in the FVIII protein, (2)

either the XTEN sequence or the FVIII protein is linked to the Ig constant region or a portion thereof, (3) the VWF fragment is linked to the second Ig constant region or a portion thereof, and (4) the Ig constant region or a portion thereof is associated with the second Ig constant region or a portion thereof. In one embodiment, the Ig constant region or a portion thereof linked to the FVII protein or the XTEN sequence is further linked to the VWF fragment by a linker, *e.g.,* a processable linker. In another embodiment, the additional Ig constant region or a portion thereof useful for chimeric proteins of the invention can further be linked to the FVIII protein or the Ig constant region or a portion thereof by an optional linker, *e.g.,* a processable linker. In some embodiments, a pair of the Ig constant region or a portion thereof and the additional Ig constant region or a portion thereof, each of which are linked to the VWF fragment and the FVIII protein, provides a bond stronger than the non-covalent bond between the FVIII protein and the VWF fragment, *i.e.,* a covalent bond, *e.g.,* a disulfide bond, thereby preventing endogenous VWF from replacing the VWF fragment *in vivo*. In other embodiments, either or both of the Ig constant region or a portion thereof and the additional Ig constant region or a portion thereof are capable of extending a half-life of the FVIII protein or the VWF fragment. In other embodiments, the additional Ig constant region or a portion thereof comprises a second Fc region or an FcRn binding partner. The Ig constant region or a portion thereof and the additional Ig constant region or a portion thereof in the chimeric proteins are identical or different.

[0130] In certain embodiments, the Ig constant region or a portion thereof and the additional Ig constant region or a portion thereof are associated by a chemical association or a physical association. In one embodiment, the chemical association, *i.e.,* (:), is at least one non-peptide bond. In certain embodiments, the chemical association, *i.e.,* (:), is a covalent bond. In other embodiments, the chemical association, *i.e.,* (:), is a non-covalent interaction, *e.g.,* an ionic interaction, a hydrophobic interaction, a hydrophilic interaction, a Van der Waals interaction, or a hydrogen bond. In other embodiments, (:) is a non-peptide covalent bond. In still other embodiments, (:) is a peptide bond. In yet other embodiments, (:) represents a physical association between two sequences, wherein a portion of a first sequence is in close proximity to a second sequence

such that the first sequence shields or blocks a portion of the second sequence from interacting with another moiety. In some embodiments, the association between the Ig constant region or a portion thereof and the additional Ig constant region or a portion thereof can be a covalent bond, *e.g.,* a disulfide bond, which prevents replacement the VWF fragment or the polypeptide containing the VWF fragment with endogenous VWF. Therefore, preventing interaction between the FVIII protein and endogenous VWF reduces or eliminates this half-life limiting factor for the FVIII protein, and thus the half-life of the FVIII protein is extended compared to a FVIII protein without the VWF protein or wild-type FVIII.

[0131] In other aspects, a chimeric protein comprises a formula comprising:

(1) FVIII(X1)-L1-F1:V-L2-X2-L3-F2;

(2) FVIII(X1)-L1-F1:F2-L3-X2-L2-V;

(3) F1-L1-FVIII(X1):V-L2-X2-L3-F2;

(4) F1-L1-FVIII(X1):F2-L3-X2-L2-V;

(5) FVIII(X1)-L1-F1-L4-V-L2-X2-L3-F2;

(6) FVIII(X1)-L1-F1-L4-F2-L3-X2-L2-V;

(7) F1-L1-FVIII(X1)-L4-V-L2-X2-L3-F2, or

(8) F1-L1-FVIII(X1)-L4- F2-L3-X2-L2-V,

wherein FVIII(X1) comprises a FVIII protein and one or more XTEN sequences, wherein the one or more XTEN sequence are linked to the N-terminus or C-terminus of the FVIII protein or inserted immediately downstream of one or more amino acids (*e.g.,* one or more XTEN insertion sites disclosed herein) in the FVIII protein;

each of L1, L2, or L3 comprises an optional linker, *e.g.,* a cleavable linker;

L4 is a linker, *e.g.,* a processable linker;

X2 comprises one or more optional XTEN sequences;

F1 comprises an Ig constant region or a portion thereof;

F2 comprises an optional additional Ig constant region or a portion thereof, and

V comprises a VWF fragment;

(-) is a peptide bond or one or more amino acids; and

(:) comprises a chemical association or a physical association. In one embodiment, (:) represents a chemical association, *e.g.*, at least one non-peptide bond. In another embodiment, the chemical association, *i.e.,* (:) is a covalent bond. In other embodiments, the chemical association, *i.e.,* (:) is a non-covalent interaction, *e.g.,* an ionic interaction, a hydrophobic interaction, a hydrophilic interaction, a Van der Waals interaction, or a hydrogen bond. In other embodiments, (:) is a non-peptide covalent bond. In still other embodiments, (:) is a peptide bond. In yet other embodiments, (:) represents a physical association between two sequences, wherein a portion of a first sequence is in close proximity to a second sequence such that the first sequence shields or blocks a portion of the second sequence from interacting with another moiety, and further that this physical association is maintained without allowing the second sequence to interact with other moieties. The orientation of the polypeptide formulas herein is listed from N-terminus (left) to C-terminus (right). For example, formula V-X-FVIII means formula NH2-V-X-FVIII -COOH. In one embodiment, the formulas described herein can comprise any additional sequences between the two moieties. For example, formula V-X-FVIII can further comprise any sequences at the N-terminus of V between V and X, between X and FVIII, or at the C-terminus of FVIII unless otherwise specified. In another embodiment, the hyphen (-) indicates a peptide bond.

[0132] In one aspect, the chimeric protein comprises two polypeptide chains, (A) a first chain comprising (i) a single chain FVIII protein (ii) an XTEN sequence, and (iii) a first Ig constant region or a portion thereof, *e.g.,* a first Fc region or FcRn binding partner, wherein the XTEN sequence is linked to the FVIII protein at the N-terminus or C-terminus or inserted immediately downstream of one or more amino acids of the FVIII protein (*e.g.,* one or more XTEN insertion sites disclosed herein) and the first Ig constant region or a portion thereof is linked to the XTEN sequence when the XTEN sequence is linked to the FVIII protein at the N-terminus or the C-terminus or the FVIII protein when the XTEN sequence is inserted within the FVIII protein, and (B) a second chain comprising (iv) a VWF fragment comprising a D' domain and a D3 domain, (v) a linker, and (vi) a second Ig constant region or a portion thereof, *e.g.,* a second Fc region or a second FcRn binding partner, wherein the VWF fragment is linked to the linker, *e.g.,* a

cleavable linker, which is further linked to the second Ig constant region or a portion thereof, and wherein the first polypeptide chain and the second polypeptide chain are associated with each other, *e.g.,* a covalent bond, *e.g.,* a disulfide bond. In one embodiment, the linker is a cleavable linker described elsewhere herein, *e.g.,* a thrombin cleavable linker. In some embodiments, the second chain comprises one or more XTEN sequences between (iv) and (v) or (v) and (vi).

[0133] In other aspects, the chimeric protein comprises one polypeptide chain comprising (i) a single chain FVIII protein (ii) an XTEN sequence, (iii) a first Ig constant region or a portion thereof, *e.g.,* a first Fc region or a first FcRn binding partner, (iv) a first linker, (v) a VWF fragment comprising a D' domain and a D3 domain, (vi) a second linker, and (vii) a second Ig constant region or a portion thereof, *e.g.,* a second Fc region or a second FcRn binding partner, wherein (i) to (vii) are linked in the order or in any orders. In one embodiment, the first linker is a processable linker, which can be intracellularly processed or cleaved after expression and makes the single polypeptide chain into two polypeptide chains. In another embodiment, the second linker is a cleavable linker described herein, *e.g.,* a thrombin cleavable linker. The XTEN sequence used herein can be linked to the FVIII protein by an optional linker at the N-terminus or the C terminus of the FVIII protein or inserted immediately downstream of one or more amino acids (*e.g.,* one or more XTEN insertion sites) in the FVIII protein.

[0134] In certain aspects, a chimeric protein comprises three polypeptide chains, (A) a first polypeptide chain comprising (i) a heavy chain of a FVIII protein and (ii) an XTEN sequence, which are linked to each other and (B) a second polypeptide chain comprising (iii) a light chain of the FVIII protein and (iv) a first Ig constant region or a portion thereof, *e.g.,* a first Fc region or a first FcRn binding partner, which are linked to each other, and (C) a third polypeptide chain comprising (v) a VWF fragment comprising a D' domain and a D3 domain, (vi) a linker, and (vii) a second Ig constant region or a portion thereof, *e.g.,* a second Fc region or a second FcRn binding partner, wherein the second chain is associated with the first chain and the third chain. In one embodiment, the association between the first chain and the second chain is a chemical association or a physical association. For example, the association between the first chain and the

second chain can be a metal bond. In another embodiment, the association between the second chain and the third chain is also a chemical association or a physical association, *e.g.,* a covalent bond or a non-covalent bond. In certain embodiments, the association between the second chain and the third chain is through the two Ig constant regions or a portion thereof and is a disulfide bond. The bonding between the second chain and the third chain prevents or inhibits binding of the FVIII protein with endogenous VWF, thus preventing the FVIII protein being cleared by the VWF clearance pathway. In some embodiments, the linker is a processable linker, which is intracellularly cleaved after expression in a host cell. The XTEN sequence used herein is linked to the FVIII protein by an optional linker at the N-terminus or C terminus of the FVIII protein or inserted immediately downstream of one or more amino acids (*e.g.,* one or more XTEN insertion sites) in the FVIII protein.

[0135] In certain embodiments, the VWF fragment is directly linked to the FVIII protein, which comprises one or more XTENs, by a peptide bond or a linker. As one way of linking the VWF fragment and the FVIII protein, in which one or more XTENs are inserted or linked, through a direct link (*e.g.* a peptide bond) or a linker, an enzymatic ligation (*e.g.,* sortase) can be employed. For example, sortase refers to a group of prokaryotic enzymes that modify surface proteins by recognizing and cleaving a carboxyl-terminal sorting signal. For most substrates of sortase enzymes, the recognition signal consists of the motif LPXTG (Leu-Pro-any-Thr-Gly (SEQ ID NO: 51), then a highly hydrophobic transmembrane sequence, then a cluster of basic residues such as arginine. Cleavage occurs between the Thr and Gly, with transient attachment through the Thr residue to the active site Cys residue of a ligation partner, followed by transpeptidation that attaches the protein covalently to the cell wall. In some embodiments, the ligation partner contains Gly(n). In other embodiments, the chimeric protein further comprises a sortase recognition motif. In some embodiments, the VWF fragment is attached to FVIII comprising one or more XTENs inserted within or linked to using sortase mediated *in vitro* protein ligation.

[0136] In one embodiment, a VWF fragment linked to a sortase recognition motif by an optional linker can be fused to a FVIII protein linked to Gly(n) by a sortase, wherein n can be any integer and wherein one or more XTENs are inserted within

or linked to the FVIII protein. A ligation construct comprises the VWF fragment (N-terminal portion of the construct) and the FVIII protein, in which one or more XTENs are inserted or linked (C-terminal portion of the construct), wherein the sortase recognition motif is inserted in between. Another ligation construct comprises the VWF fragment (N-terminal portion of the construct, the linker, the sortase recognition motif, and the FVIII protein, in which one or more XTENs are inserted or linked (C-terminal portion of the construct). In another embodiment, a FVIII protein linked to a sortase recognition motif by an optional linker can be fused to a VWF fragment linked to Gly(n) by a sortase, wherein n is any integer. A resulting ligation construct comprises the FVIII protein (N-terminal portion of the construct), in which one or more XTENs are inserted or linked, and the VWF fragment (C-terminal portion of the construct), wherein the sortase recognition motif is inserted in between. Another resulting ligation construct comprises the FVIII protein (N-terminal portion of the construct), in which one or more XTENs are inserted or linked, the linker, the sortase recognition motif, and the VWF fragment (C-terminal portion of the construct). In other embodiments, a VWF fragment linked to a sortase recognition motif by a first optional linker can be fused to a heterologous moiety, *e.g.,* an immunoglobulin constant region or a portion thereof, *e.g.,* an Fc region, linked to a thrombin cleavage site by a second optional linker. A resulting construct can comprise the VWF fragment (N-terminal portion), the first linker, the sortase recognition motif, the protease cleavage site, the second optional linker, and the heterologous moiety.

[0137] In some embodiments, the VWF fragment is associated with the FVIII protein. The association between the VWF fragment and the FVIII protein can be a chemical association or a physical association. The chemical association can be a non-covalent interaction, *e.g.,* an ionic interaction, a hydrophobic interaction, a hydrophilic interaction, a Van der Waals interaction, or a hydrogen bond. In yet other embodiments, the association between the FVIII protein and the VWF fragment is a physical association between two sequences, *e.g.,* due to an additional association between the sequence having the FVIII protein and the sequence having the VWF fragment, wherein a portion of a first sequence is in close proximity to a second sequence such that the first sequence shields or blocks a portion of the second sequence from interacting with another moiety.

[0138] As a result of preventing or inhibiting endogenous VWF interaction with the FVIII protein by the VWF fragment, the chimeric protein described herein have an extended half-life compared to wild-type FVIII or the corresponding chimeric protein without the VWF fragment. In one embodiment, the half-life of the FVIII protein is extended at least about 1.5 times, at least about 2 times, at least about 2.5 times, at least about 3 times, at least about 4 times, at least about 5 times, at least about 6 times, at least about 7 times, at least about 8 times, at least about 9 times, at least about 10 times, at least about 11 times, or at least about 12 times longer than a FVIII protein without the VWF fragment. In another embodiment, the half-life of the FVIII protein is at least about 10 hours, at least about 11 hours, at least about 12 hours, at least about 13 hours, at least about 14 hours, at least about 15 hours, at least about 16 hours, at least about 17 hours, at least about 18 hours, at least about 19 hours, at least about 20 hours, at least about 21 hours, at least about 22 hours, at least about 23 hours, at least about 24 hours, at least about 36 hours, at least about 48 hours, at least about 60 hours, at least about 72 hours, at least about 84 hours, at least about 96 hours, or at least about 108 hours. In a particular embodiment, the half-life of the FVIII protein is extended at least 10 hours, at least about 11 hours, at least about 12 hours, at least about 13 hours, at least about 14 hours, at least about 15 hours, at least about 16 hours, at least about 17 hours, at least about 18 hours, at least about 19 hours, at least about 20 hours, at least about 21 hours, at least about 22 hours, at least about 23 hours, at least about 24 hours, at least about 25 hours, at least about 26 hours, or at least about 27 hours in HemA mice.

A) Von Willebrand Factor (VWF) Fragments

[0139] VWF (also known as F8VWF) is a large multimeric glycoprotein present in blood plasma and produced constitutively in endothelium (in the Weibel-Palade bodies), megakaryocytes (α-granules of platelets), and subendothelian connective tissue. The basic VWF monomer is a 2813 amino acid protein. Every monomer contains a number of specific domains with a specific function, the D'/D3 domain (which binds to Factor VIII), the A1 domain (which binds to platelet GPIb-receptor, heparin, and/or possibly collagen), the A3 domain (which binds to collagen), the C1 domain (in which the RGD domain binds to platelet integrin αIIbβ3 when this is activated), and the "cysteine knot" domain at the C-terminal

end of the protein (which VWF shares with platelet-derived growth factor (PDGF), transforming growth factor-β (TGFβ) and β-human chorionic gonadotropin (βHCG).

[0140] The term "a VWF fragment" as used herein includes, but is not limited to, functional VWF fragments comprising a D' domain and a D3 domain, which are capable of inhibiting binding of endogenous VWF to FVIII. In one embodiment, the VWF fragment binds to the FVIII protein. In another embodiment, the VWF fragment blocks the VWF binding site on the FVIII protein, thereby inhibiting interaction of the FVIII protein with endogenous VWF. The VWF fragments include derivatives, variants, mutants, or analogues that retain these activities of VWF.

[0141] The 2813 monomer amino acid sequence for human VWF is reported as Accession Number _NP_000543.2__ in Genbank. The nucleotide sequence encoding the human VWF is reported as Accession Number __NM_000552.3_ in Genbank. The nucleotide sequence of human VWF is designated as SEQ ID NO: 1. SEQ ID NO: 2 is the amino acid sequence encoded by SEQ ID NO: 1. Each domain of VWF is listed in Table 1.

TABLE 1. VWF Sequences

VWF domains	Amino acid Sequence
VWF Signal Peptide (Amino acids 1 to 22 of SEQ ID NO: 2)	1 MIPARFAGVL LALALILPGT LC 22
VWF D1D2 region (Amino acids 23 to 763 of SEQ ID NO: 2)	23 **AEGTRGRS** **STARCSLFGS DFVNTFDGSM** 51 **YSFAGYCSYL LAGGCQKRSF SIIGDFQNGK** **RVSLSVYLGE FFDIHLFVNG** 101 **TVTQGDQRVS MPYASKGLYL ETEAGYYKLS** **GEAYGFVARI DGSGNFQVLL** 151 **SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL** **TSDPYDFANS WALSSGEQWC** 201 **ERASPPSSSC NISSGEMQKG LWEQCQLLKS** **TSVFARCHPL VDPEPFVALC** 251 **EKTLCECAGG LECACPALLE YARTCAQEGM** **VLYGWTDHSA CSPVCPAGME** 301 **YRQCVSPCAR TCQSLHINEM CQERCVDGCS** **CPEGQLLDEG LCVESTECPC** 351 **VHSGKRYPPG TSLSRDCNTC ICRNSQWICS** **NEECPGECLV TGQSHFKSFD** 401 **NRYFTFSGIC QYLLARDCQD HSFSIVIETV**

	QCADDRDAVC TRSVTVRLPG 451 **LHNSLVKLKH GAGVAMDGQD IQLPLLKGDL** **RIQHTVTASV RLSYGEDLQM** 501 **DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN** **QGDDFLTPSG LAEPRVEDFG** 551 **NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS** **EEACAVLTSP TFEACHRAVS** 601 **PLPYLRNCRY DVCSCSDGRE CLCGALASYA** **AACAGRGVRV AWREPGRCEL** 651 **NCPKGQVYLQ CGTPCNLTCR SLSYPDEECN** **EACLEGCFCP PGLYMDERGD** 701 **CVPKAQCPCY YDGEIFQPED IFSDHHTMCY** **CEDGFMHCTM SGVPGSLLPD** 751 **AVLSSPLSHR SKR** 763
VWF D' Domain	764 SLSCRPP MVKLVCPADN LRAEGLECTK TCQNYDLECM 801 SMGCVSGCLC PPGMVRHENR CVALERCPCF HQGKEYAPGE TVKIGCNTCV 851 CRDRKWNCTD HVCDAT 866
VWF D3 Domain	867 CSTI GMAHYLTFDG LKYLFPGECQ YVLVQDYCGS 901 NPGTFRILVG NKGCSHPSVK CKKRVTILVE GGEIELFDGE VNVKRPMKDE 951 THFEVVESGR YIILLLGKAL SVVWDRHLSI SVVLKQTYQE KVCGLCGNFD 1001 GIQNNDLTSS NLQVEEDPVD FGNSWKVSSQ CADTRKVPLD SSPATCHNNI 1051 MKQTMVDSSC RILTSDVFQD CNKLVDPEPY LDVCIYDTCS CESIGDCACF 1101 CDTIAAYAHV CAQHGKVVTW RTATLCPQSC EERNLRENGY ECEWRYNSCA 1151 PACQVTCQHP EPLACPVQCV EGCHAHCPPG KILDELLQTC VDPEDCPVCE 1201 VAGRRFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP 1240
VWF A1 Domain	1241 GGLVVPPTDA 1251 PVSPTTLYVE DISEPPLHDF YCSRLLDLVF LLDGSSRLSE AEFEVLKAFV 1301 VDMMERLRIS QKWVRVAVVE YHDGSHAYIG LKDRKRPSEL RRIASQVKYA 1351 GSQVASTSEV LKYTLFQIFS KIDRPEASRI ALLLMASQEP QRMSRNFVRY 1401 VQGLKKKKVI VIPVGIGPHA NLKQIRLIEK

	QAPENKAFVL SSVDELEQQR 1451 DEIVSYLCDL APEAPPPTLP PDMAQVTVG 1479
	1480 P GLLGVSTLGP KRNSMVLDVA 1501 FVLEGSDKIG EADFNRSKEF MEEVIQRMDV GQDSIHVTVL QYSYMVTVEY 1551 PFSEAQSKGD ILQRVREIRY QGGNRTNTGL ALRYLSDHSF LVSQGDREQA 1600 1601 PNLVYMVTGN PASDEIKRLP GDIQVVPIGV GPNANVQELE RIGWPNAPIL 1651 IQDFETLPRE APDLVLQRCC SGEGLQIPTL SPAPDCSQPL DVILLLDGSS 1701 SFPASYFDEM KSFAKAFISK ANIGPRLTQV SVLQYGSITT IDVPWNVVPE 1751 KAHLLSLVDV MQREGGPSQI GDALGFAVRY LTSEMHGARP GASKAVVILV 1801 TDVSVDSVDA AADAARSNRV TVFPIGIGDR YDAAQLRILA GPAGDSNVVK 1851 LQRIEDLPTM VTLGNSFLHK LCSGFVRICM DEDGNEKRPG DVWTLPDQCH 1901 TVTCQPDGQT LLKSHRVNCD RGLRPSCPNS QSPVKVEETC GCRWTCPCVC 1951 TGSSTRHIVT FDGQNFKLTG SCSYVLFQNK EQDLEVILHN GACSPGARQG 2001 CMKSIEVKHS ALSVEXHSDM EVTVNGRLVS VPYVGGNMEV NVYGAIMHEV 2051 RFNHLGHIFT FTPQNNEFQL QLSPKTFASK TYGLCGICDE NGANDFMLRD 2101 GTVTTDWKTL VQEWTVQRPG QTCQPILEEQ CLVPDSSHCQ VLLLPLFAEC 2151 HKVLAPATFY AICQQDSCHQ EQVCEVIASY AHLCRTNGVC VDWRTPDFCA 2201 MSCPPSLVYN HCEHGCPRHC DGNVSSCGDH PSEGCFCPPD KVMLEGSCVP 2251 EEACTQCIGE DGVQHQFLEA WVPDHQPCQI CTCLSGRKVN CTTQPCPTAK 2301 APTCGLCEVA RLRQNADQCC PEYECVCDPV SCDLPPVPHC ERGLQPTLTN 2351 PGECRPNFTC ACRKEECKRV SPPSCPPHRL PTLRKTQCCD EYECACNCVN 2401 STVSCPLGYL ASTATNDCGC TTTTCLPDKV CVHRSTIYPV GQFWEEGCDV 2451 CTCTDMEDAV MGLRVAQCSQ KPCEDSCRSG FTYVLHEGEC CGRCLPSACE 2501 VVTGSPRGDS QSSWKSVGSQ WASPENPCLI NECVRVKEEV FIQQRNVSCP 2551 QLEVPVCPSG FQLSCKTSAC CPSCRCERME ACMLNGTVIG PGKTVMIDVC

	2601 TTCRCMVQVG VISGFKLECR KTTCNPCPLG YKEENNTGEC CGRCLPTACT 2651 IQLRGGQIMT LKRDETLQDG CDTHFCKVNE RGEYFWEKRV TGCPPFDEHK 2701 CLAEGGKIMK IPGTCCDTCE EPECNDITAR LQYVKVGSCK SEVEVDIHYC 2751 QGKCASKAMY SIDINDVQDQ CSCCSPTRTE PMQVALHCTN GSVVYHEVLN 2801 AMECKCSPRK CSK
	Nucleotide Sequence (SEQ ID NO: 1)
Full-length VWF	1 ATGATTCCTG CCAGATTTGC CGGGGTGCTG CTTGCTCTGG CCCTCATTTT 51 GCCAGGGACC CTTTGTGCAG AAGGAACTCG CGGCAGGTCA TCCACGGCCC 101 GATGCAGCCT TTTCGGAAGT GACTTCGTCA ACACCTTTGA TGGGAGCATG 151 TACAGCTTTG CGGGATACTG CAGTTACCTC CTGGCAGGGG GCTGCCAGAA 201 ACGCTCCTTC TCGATTATTG GGGACTTCCA GAATGGCAAG AGAGTGAGCC 251 TCTCCGTGTA TCTTGGGGAA TTTTTTGACA TCCATTTGTT TGTCAATGGT 301 ACCGTGACAC AGGGGGACCA AAGAGTCTCC ATGCCCTATG CCTCCAAAGG 351 GCTGTATCTA GAAACTGAGG CTGGGTACTA CAAGCTGTCC GGTGAGGCCT 401 ATGGCTTTGT GGCCAGGATC GATGGCAGCG GCAACTTTCA AGTCCTGCTG 451 TCAGACAGAT ACTTCAACAA GACCTGCGGG CTGTGTGGCA ACTTTAACAT 501 CTTTGCTGAA GATGACTTTA TGACCCAAGA AGGGACCTTG ACCTCGGACC 551 CTTATGACTT TGCCAACTCA TGGGCTCTGA GCAGTGGAGA ACAGTGGTGT 601 GAACGGGCAT CTCCTCCCAG CAGCTCATGC AACATCTCCT CTGGGGAAAT 651 GCAGAAGGGC CTGTGGGAGC AGTGCCAGCT TCTGAAGAGC ACCTCGGTGT 701 TTGCCCGCTG CCACCCTCTG GTGGACCCCG AGCCTTTTGT GGCCCTGTGT 751 GAGAAGACTT TGTGTGAGTG TGCTGGGGGG CTGGAGTGCG CCTGCCCTGC 801 CCTCCTGGAG TACGCCCGGA CCTGTGCCCA GGAGGGAATG GTGCTGTACG 851 GCTGGACCGA CCACAGCGCG TGCAGCCCAG TGTGCCCTGC TGGTATGGAG 901 TATAGGCAGT GTGTGTCCCC TTGCGCCAGG ACCTGCCAGA GCCTGCACAT 951 CAATGAAATG TGTCAGGAGC

	GATGCGTGGA TGGCTGCAGC TGCCCTGAGG 1001 GACAGCTCCT GGATGAAGGC CTCTGCGTGG AGAGCACCGA GTGTCCCTGC 1051 GTGCATTCCG GAAAGCGCTA CCCTCCCGGC ACCTCCCTCT CTCGAGACTG 1101 CAACACCTGC ATTTGCCGAA ACAGCCAGTG GATCTGCAGC AATGAAGAAT 1151 GTCCAGGGGA GTGCCTTGTC ACTGGTCAAT CCCACTTCAA GAGCTTTGAC 1201 AACAGATACT TCACCTTCAG TGGGATCTGC CAGTACCTGC TGGCCCGGGA 1251 TTGCCAGGAC CACTCCTTCT CCATTGTCAT TGAGACTGTC CAGTGTGCTG 1301 ATGACCGCGA CGCTGTGTGC ACCCGCTCCG TCACCGTCCG GCTGCCTGGC 1351 CTGCACAACA GCCTTGTGAA ACTGAAGCAT GGGGCAGGAG TTGCCATGGA 1401 TGGCCAGGAC ATCCAGCTCC CCCTCCTGAA AGGTGACCTC CGCATCCAGC 1451 ATACAGTGAC GGCCTCCGTG CGCCTCAGCT ACGGGGAGGA CCTGCAGATG 1501 GACTGGGATG GCCGCGGGAG GCTGCTGGTG AAGCTGTCCC CCGTCTATGC 1551 CGGGAAGACC TGCGGCCTGT GTGGGAATTA CAATGGCAAC CAGGGCGACG 1601 ACTTCCTTAC CCCCTCTGGG CTGGCRGAGC CCCGGGTGGA GGACTTCGGG 1651 AACGCCTGGA AGCTGCACGG GGACTGCCAG GACCTGCAGA AGCAGCACAG 1701 CGATCCCTGC GCCCTCAACC CGCGCATGAC CAGGTTCTCC GAGGAGGCGT 1751 GCGCGGTCCT GACGTCCCCC ACATTCGAGG CCTGCCATCG TGCCGTCAGC 1801 CCGCTGCCCT ACCTGCGGAA CTGCCGCTAC GACGTGTGCT CCTGCTCGGA 1851 CGGCCGCGAG TGCCTGTGCG GCGCCCTGGC CAGCTATGCC GCGGCCTGCG 1901 CGGGGAGAGG CGTGCGCGTC GCGTGGCGCG AGCCAGGCCG CTGTGAGCTG 1951 AACTGCCCGA AAGGCCAGGT GTACCTGCAG TGCGGGACCC CCTGCAACCT 2001 GACCTGCCGC TCTCTCTCTT ACCCGGATGA GGAATGCAAT GAGGCCTGCC 2051 TGGAGGGCTG CTTCTGCCCC CCAGGGCTCT ACATGGATGA GAGGGGGGAC 2101 TGCGTGCCCA AGGCCCAGTG CCCCTGTTAC TATGACGGTG AGATCTTCCA 2151 GCCAGAAGAC ATCTTCTCAG ACCATCACAC CATGTGCTAC TGTGAGGATG 2201 GCTTCATGCA CTGTACCATG AGTGGAGTCC CCGGAAGCTT GCTGCCTGAC

	2251 GCTGTCCTCA GCAGTCCCCT GTCTCATCGC AGCAAAAGGA GCCTATCCTG 2301 TCGGCCCCCC ATGGTCAAGC TGGTGTGTCC CGCTGACAAC CTGCGGGCTG 2351 AAGGGCTCGA GTGTACCAAA ACGTGCCAGA ACTATGACCT GGAGTGCATG 2401 AGCATGGGCT GTGTCTCTGG CTGCCTCTGC CCCCCGGGCA TGGTCCGGCA 2451 TGAGAACAGA TGTGTGGCCC TGGAAAGGTG TCCCTGCTTC CATCAGGGCA 2501 AGGAGTATGC CCCTGGAGAA ACAGTGAAGA TTGGCTGCAA CACTTGTGTC 2551 TGTCGGGACC GGAAGTGGAA CTGCACAGAC CATGTGTGTG ATGCCACGTG 2601 CTCCACGATC GGCATGGCCC ACTACCTCAC CTTCGACGGG CTCAAATACC 2651 TGTTCCCCGG GGAGTGCCAG TACGTTCTGG TGCAGGATTA CTGCGGCAGT 2701 AACCCTGGGA CCTTTCGGAT CCTAGTGGGG AATAAGGGAT GCAGCCACCC 2751 CTCAGTGAAA TGCAAGAAAC GGGTCACCAT CCTGGTGGAG GGAGGAGAGA 2801 TTGAGCTGTT TGACGGGGAG GTGAATGTGA AGAGGCCCAT GAAGGATGAG 2851 ACTCACTTTG AGGTGGTGGA GTCTGGCCGG TACATCATTC TGCTGCTGGG 2901 CAAAGCCCTC TCCGTGGTCT GGGACCGCCA CCTGAGCATC TCCGTGGTCC 2951 TGAAGCAGAC ATACCAGGAG AAAGTGTGTG GCCTGTGTGG GAATTTTGAT 3001 GGCATCCAGA ACAATGACCT CACCAGCAGC AACCTCCAAG TGGAGGAAGA 3051 CCCTGTGGAC TTTGGGAACT CCTGGAAAGT GAGCTCGCAG TGTGCTGACA 3101 CCAGAAAAGT GCCTCTGGAC TCATCCCCTG CCACCTGCCA TAACAACATC 3151 ATGAAGCAGA CGATGGTGGA TTCCTCCTGT AGAATCCTTA CCAGTGACGT 3201 CTTCCAGGAC TGCAACAAGC TGGTGGACCC CGAGCCATAT CTGGATGTCT 3251 GCATTTACGA CACCTGCTCC TGTGAGTCCA TTGGGGACTG CGCCTGCTTC 3301 TGCGACACCA TTGCTGCCTA TGCCCACGTG TGTGCCCAGC ATGGCAAGGT 3351 GGTGACCTGG AGGACGGCCA CATTGTGCCC CCAGAGCTGC GAGGAGAGGA 3401 ATCTCCGGGA GAACGGGTAT GAGTGTGAGT GGCGCTATAA CAGCTGTGCA 3451 CCTGCCTGTC AAGTCACGTG TCAGCACCCT GAGCCACTGG CCTGCCCTGT 3501 GCAGTGTGTG GAGGGCTGCC

	ATGCCCACTG CCCTCCAGGG AAAATCCTGG 3551 ATGAGCTTTT GCAGACCTGC GTTGACCCTG AAGACTGTCC AGTGTGTGAG 3601 GTGGCTGGCC GGCGTTTTGC CTCAGGAAAG AAAGTCACCT TGAATCCCAG 3651 TGACCCTGAG CACTGCCAGA TTTGCCACTG TGATGTTGTC AACCTCACCT 3701 GTGAAGCCTG CCAGGAGCCG GGAGGCCTGG TGGTGCCTCC CACAGATGCC 3751 CCGGTGAGCC CCACCACTCT GTATGTGGAG GACATCTCGG AACCGCCGTT 3801 GCACGATTTC TACTGCAGCA GGCTACTGGA CCTGGTCTTC CTGCTGGATG 3851 GCTCCTCCAG GCTGTCCGAG GCTGAGTTTG AAGTGCTGAA GGCCTTTGTG 3901 GTGGACATGA TGGAGCGGCT GCGCATCTCC CAGAAGTGGG TCCGCGTGGC 3951 CGTGGTGGAG TACCACGACG GCTCCCACGC CTACATCGGG CTCAAGGACC 4001 GGAAGCGACC GTCAGAGCTG CGGCGCATTG CCAGCCAGGT GAAGTATGCG 4051 GGCAGCCAGG TGGCCTCCAC CAGCGAGGTC TTGAAATACA CACTGTTCCA 4101 AATCTTCAGC AAGATCGACC GCCCTGAAGC CTCCCGCATC GCCCTGCTCC 4151 TGATGGCCAG CCAGGAGCCC CAACGGATGT CCCGGAACTT TGTCCGCTAC 4201 GTCCAGGGCC TGAAGAAGAA GAAGGTCATT GTGATCCCGG TGGGCATTGG 4251 GCCCCATGCC AACCTCAAGC AGATCCGCCT CATCGAGAAG CAGGCCCCTG 4301 AGAACAAGGC CTTCGTGCTG AGCAGTGTGG ATGAGCTGGA GCAGCAAAGG 4351 GACGAGATCG TTAGCTACCT CTGTGACCTT GCCCCTGAAG CCCCTCCTCC 4401 TACTCTGCCC CCCGACATGG CACAAGTCAC TGTGGGCCCG GGGCTCTTGG 4451 GGGTTTCGAC CCTGGGGCCC AAGAGGAACT CCATGGTTCT GGATGTGGCG 4501 TTCGTCCTGG AAGGATCGGA CAAAATTGGT GAAGCCGACT TCAACAGGAG 4551 CAAGGAGTTC ATGGAGGAGG TGATTCAGCG GATGGATGTG GGCCAGGACA 4601 GCATCCACGT CACGGTGCTG CAGTACTCCT ACATGGTGAC CGTGGAGTAC 4651 CCCTTCAGCG AGGCACAGTC CAAAGGGGAC ATCCTGCAGC GGGTGCGAGA 4701 GATCCGCTAC CAGGGCGGCA ACAGGACCAA CACTGGGCTG GCCCTGCGGT 4751 ACCTCTCTGA CCACAGCTTC TTGGTCAGCC AGGGTGACCG GGAGCAGGCG

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4801  CCCAACCTGG TCTACATGGT
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5951  TGGAGGTGAT TCTCCATAAT
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6001  TGCATGAAAT CCATCGAGGT
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6051  CAGTGACATG GAGGTGACGG
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	TGAATGGGAG ACTGGTCTCT GTTCCTTACG 6101 TGGGTGGGAA CATGGAAGTC AACGTTTATG GTGCCATCAT GCATGAGGTC 6151 AGATTCAATC ACCTTGGTCA CATCTTCACA TTCACTCCAC AAAACAATGA 6201 GTTCCAACTG CAGCTCAGCC CCAAGACTTT TGCTTCAAAG ACGTATGGTC 6251 TGTGTGGGAT CTGTGATGAG AACGGAGCCA ATGACTTCAT GCTGAGGGAT 6301 GGCACAGTCA CCACAGACTG GAAAACACTT GTTCAGGAAT GGACTGTGCA 6351 GCGGCCAGGG CAGACGTGCC AGCCCATCCT GGAGGAGCAG TGTCTTGTCC 6401 CCGACAGCTC CCACTGCCAG GTCCTCCTCT TACCACTGTT TGCTGAATGC 6451 CACAAGGTCC TGGCTCCAGC CACATTCTAT GCCATCTGCC AGCAGGACAG 6501 TTGCCACCAG GAGCAAGTGT GTGAGGTGAT CGCCTCTTAT GCCCACCTCT 6551 GTCGGACCAA CGGGGTCTGC GTTGACTGGA GGACACCTGA TTTCTGTGCT 6601 ATGTCATGCC CACCATCTCT GGTCTACAAC CACTGTGAGC ATGGCTGTCC 6651 CCGGCACTGT GATGGCAACG TGAGCTCCTG TGGGGACCAT CCCTCCGAAG 6701 GCTGTTTCTG CCCTCCAGAT AAAGTCATGT TGGAAGGCAG CTGTGTCCCT 6751 GAAGAGGCCT GCACTCAGTG CATTGGTGAG GATGGAGTCC AGCACCAGTT 6801 CCTGGAAGCC TGGGTCCCGG ACCACCAGCC CTGTCAGATC TGCACATGCC 6851 TCAGCGGGCG GAAGGTCAAC TGCACAACGC AGCCCTGCCC CACGGCCAAA 6901 GCTCCCACGT GTGGCCTGTG TGAAGTAGCC CGCCTCCGCC AGAATGCAGA 6951 CCAGTGCTGC CCCGAGTATG AGTGTGTGTG TGACCCAGTG AGCTGTGACC 7001 TGCCCCCAGT GCCTCACTGT GAACGTGGCC TCCAGCCCAC ACTGACCAAC 7051 CCTGGCGAGT GCAGACCCAA CTTCACCTGC GCCTGCAGGA AGGAGGAGTG 7101 CAAAAGAGTG TCCCCACCCT CCTGCCCCCC GCACCGTTTG CCCACCCTTC 7151 GGAAGACCCA GTGCTGTGAT GAGTATGAGT GTGCCTGCAA CTGTGTCAAC 7201 TCCACAGTGA GCTGTCCCCT TGGGTACTTG GCCTCAACCG CCACCAATGA 7251 CTGTGGCTGT ACCACAACCA CCTGCCTTCC CGACAAGGTG TGTGTCCACC 7301 GAAGCACCAT CTACCCTGTG GGCCAGTTCT GGGAGGAGGG CTGCGATGTG

	7351 TGCACCTGCA CCGACATGGA GGATGCCGTG ATGGGCCTCC GCGTGGCCCA 7401 GTGCTCCCAG AAGCCCTGTG AGGACAGCTG TCGGTCGGGC TTCACTTACG 7451 TTCTGCATGA AGGCGAGTGC TGTGGAAGGT GCCTGCCATC TGCCTGTGAG 7501 GTGGTGACTG GCTCACCGCG GGGGGACTCC CAGTCTTCCT GGAAGAGTGT 7551 CGGCTCCCAG TGGGCCTCCC CGGAGAACCC CTGCCTCATC AATGAGTGTG 7601 TCCGAGTGAA GGAGGAGGTC TTTATACAAC AAAGGAACGT CTCCTGCCCC 7651 CAGCTGGAGG TCCCTGTCTG CCCCTCGGGC TTTCAGCTGA GCTGTAAGAC 7701 CTCAGCGTGC TGCCCAAGCT GTCGCTGTGA GCGCATGGAG GCCTGCATGC 7751 TCAATGGCAC TGTCATTGGG CCCGGGAAGA CTGTGATGAT CGATGTGTGC 7801 ACGACCTGCC GCTGCATGGT GCAGGTGGGG GTCATCTCTG GATTCAAGCT 7851 GGAGTGCAGG AAGACCACCT GCAACCCCTG CCCCCTGGGT TACAAGGAAG 7901 AAAATAACAC AGGTGAATGT TGTGGGAGAT GTTTGCCTAC GGCTTGCACC 7951 ATTCAGCTAA GAGGAGGACA GATCATGACA CTGAAGCGTG ATGAGACGCT 8001 CCAGGATGGC TGTGATACTC ACTTCTGCAA GGTCAATGAG AGAGGAGAGT 8051 ACTTCTGGGA GAAGAGGGTC ACAGGCTGCC CACCCTTTGA TGAACACAAG 8101 TGTCTTGCTG AGGGAGGTAA AATTATGAAA ATTCCAGGCA CCTGCTGTGA 8151 CACATGTGAG GAGCCTGAGT GCAACGACAT CACTGCCAGG CTGCAGTATG 8201 TCAAGGTGGG AAGCTGTAAG TCTGAAGTAG AGGTGGATAT CCACTACTGC 8251 CAGGGCAAAT GTGCCAGCAA AGCCATGTAC TCCATTGACA TCAACGATGT 8301 GCAGGACCAG TGCTCCTGCT GCTCTCCGAC ACGGACGGAG CCCATGCAGG 8351 TGGCCCTGCA CTGCACCAAT GGCTCTGTTG TGTACCATGA GGTTCTCAAT 8401 GCCATGGAGT GCAAATGCTC CCCCAGGAAG TGCAGCAAGT GA

[0142] The VWF fragment as used herein can be a VWF fragment comprising a D' domain and a D3 domain of VWF, wherein the VWF fragment binds to Factor

VIII (FVIII) and inhibits binding of endogenous VWF (full-length VWF) to FVIII. The VWF fragment comprising the D' domain and the D3 domain can further comprise a VWF domain selected from the group consisting of an A1 domain, an A2 domain, an A3 domain, a D1 domain, a D2 domain, a D4 domain, a B1 domain, a B2 domain, a B3 domain, a C1 domain, a C2 domain, a CK domain, one or more fragments thereof, and any combinations thereof. In one embodiment, a VWF fragment comprises, consists essentially of, or consists of: (1) the D' and D3 domains of VWF or fragments thereof; (2) the D1, D', and D3 domains of VWF or fragments thereof; (3) the D2, D', and D3 domains of VWF or fragments thereof; (4) the D1, D2, D', and D3 domains of VWF or fragments thereof; or (5) the D1, D2, D', D3, and A1 domains of VWF or fragments thereof. The VWF fragment described herein does not contain a site binding to a VWF clearance receptor. In another embodiment, the VWF fragment described herein is not amino acids 764 to 1274 of SEQ ID NO: 2. The VWF fragment of the present invention can comprise any other sequences linked to or fused to the VWF fragment. For example, a VWF fragment described herein can further comprise a signal peptide.

[0143] In one embodiment, the VWF fragment binds to or is associated with a FVIII protein. By binding to or associating with a FVIII protein, a VWF fragment of the invention protects FVIII from protease cleavage and FVIII activation, stabilizes the heavy chain and light chain of FVIII, and prevents clearance of FVIII by scavenger receptors. In another embodiment, the VWF fragment binds to or associates with a FVIII protein and blocks or prevents binding of the FVIII protein to phospholipid and activated Protein C. By preventing or inhibiting binding of the FVIII protein with endogenous, full-length VWF, the VWF fragment of the invention reduces the clearance of FVIII by VWF clearance receptors and thus extends half-life of the FVIII protein. In one embodiment, the half-life extension of a FVIII protein is thus due to the binding of or associating with the VWF fragment lacking a VWF clearance receptor binding site to the FVIII protein and shielding or protecting of the FVIII protein by the VWF fragment from endogenous VWF which contains the VWF clearance receptor binding site. The FVIII protein bound to or protected by the VWF fragment can also allow recycling of a FVIII protein. By eliminating the VWF clearance

pathway receptor binding sites contained in the full length VWF molecule, the FVIII/VWF heterodimers of the invention are shielded from the VWF clearance pathway, further extending FVIII half-life.

[0144] In one embodiment, a VWF fragment of the present invention comprises the D' domain and the D3 domain of VWF, wherein the D' domain is at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 764 to 866 of SEQ ID NO: 2, wherein the VWF fragment prevents binding of endogenous VWF to FVIII. In another embodiment, a VWF fragment comprises the D' domain and the D3 domain of VWF, wherein the D3 domain is at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 867 to 1240 of SEQ ID NO: 2, wherein the VWF fragment prevents binding of endogenous VWF to FVIII. In some embodiments, a VWF fragment described herein comprises, consists essentially of, or consists of the D' domain and D3 domain of VWF, which are at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 764 to 1240 of SEQ ID NO: 2, wherein the VWF fragment prevents binding of endogenous VWF to FVIII. In other embodiments, a VWF fragment comprises, consists essentially of, or consists of the D1, D2, D', and D3 domains at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 23 to 1240 of SEQ ID NO: 2, wherein the VWF fragment prevents binding of endogenous VWF to FVIII. In still other embodiments, the VWF fragment further comprises a signal peptide operably linked thereto.

[0145] In some embodiments, a VWF fragment of the invention consists essentially of or consists of (1) the D'D3 domain, the D1D'D3 domain, D2D'D3 domain, or D1D2D'D3 domain and (2) an additional VWF sequence up to about 10 amino acids (*e.g.*, any sequences from amino acids 764 to 1240 of SEQ ID NO: 2 to amino acids 764 to 1250 of SEQ ID NO: 2), up to about 15 amino acids (*e.g.*, any sequences from amino acids 764 to 1240 of SEQ ID NO: 2 to amino acids 764 to 1255 of SEQ ID NO: 2), up to about 20 amino acids (*e.g.*, any sequences from amino acids 764 to 1240 of SEQ ID NO: 2 to amino acids 764 to 1260 of SEQ ID NO: 2), up to about 25 amino acids (*e.g.*, any sequences from amino acids 764 to 1240 of SEQ ID NO: 2 to amino acids 764 to 1265 of SEQ ID NO: 2), or up to about 30 amino acids (*e.g.*, any sequences from amino acids 764 to 1240 of SEQ

ID NO: 2 to amino acids 764 to 1260 of SEQ ID NO: 2). In a particular embodiment, the VWF fragment comprising or consisting essentially of the D' domain and the D3 domain is neither amino acids 764 to 1274 of SEQ ID NO: 2 nor the full-length mature VWF. In some embodiments, the D1D2 domain is expressed in trans with the D'D3 domain. In some embodiments, the D1D2 domain is expressed in cis with the D'D3 domain.

[0146] In other embodiments, the VWF fragment comprising the D'D3 domains linked to the D1D2 domains further comprises an intracellular cleavage site, *e.g.,* (a cleavage site by PACE (furin) or PC5), allowing cleavage of the D1D2 domains from the D'D3 domains upon expression. Non-limiting examples of the intracellular cleavage site are disclosed elsewhere herein.

[0147] In yet other embodiments, a VWF fragment comprises the D' domain and the D3 domain, but does not comprise an amino acid sequence selected from the group consisting of (1) amino acids 1241 to 2813 of SEQ ID NO: 2, (2) amino acids 1270 to amino acids 2813 of SEQ ID NO: 2, (3) amino acids 1271 to amino acids 2813 of SEQ ID NO: 2, (4) amino acids 1272 to amino acids 2813 of SEQ ID NO: 2, (5) amino acids 1273 to amino acids 2813 of SEQ ID NO: 2, (6) amino acids 1274 to amino acids 2813 of SEQ ID NO: 2, and any combinations thereof.

[0148] In still other embodiments, a VWF fragment of the present invention comprises, consists essentially of, or consists of an amino acid sequence corresponding to the D' domain, D3 domain, and A1 domain, wherein the amino acid sequence is at least 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acid 764 to 1479 of SEQ ID NO: 2, wherein the VWF fragment prevents binding of endogenous VWF to FVIII. In a particular embodiment, the VWF fragment is not amino acids 764 to 1274 of SEQ ID NO: 2.

[0149] In some embodiments, a VWF fragment of the invention comprises the D' domain and the D3 domain, but does not comprise at least one VWF domain selected from the group consisting of (1) an A1 domain, (2) an A2 domain, (3) an A3 domain, (4) a D4 domain, (5) a B1 domain, (6) a B2 domain, (7) a B3 domain, (8) a C1 domain, (9) a C2 domain, (10) a CK domain, (11) a CK domain and C2 domain, (12) a CK domain, a C2 domain, and a C1 domain, (13) a CK domain, a C2 domain, a C1 domain, a B3 domain, (14) a CK domain, a C2 domain, a C1 domain, a B3 domain, a B2 domain, (15) a CK domain, a C2 domain, a C1

domain, a B3 domain, a B2 domain, and a B1 domain, (16) a CK domain, a C2 domain, a C1 domain, a B3 domain, a B2 domain, a B1 domain, and a D4 domain, (17) a CK domain, a C2 domain, a C1 domain, a B3 domain, a B2 domain, a B1 domain, a D4 domain, and an A3 domain, (18) a CK domain, a C2 domain, a C1 domain, a B3 domain, a B2 domain, a B1 domain, a D4 domain, an A3 domain, and an A2 domain, (19) a CK domain, a C2 domain, a C1 domain, a B3 domain, a B2 domain, a B1 domain, a D4 domain, an A3 domain, an A2 domain, and an A1 domain, and (20) any combinations thereof.

[0150] In yet other embodiments, the VWF fragment comprises the D'D3 domains and one or more domains or modules. Examples of such domains or modules include, but are not limited to, the domains and modules disclosed in Zhour et al., Blood published online April 6, 2012: DOI 10.1182/blood-2012-01-405134. For example, the VWF fragment can comprise the D'D3 domain and one or more domains or modules selected from the group consisting of A1 domain, A2 domain, A3 domain, D4N module, VWD4 module, C8-4 module, TIL-4 module, C1 module, C2 module, C3 module, C4 module, C5 module, C5 module, C6 module, and any combinations thereof.

[0151] In still other embodiments, the VWF fragment is linked to a heterologous moiety, wherein the heterologous moiety is linked to the N-terminus or the C-terminus of the VWF fragment or inserted immediately downstream of one or more amino acids (*e.g.,* one or more XTEN insertion sites) in the FVIII protein in the VWF fragment. For example, the insertion sites for the heterologous moiety in the VWF fragment can be in the D' domain, the D3 domain, or both. The heterologous moiety can be a half-life extender.

[0152] In certain embodiments, a VWF fragment of the invention forms a multimer, *e.g.,* dimer, trimer, tetramer, pentamer, hexamer, heptamer, or the higher order multimers. In other embodiments, the VWF fragment is a monomer having only one VWF fragment. In some embodiments, the VWF fragment of the present invention can have one or more amino acid substitutions, deletions, additions, or modifications. In one embodiment, the VWF fragment can include amino acid substitutions, deletions, additions, or modifications such that the VWF fragment is not capable of forming a disulfide bond or forming a dimer or a multimer. In another embodiment, the amino acid substitution is within the D'

domain and the D3 domain. In a particular embodiment, a VWF fragment of the invention contains at least one amino acid substitution at a residue corresponding to residue 1099, residue 1142, or both residues 1099 and 1142 of SEQ ID NO: 2. The at least one amino acid substitution can be any amino acids that are not occurring naturally in the wild type VWF. For example, the amino acid substitution can be any amino acids other than cysteine, *e.g.,* Isoleucine, Alanine, Leucine, Asparagine, Lysine, Aspartic acid, Methionine, Phenylalanine, Glutamic acid, Threonine, Glutamine, Tryptophan, Glycine, Valine, Proline, Serine, Tyrosine, Arginine, or Histidine. In another example, the amino acid substitution has one or more amino acids that prevent or inhibit the VWF fragments from forming multimers.

[0153] In certain embodiments, the VWF fragment useful herein can be further modified to improve its interaction with FVIII, *e.g.,* to improve binding affinity to FVIII. As a non-limiting example, the VWF fragment comprises a serine residue at the residue corresponding to amino acid 764 of SEQ ID NO: 2 and a lysine residue at the residue corresponding to amino acid 773 of SEQ ID NO: 2. Residues 764 and/or 773 can contribute to the binding affinity of the VWF fragments to FVIII. In other embodiments, the VWF fragments useful for the invention can have other modifications, *e.g.,* the protein can be pegylated, glycosylated, hesylated, or polysialylated.

B) XTEN Sequences

[0154] As used here "XTEN sequence" refers to extended length polypeptides with non-naturally occurring, substantially non-repetitive sequences that are composed mainly of small hydrophilic amino acids, with the sequence having a low degree or no secondary or tertiary structure under physiologic conditions. As a chimeric protein partner, XTENs can serve as a carrier, conferring certain desirable pharmacokinetic, physicochemical and pharmaceutical properties when linked to a VWF fragment or a FVIII sequence of the invention to create a chimeric protein. Such desirable properties include but are not limited to enhanced pharmacokinetic parameters and solubility characteristics. As used herein, "XTEN" specifically excludes antibodies or antibody fragments such as single-chain antibodies or Fc fragments of a light chain or a heavy chain.

[0155] In some embodiments, the XTEN sequence of the invention is a peptide or a polypeptide having greater than about 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1200, 1400, 1600, 1800, or 2000 amino acid residues. In certain embodiments, XTEN is a peptide or a polypeptide having greater than about 20 to about 3000 amino acid residues, greater than 30 to about 2500 residues, greater than 40 to about 2000 residues, greater than 50 to about 1500 residues, greater than 60 to about 1000 residues, greater than 70 to about 900 residues, greater than 80 to about 800 residues, greater than 90 to about 700 residues, greater than 100 to about 600 residues, greater than 110 to about 500 residues, or greater than 120 to about 400 residues.

[0156] The XTEN sequence of the invention can comprise one or more sequence motif of 9 to 14 amino acid residues or an amino acid sequence at least 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the sequence motif, wherein the motif comprises, consists essentially of, or consists of 4 to 6 types of amino acids selected from the group consisting of glycine (G), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P). *See* US 2010-0239554 A1.

[0157] In some embodiments, the XTEN comprises non-overlapping sequence motifs in which about 80%, or at least about 85%, or at least about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, or about 99% or about 100% of the sequence consists of multiple units of non-overlapping sequences selected from a single motif family selected from Table 2A, resulting in a family sequence. As used herein, "family" means that the XTEN has motifs selected only from a single motif category from Table 2A; *i.e.,* AD, AE, AF, AG, AM, AQ, BC, or BD XTEN, and that any other amino acids in the XTEN not from a family motif are selected to achieve a needed property, such as to permit incorporation of a restriction site by the encoding nucleotides, incorporation of a cleavage sequence, or to achieve a better linkage to FVIII or VWF. In some embodiments of XTEN families, an XTEN sequence comprises multiple units of non-overlapping sequence motifs of the AD motif family, or of the AE motif family, or of the AF motif family, or of the AG motif family, or of the AM motif family, or of the AQ motif family, or of the BC family,

or of the BD family, with the resulting XTEN exhibiting the range of homology described above. In other embodiments, the XTEN comprises multiple units of motif sequences from two or more of the motif families of Table 2A. These sequences can be selected to achieve desired physical/chemical characteristics, including such properties as net charge, hydrophilicity, lack of secondary structure, or lack of repetitiveness that are conferred by the amino acid composition of the motifs, described more fully below. In the embodiments hereinabove described in this paragraph, the motifs incorporated into the XTEN can be selected and assembled using the methods described herein to achieve an XTEN of about 36 to about 3000 amino acid residues.

Table 2A. XTEN Sequence Motifs of 12 Amino Acids and Motif Families

Motif Family*	MOTIF SEQUENCE
AD	GESPGGSSGSES
AD	GSEGSSGPGESS
AD	GSSESGSSEGGP
AD	GSGGEPSESGSS
AE, AM	GSPAGSPTSTEE
AE, AM, AQ	GSEPATSGSETP
AE, AM, AQ	GTSESATPESGP
AE, AM, AQ	GTSTEPSEGSAP
AF, AM	GSTSESPSGTAP
AF, AM	GTSTPESGSASP
AF, AM	GTSPSGESSTAP
AF, AM	GSTSSTAESPGP
AG, AM	GTPGSGTASSSP
AG, AM	GSSTPSGATGSP
AG, AM	GSSPSASTGTGP
AG, AM	GASPGTSSTGSP
AQ	GEPAGSPTSTSE
AQ	GTGEPSSTPASE
AQ	GSGPSTESAPTE
AQ	GSETPSGPSETA
AQ	GPSETSTSEPGA
AQ	GSPSEPTEGTSA
BC	GSGASEPTSTEP
BC	GSEPATSGTEPS
BC	GTSEPSTSEPGA
BC	GTSTEPSEPGSA
BD	GSTAGSETSTEA
BD	GSETATSGSETA
BD	GTSESATSESGA
BD	GTSTEASEGSAS

- Denotes individual motif sequences that, when used together in various permutations, results in a "family sequence"

[0158] XTEN can have varying lengths for insertion into or linkage to FVIII or VWF. In one embodiment, the length of the XTEN sequence(s) is chosen based on the property or function to be achieved in the fusion protein. Depending on the intended property or function, XTEN can be short or intermediate length sequence or longer sequence that can serve as carriers. In certain embodiments, the XTEN include short segments of about 6 to about 99 amino acid residues, intermediate lengths of about 100 to about 399 amino acid residues, and longer lengths of about 400 to about 1000 and up to about 3000 amino acid residues. Thus, the XTEN inserted into or linked to FVIII or VWF can have lengths of about 6, about 12, about 36, about 40, about 42, about 72, about 96, about 144, about 288, about 400, about 500, about 576, about 600, about 700, about 800, about 864, about 900, about 1000, about 1500, about 2000, about 2500, or up to about 3000 amino acid residues in length. In other embodiments, the XTEN sequences is about 6 to about 50, about 50 to about 100, about 100 to 150, about 150 to 250, about 250 to 400, about 400 to about 500, about 500 to about 900, about 900 to 1500, about 1500 to 2000, or about 2000 to about 3000 amino acid residues in length. The precise length of an XTEN inserted into or linked to FVIII or VWF can vary without adversely affecting the activity of the FVIII or VWF. In one embodiment, one or more of the XTEN used herein has 36 amino acids, 42 amino acids, 72 amino acids, 144 amino acids, 288 amino acids, 576 amino acids, or 864 amino acids in length and can be selected from one or more of the XTEN family sequences; *i.e.,* AD, AE, AF, AG, AM, AQ, BC or BD.

[0159] In some embodiments, the XTEN sequence used in the invention is at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to a sequence selected from the group consisting of AE42, AG42, AE48, AM48, AE72, AG72, AE108, AG108, AE144, AF144, AG144, AE180, AG180, AE216, AG216, AE252, AG252, AE288, AG288, AE324, AG324, AE360, AG360, AE396, AG396, AE432, AG432, AE468, AG468, AE504, AG504, AF504, AE540, AG540, AF540, AD576, AE576, AF576, AG576, AE612, AG612, AE624, AE648, AG648, AG684, AE720, AG720, AE756, AG756, AE792, AG792, AE828, AG828, AD836, AE864, AF864,

AG864, AM875, AE912, AM923, AM1318, BC864, BD864, AE948, AE1044, AE1140, AE1236, AE1332, AE1428, AE1524, AE1620, AE1716, AE1812, AE1908, AE2004A, AG948, AG1044, AG1140, AG1236, AG1332, AG1428, AG1524, AG1620, AG1716, AG1812, AG1908, and AG2004. *See* US 2010-0239554 A1.

[0160] In one embodiment, the XTEN sequence is at least 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of AE42 (SEQ ID NO: 36), AE72 (SEQ ID NO: 127), AE144_2A (SEQ IDNO: 128), AE144_3B (SEQ ID NO: 129), AE144_4A (SEQ ID NO: 130), AE144_5A (SEQ IDNO: 131), AE144_6B (SEQ IDNO: 132), AG144_A (SEQ ID NO: 133), AG144_B (SEQ IDNO: 134), AG144_C (SEQ ID NO: 135), AG144_F (SEQ IDNO: 136), AE864 (SEQ ID NO: 43), AE576 (SEQ ID NO: 41), AE288 (SEQ IDNO: 39), AE288_2 (SEQ ID NO: 137), AE144 (SEQ ID NO: 37), AG864 (SEQ ID NO: 44), AG576 (SEQ ID NO: 42), AG288 (SEQ ID NO: 40), AG144 (SEQ ID NO: 38), and any combinations thereof.

[0161] In some embodiments, less than 100% of amino acids of an XTEN are selected from glycine (G), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P), or less than 100% of the sequence consists of the sequence motifs from Table 2A or the XTEN sequences of Table 2B. In such embodiments, the remaining amino acid residues of the XTEN are selected from any of the other 14 natural L-amino acids, but may be preferentially selected from hydrophilic amino acids such that the XTEN sequence contains at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least about 99% hydrophilic amino acids. The content of hydrophobic amino acids in the XTEN utilized in the conjugation constructs may be less than 5%, or less than 2%, or less than 1% hydrophobic amino acid content. Hydrophobic residues that are less favored in construction of XTEN include tryptophan, phenylalanine, tyrosine, leucine, isoleucine, valine, and methionine. Additionally, XTEN sequences may contain less than 5% or less than 4% or less than 3% or less than 2% or less than 1% or none of the following amino acids: methionine (for example, to avoid oxidation), or asparagine and glutamine (to avoid desamidation).

[0162] In another embodiment, the XTEN sequence is selected from the group consisting of AE42 (SEQ ID NO: 36), AE72 (SEQ ID NO: 127), AE144_2A

(SEQ IDNO: 128), AE144_3B (SEQ ID NO: 129), AE144_4A (SEQ ID NO: 130), AE144_5A (SEQ IDNO: 131), AE144_6B (SEQ IDNO: 132), AG144_A (SEQ ID NO: 133), AG144_B (SEQ IDNO: 134), AG144_C (SEQ ID NO: 135), AG144_F (SEQ IDNO: 136), AE864 (SEQ ID NO: 43), AE576 (SEQ ID NO: 41), AE288 (SEQ IDNO: 39), AE288_2 (SEQ ID NO: 137), AE144 (SEQ ID NO: 37), AG864 (SEQ ID NO: 44), AG576 (SEQ ID NO: 42), AG288 (SEQ ID NO: 40), AG144 (SEQ ID NO: 38), and any combinations thereof. In a specific embodiment, the XTEN sequence is AE288. The amino acid sequences for certain XTEN sequences of the invention are shown in Table 2B.

TABLE 2B. XTEN Sequences

XTEN	Amino Acid Sequence
AE42 SEQ ID NO: 36	GAPGSPAGSPTSTEEGTSESATPESGPGSEPATSGSETPASS
AE72 SEQ ID NO: 127	GAPTSESATPESGPGSEPATSGSETPGTSESATPESGPGSEPA TSGSETPGTSESATPESGPGTSTEPSEGSAPGASS
AE144 SEQ ID NO:37	GSEPATSGSETPGTSESATPESGPGSEPATSGSETPGSPAGSPTSTE EGTSTEPSEG SAPGSEPATSGSETPGSEPATSGSETPGSEPATSGSETPGTSTEPSE GSAPGTSESA PESGPGSEPATSGSETPGTSTEPSEGSAP
AE144_2A (SEQ ID NO: 128)	TSTEPSEGSAPGSPAGSPTSTEEGTSTEPSEGSAPGTSTEPSEGSAP GTSESATPESGPGTSTEPSEGSAPGTSESATPESGPGSEPATSGSET PGTSTEPSEGSAPGTSTEPSEGSAPGTSESATPESGPGTSESATPES GPG
AE144_3B (SEQ ID NO: 129)	SPAGSPTSTEEGTSESATPESGPGSEPATSGSETPGTSESATPESGP GTSTEPSEGSAPGTSTEPSEGSAPGTSTEPSEGSAPGTSTEPSEGSA PGTSTEPSEGSAPGTSTEPSEGSAPGSPAGSPTSTEEGTSTEPSEGS APG
AE144_4A (SEQ ID NO: 130)	TSESATPESGPGSEPATSGSETPGTSESATPESGPGSEPATSGSETP GTSESATPESGPGTSTEPSEGSAPGTSESATPESGPGSPAGSPTSTE EGSPAGSPTSTEEGSPAGSPTSTEEGTSESATPESGPGTSTEPSEGS APG
AE144_5A (SEQ ID NO:	TSESATPESGPGSEPATSGSETPGTSESATPESGPGSEPATSGSETP GTSESATPESGPGTSTEPSEGSAPGSPAGSPTSTEEGTSESATPESG PGSEPATSGSETPGTSESATPESGPGSPAGSPTSTEEGSPAGSPTST

131)	EEG
AE144_6B (SEQ ID NO: 132)	TSTEPSEGSAPGTSESATPESGPGTSESATPESGPGTSESATPESGP GSEPATSGSETPGSEPATSGSETPGSPAGSPTSTEEGTSTEPSEGSA PGTSTEPSEGSAPGSEPATSGSETPGTSESATPESGPGTSTEPSEGS APG
AG144 SEQ ID NO:38	GTPGSGTASSSPGSSTPSGATGSPGSSPSASTGTGPGSSPSASTGTG PGASPGTSST GSPGASPGTSSTGSPGSSTPSGATGSPGSSPSASTGTGPGASPGTSS TGSPGSSPSA STGTGPGTPGSGTASSSPGSSTPSGATGSP
AG144_A (SEQ ID NO: 133)	GASPGTSSTGSPGSSPSASTGTGPGSSPSASTGTGPGTPGSGTASSS PGSSTPSGATGSPGSSPSASTGTGPGASPGTSSTGSPGTPGSGTASS SPGSSTPSGATGSPGTPGSGTASSSPGASPGTSSTGSPGASPGTSST GSP
AG144_B (SEQ ID NO: 134)	GTPGSGTASSSPGSSTPSGATGSPGASPGTSSTGSPGTPGSGTASSS PGSSTPSGATGSPGSSPSASTGTGPGSSPSASTGTGPGSSTPSGATG SPGSSTPSGATGSPGASPGTSSTGSPGASPGTSSTGSPGASPGTSST GSP
AG144_C (SEQ ID NO: 135)	GTPGSGTASSSPGASPGTSSTGSPGASPGTSSTGSPGASPGTSSTGS PGSSPSASTGTGPGTPGSGTASSSPGASPGTSSTGSPGASPGTSSTG SPGASPGTSSTGSPGSSTPSGATGSPGSSTPSGATGSPGASPGTSST GSP
AG144_F (SEQ ID NO: 136)	GSSPSASTGTGPGSSPSASTGTGPGASPGTSSTGSPGASPGTSSTGS PGSSTPSGATGSPGSSPSASTGTGPGASPGTSSTGSPGSSPSASTGT GPGTPGSGTASSSPGSSTPSGATGSPGSSTPSGATGSPGASPGTSST GSP
AE288 SEQ ID NO:39	GTSESATPESGPGSEPATSGSETPGTSESATPESGPGSEPATSGSET PGTSESATPESG PGTSTEPSEGSAPGSPAGSPTSTEEGTSESATPESGPGSEPATSGSE TPGTSESATPES GPGSPAGSPTSTEEGSPAGSPTSTEEGTSTEPSEGSAPGTSESATPE SGPGTSESATPE SGPGTSESATPESGPGSEPATSGSETPGSEPATSGSETPGSPAGSPT STEEGTSTEPSE GSAPGTSTEPSEGSAPGSEPATSGSETPGTSESATPESGPGTSTEPS EGSAP
AE288_2 (SEQ ID NO: 137)	GSPAGSPTSTEEGTSESATPESGPGSEPATSGSETPGTSESATPESG PGTSTEPSEGSAPGTSTEPSEGSAPGTSTEPSEGSAPGTSTEPSEGS APGTSTEPSEGSAPGTSTEPSEGSAPGSPAGSPTSTEEGTSTEPSEG SAPGTSESATPESGPGSEPATSGSETPGTSESATPESGPGSEPATSG SETPGTSESATPESGPGTSTEPSEGSAPGTSESATPESGPGSPAGSP TSTEEGSPAGSPTSTEEGSPAGSPTSTEEGTSESATPESGPGTSTEP SEGSAP
AG288	PGASPGTSSTGSPGASPGTSSTGSPGTPGSGTASSSPGSSTPSGATG

SEQ ID NO:40	SPGTPGSGTASS SPGSSTPSGATGSPGTPGSGTASSSPGSSTPSGATGSPGSSTPSGAT GSPGSSPSASTG TGPGSSPSASTGTGPGASPGTSSTGSPGTPGSGTASSSPGSSTPSGA TGSPGSSPSAST GTGPGSSPSASTGTGPGASPGTSSTGSPGASPGTSSTGSPGSSTPSG ATGSPGSSPSAS TGTGPGASPGTSSTGSPGSSPSASTGTGPGTPGSGTASSSPGSSTPS GATGS
AE576 SEQ ID NO:41	GSPAGSPTSTEEGTSESATPESGPGTSTEPSEGSAPGSPAGSPTSTE EGTSTEPSEGSA PGTSTEPSEGSAPGTSESATPESGPGSEPATSGSETPGSEPATSGSE TPGSPAGSPTST EEGTSESATPESGPGTSTEPSEGSAPGTSTEPSEGSAPGSPAGSPTS TEEGTSTEPSEG SAPGTSTEPSEGSAPGTSESATPESGPGTSTEPSEGSAPGTSESATP ESGPGSEPATSG SETPGTSTEPSEGSAPGTSTEPSEGSAPGTSESATPESGPGTSESAT PESGPGSPAGSP TSTEEGTSESATPESGPGSEPATSGSETPGTSESATPESGPGTSTEP SEGSAPGTSTEP SEGSAPGTSTEPSEGSAPGTSTEPSEGSAPGTSTEPSEGSAPGTSTE PSEGSAPGSPAG SPTSTEEGTSTEPSEGSAPGTSESATPESGPGSEPATSGSETPGTSE SATPESGPGSEP ATSGSETPGTSESATPESGPGTSTEPSEGSAPGTSESATPESGPGSP AGSPTSTEEGSP AGSPTSTEEGSPAGSPTSTEEGTSESATPESGPGTSTEPSEGSAP
AG576 SEQ ID NO:42	PGTPGSGTASSSPGSSTPSGATGSPGSSPSASTGTGPGSSPSASTGT GPGSSTPSGATG SPGSSTPSGATGSPGASPGTSSTGSPGASPGTSSTGSPGASPGTSST GSPGTPGSGTAS SSPGASPGTSSTGSPGASPGTSSTGSPGASPGTSSTGSPGSSPSAST GTGPGTPGSGTA SSSPGASPGTSSTGSPGASPGTSSTGSPGASPGTSSTGSPGSSTPSG ATGSPGSSTPSG ATGSPGASPGTSSTGSPGTPGSGTASSSPGSSTPSGATGSPGSSTPS GATGSPGSSTPS GATGSPGSSPSASTGTGPGASPGTSSTGSPGASPGTSSTGSPGTPGS GTASSSPGASPG TSSTGSPGASPGTSSTGSPGASPGTSSTGSPGASPGTSSTGSPGTPG SGTASSSPGSST PSGATGSPGTPGSGTASSSPGSSTPSGATGSPGTPGSGTASSSPGSS TPSGATGSPGSS TPSGATGSPGSSPSASTGTGPGSSPSASTGTGPGASPGTSSTGSPGT PGSGTASSSPGS STPSGATGSPGSSPSASTGTGPGSSPSASTGTGPGASPGTSSTGS
AE864	GSPAGSPTSTEEGTSESATPESGPGTSTEPSEGSAPGSPAGSPTSTE EGTSTEPSEGSA

SEQ ID NO:43	PGTSTEPSEGSAPGTSESATPESGPGSEPATSGSETPGSEPATSGSE TPGSPAGSPTST EEGTSESATPESGPGTSTEPSEGSAPGTSTEPSEGSAPGSPAGSPTS TEEGTSTEPSEG SAPGTSTEPSEGSAPGTSESATPESGPGTSTEPSEGSAPGTSESATP ESGPGSEPATSG SETPGTSTEPSEGSAPGTSTEPSEGSAPGTSESATPESGPGTSESAT PESGPGSPAGSP TSTEEGTSESATPESGPGSEPATSGSETPGTSESATPESGPGTSTEP SEGSAPGTSTEP SEGSAPGTSTEPSEGSAPGTSTEPSEGSAPGTSTEPSEGSAPGTSTE PSEGSAPGSPAG SPTSTEEGTSTEPSEGSAPGTSESATPESGPGSEPATSGSETPGTSE SATPESGPGSEP ATSGSETPGTSESATPESGPGTSTEPSEGSAPGTSESATPESGPGSP AGSPTSTEEGSP AGSPTSTEEGSPAGSPTSTEEGTSESATPESGPGTSTEPSEGSAPGT SESATPESGPGS EPATSGSETPGTSESATPESGPGSEPATSGSETPGTSESATPESGPG TSTEPSEGSAPG SPAGSPTSTEEGTSESATPESGPGSEPATSGSETPGTSESATPESGP GSPAGSPTSTEE GSPAGSPTSTEEGTSTEPSEGSAPGTSESATPESGPGTSESATPESG PGTSESATPESG PGSEPATSGSETPGSEPATSGSETPGSPAGSPTSTEEGTSTEPSEGS APGTSTEPSEGS APGSEPATSGSETPGTSESATPESGPGTSTEPSEGSAP
AG864 SEQ ID NO:44	GASPGTSSTGSPGSSPSASTGTGPGSSPSASTGTGPGTPGSGTASSS PGSSTPSGATGS PGSSPSASTGTGPGASPGTSSTGSPGTPGSGTASSSPGSSTPSGATG SPGTPGSGTASS SPGASPGTSSTGSPGASPGTSSTGSPGTPGSGTASSSPGSSTPSGAT GSPGASPGTSST GSPGTPGSGTASSSPGSSTPSGATGSPGSSPSASTGTGPGSSPSAST GTGPGSSTPSGA TGSPGSSTPSGATGSPGASPGTSSTGSPGASPGTSSTGSPGASPGTS STGSPGTPGSGT ASSSPGASPGTSSTGSPGASPGTSSTGSPGASPGTSSTGSPGSSPSA STGTGPGTPGSG TASSSPGASPGTSSTGSPGASPGTSSTGSPGASPGTSSTGSPGSSTP SGATGSPGSSTP SGATGSPGASPGTSSTGSPGTPGSGTASSSPGSSTPSGATGSPGSST PSGATGSPGSST PSGATGSPGSSPSASTGTGPGASPGTSSTGSPGASPGTSSTGSPGTP GSGTASSSPGAS PGTSSTGSPGASPGTSSTGSPGASPGTSSTGSPGASPGTSSTGSPGT PGSGTASSSPGS STPSGATGSPGTPGSGTASSSPGSSTPSGATGSPGTPGSGTASSSPG SSTPSGATGSPG SSTPSGATGSPGSSPSASTGTGPGSSPSASTGTGPGASPGTSSTGSP GTPGSGTASSSP

	GSSTPSGATGSPGSSPSASTGTGPGSSPSASTGTGPGASPGTSSTGS PGASPGTSSTGS PGSSTPSGATGSPGSSPSASTGTGPGASPGTSSTGSPGSSPSASTGT GPGTPGSGTASS SPGSSTPSGATGSPGSSTPSGATGSPGASPGTSSTGSP

[0163] In further embodiments, the XTEN sequence used in the invention affects the physical or chemical property, *e.g.,* pharmacokinetics, of the chimeric protein of the present invention. The XTEN sequence used in the present invention can exhibit one or more of the following advantageous properties: conformational flexibility, enhanced aqueous solubility, high degree of protease resistance, low immunogenicity, low binding to mammalian receptors, or increased hydrodynamic (or Stokes) radii. In a specific embodiment, the XTEN sequence linked to a FVIII protein in this invention increases pharmacokinetic properties such as longer terminal half-life or increased area under the curve (AUC), so that the chimeric protein described herein stays *in vivo* for an increased period of time compared to wild type FVIII. In further embodiments, the XTEN sequence used in this invention increases pharmacokinetic properties such as longer terminal half-life or increased area under the curve (AUC), so that FVIII protein stays *in vivo* for an increased period of time compared to wild type FVIII.

[0164] A variety of methods and assays can be employed to determine the physical/chemical properties of proteins comprising the XTEN sequence. Such methods include, but are not limited to analytical centrifugation, EPR, HPLC-ion exchange, HPLC-size exclusion, HPLC-reverse phase, light scattering, capillary electrophoresis, circular dichroism, differential scanning calorimetry, fluorescence, HPLC-ion exchange, HPLC-size exclusion, IR, NMR, Raman spectroscopy, refractometry, and UV/Visible spectroscopy. Additional methods are disclosed in Amau *et al., Prot Expr and Purif* 48, 1-13 (2006).

[0165] Additional examples of XTEN sequences that can be used according to the present invention and are disclosed in US Patent Publication Nos. 2010/0239554 A1, 2010/0323956 A1, 2011/0046060 A1, 2011/0046061 A1, 2011/0077199 A1, or 2011/0172146 A1, or International Patent Publication Nos. WO 2010091122 A1, WO 2010144502 A2, WO 2010144508 A1, WO 2011028228 A1, WO 2011028229 A1, or WO 2011028344 A2.

C) Factor VIII (FVIII) Protein

[0166] "A FVIII protein" as used herein means a functional FVIII polypeptide in its normal role in coagulation, unless otherwise specified. The term a FVIII protein includes a functional fragment, variant, analog, or derivative thereof that retains the function of full-length wild-type Factor VIII in the coagulation pathway. "A FVIII protein" is used interchangeably with FVIII polypeptide (or protein) or FVIII. Examples of the FVIII functions include, but not limited to, an ability to activate coagulation, an ability to act as a cofactor for factor IX, or an ability to form a tenase complex with factor IX in the presence of Ca^{2+} and phospholipids, which then converts Factor X to the activated form Xa. The FVIII protein can be the human, porcine, canine, rat, or murine FVIII protein. In addition, comparisons between FVIII from humans and other species have identified conserved residues that are likely to be required for function (Cameron *et al., Thromb. Haemost.* 79:317-22 (1998); US 6,251,632).

[0167] A number of tests are available to assess the function of the coagulation system: activated partial thromboplastin time (aPTT) test, chromogenic assay, ROTEM assay, prothrombin time (PT) test (also used to determine INR), fibrinogen testing (often by the Clauss method), platelet count, platelet function testing (often by PFA-100), TCT, bleeding time, mixing test (whether an abnormality corrects if the patient's plasma is mixed with normal plasma), coagulation factor assays, antiphospholipid antibodies, D-dimer, genetic tests (*e.g.*, factor V Leiden, prothrombin mutation G20210A), dilute Russell's viper venom time (dRVVT), miscellaneous platelet function tests, thromboelastography (TEG or Sonoclot), thromboelastometry (TEM®, *e.g.*, ROTEM®), or euglobulin lysis time (ELT).

[0168] The aPTT test is a performance indicator measuring the efficacy of both the "intrinsic" (also referred to the contact activation pathway) and the common coagulation pathways. This test is commonly used to measure clotting activity of commercially available recombinant clotting factors, *e.g.*, FVIII or FIX. It is used in conjunction with prothrombin time (PT), which measures the extrinsic pathway.

[0169] ROTEM analysis provides information on the whole kinetics of haemostasis: clotting time, clot formation, clot stability and lysis. The different parameters in thromboelastometry are dependent on the activity of the plasmatic coagulation system, platelet function, fibrinolysis, or many factors which

influence these interactions. This assay can provide a complete view of secondary haemostasis.

[0170] The FVIII polypeptide and polynucleotide sequences are known, as are many functional fragments, mutants and modified versions. Examples of human FVIII sequences (full-length) are shown below.

TABLE 3. Amino Acid Sequence of Full-length Factor VIII

(Full-length FVIII (FVIII signal peptide underlined; FVIII heavy chain is double underlined; B domain is italicized; and FVIII light chain is in plain text)

Signal Peptide: (SEQ ID NO: 3)
MQIELSTCFFLCLLRFCFS

Mature Factor VIII (SEQ ID NO: 4)*
ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSVVYKKTLFVEFT
DHLFNIAKPRPPWMGLLGPTIQAEVYDTVVITLKNMASHPVSLHAVGVSYWKASE
GAEYDDQTSQREKEDDKVFPGGSHTYVWQVLKENGPMASDPLCLTYSYLSHVDLV
KDLNSGLIGALLVCREGSLAKEKTQTLHKFILLFAVFDEGKSWHSETKNSLMQDR
DAASARAWPKMHTVNGYVNRSLPGLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHT
FLVRNHRQASLEISPITFLTAQTLLMDLGQFLLFCHISSHQHDGMEAYVKVDSCP
EEPQLRMKNNEEAEDYDDDLTDSEMDVVRFDDDNSPSFIQIRSVAKKHPKTWVHY
IAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGRKYKKVRFMAYTDETFKTRE
AIQHESGILGPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRPLYSRRLPKGV
KHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGLIGP
LLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAGVQLED
PEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKH
KMVYEDTLTLFPFSGETVFMSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNT
GDYYEDSYEDISAYLLSKNNAIEPR*SFSQNSRHPSTRQKQFNATTIPENDIEKTD*
PWFAHRTPMPKIQNVSSSDLLMLLRQSPTPHGLSLSDLQEAKYETFSDDPSPGAI
DSNNSLSEMTHFRPQLHHSGDMVFTPESGLQLRLNEKLGTTAATELKKLDFKVSS
TSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTTLFGKKSSPLTESGGP
LSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHGPALLTKDNAL
FKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTPL
IHDRMLMDKNATALRLNHMSNKTTSSKNMEMVQQKKEGPIPPDAQNPDMSFFKML
FLPESARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKG
EFTKDVGLKEMVFPSSRNLFLTNLDNLHENNTHNQEKKIQEEIEKKETLIQENVV
LPQIHTVTGTKNFMKNLFLLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRTKKHT
AHFSKKGEEENLEGLGNQTKQIVEKYACTTRISPNTSQQNFVTQRSKRALKQFRL
PLEETELEKRIIVDDTSTQWSKNMKHLTPSTLTQIDYNEKEKGAITQSPLSDCLT
RSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHLPAASYRKKDSGVQE
SSHFLQGAKKNNLSLAILTLEMTGDQREVGSLGTSATNSVTYKKVENTVLPKPDL
PKTSGKVELLPKVHIYQKDLFPTETSNGSPGHLDLVEGSLLQGTEGAIKWNEANR
PGKVPFLRVATESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSQEKSPEKTAFKKK
*DTILSLNACESNHAIAAINEGQNKPEIEVTWAKQGRTERLCSQNPPVLKRHQR*EI
TRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAVER
LWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGLLG

PYIRAEVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGAEPRKNFVKPNETKTY
FWKVQHHMAPTKDEFDCKAWAYFSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQ
VTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKENYRFHAINGYI
MDTLPGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYP
GVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDF
QITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGAR
QKFSSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPII
ARYIRLHPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMF
ATWSPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSM
YVKEFLISSSQDGHQWTLFFQNGKVKVFQGNQDSFTPVVNSLDPPLLTRYLRIHP
QSWVHQIALRMEVLGCEAQDLY

TABLE 4. Nucleotide Sequence Encoding Full-Length FVIII (SEQ ID NO: 5)*

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661                                                    ATG
  CAAATAGAGC TCTCCACCTG
721  CTTCTTTCTG TGCCTTTTGC GATTCTGCTT TAGTGCCACC
  AGAAGATACT ACCTGGGTGC
781  AGTGGAACTG TCATGGGACT ATATGCAAAG TGATCTCGGT
  GAGCTGCCTG TGGACGCAAG
841  ATTTCCTCCT AGAGTGCCAA AATCTTTTCC ATTCAACACC
  TCAGTCGTGT ACAAAAAGAC
901  TCTGTTTGTA GAATTCACGG ATCACCTTTT CAACATCGCT
  AAGCCAAGGC CACCCTGGAT
961  GGGTCTGCTA GGTCCTACCA TCCAGGCTGA GGTTTATGAT
  ACAGTGGTCA TTACACTTAA
1021  GAACATGGCT TCCCATCCTG TCAGTCTTCA TGCTGTTGGT
  GTATCCTACT GGAAAGCTTC
1081  TGAGGGAGCT GAATATGATG ATCAGACCAG TCAAAGGGAG
  AAAGAAGATG ATAAAGTCTT
1141  CCCTGGTGGA AGCCATACAT ATGTCTGGCA GGTCCTGAAA
  GAGAATGGTC CAATGGCCTC
1201  TGACCCACTG TGCCTTACCT ACTCATATCT TTCTCATGTG
  GACCTGGTAA AAGACTTGAA
1261  TTCAGGCCTC ATTGGAGCCC TACTAGTATG TAGAGAAGGG
  AGTCTGGCCA AGGAAAAGAC
1321  ACAGACCTTG CACAAATTTA TACTACTTTT TGCTGTATTT
  GATGAAGGGA AAAGTTGGCA
1381  CTCAGAAACA AAGAACTCCT TGATGCAGGA TAGGGATGCT
  GCATCTGCTC GGGCCTGGCC
1441  TAAAATGCAC ACAGTCAATG GTTATGTAAA CAGGTCTCTG
  CCAGGTCTGA TTGGATGCCA
1501  CAGGAAATCA GTCTATTGGC ATGTGATTGG AATGGGCACC
  ACTCCTGAAG TGCACTCAAT
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1561 ATTCCTCGAA GGTCACACAT TTCTTGTGAG GAACCATCGC
CAGGCGTCCT TGGAAATCTC
1621 GCCAATAACT TTCCTTACTG CTCAAACACT CTTGATGGAC
CTTGGACAGT TTCTACTGTT
1681 TTGTCATATC TCTTCCCACC AACATGATGG CATGGAAGCT
TATGTCAAAG TAGACAGCTG
1741 TCCAGAGGAA CCCCAACTAC GAATGAAAAA TAATGAAGAA
GCGGAAGACT ATGATGATGA
1801 TCTTACTGAT TCTGAAATGG ATGTGGTCAG GTTTGATGAT
GACAACTCTC CTTCCTTTAT
1861 CCAAATTCGC TCAGTTGCCA AGAAGCATCC TAAAACTTGG
GTACATTACA TTGCTGCTGA
1921 AGAGGAGGAC TGGGACTATG CTCCCTTAGT CCTCGCCCCC
GATGACAGAA GTTATAAAAG
1981 TCAATATTTG AACAATGGCC CTCAGCGGAT TGGTAGGAAG
TACAAAAAAG TCCGATTTAT
2041 GGCATACACA GATGAAACCT TTAAGACTCG TGAAGCTATT
CAGCATGAAT CAGGAATCTT
2101 GGGACCTTTA CTTTATGGGG AAGTTGGAGA CACACTGTTG
ATTATATTTA AGAATCAAGC
2161 AAGCAGACCA TATAACATCT ACCCTCACGG AATCACTGAT
GTCCGTCCTT TGTATTCAAG
2221 GAGATTACCA AAAGGTGTAA AACATTTGAA GGATTTTCCA
ATTCTGCCAG GAGAAATATT
2281 CAAATATAAA TGGACAGTGA CTGTAGAAGA TGGGCCAACT
AAATCAGATC CTCGGTGCCT
2341 GACCCGCTAT TACTCTAGTT TCGTTAATAT GGAGAGAGAT
CTAGCTTCAG GACTCATTGG
2401 CCCTCTCCTC ATCTGCTACA AAGAATCTGT AGATCAAAGA
GGAAACCAGA TAATGTCAGA
2461 CAAGAGGAAT GTCATCCTGT TTTCTGTATT TGATGAGAAC
CGAAGCTGGT ACCTCACAGA
2521 GAATATACAA CGCTTTCTCC CCAATCCAGC TGGAGTGCAG
CTTGAGGATC CAGAGTTCCA
2581 AGCCTCCAAC ATCATGCACA GCATCAATGG CTATGTTTTT
GATAGTTTGC AGTTGTCAGT
2641 TTGTTTGCAT GAGGTGGCAT ACTGGTACAT TCTAAGCATT
GGAGCACAGA CTGACTTCCT
2701 TTCTGTCTTC TTCTCTGGAT ATACCTTCAA ACACAAAATG
GTCTATGAAG ACACACTCAC
2761 CCTATTCCCA TTCTCAGGAG AAACTGTCTT CATGTCGATG
GAAAACCCAG GTCTATGGAT
2821 TCTGGGGTGC CACAACTCAG ACTTTCGGAA CAGAGGCATG
ACCGCCTTAC TGAAGGTTTC
2881 TAGTTGTGAC AAGAACACTG GTGATTATTA CGAGGACAGT
TATGAAGATA TTTCAGCATA
2941 CTTGCTGAGT AAAAACAATG CCATTGAACC AAGAAGCTTC
TCCCAGAATT CAAGACACCC
3001 TAGCACTAGG CAAAAGCAAT TTAATGCCAC CACAATTCCA
GAAAATGACA TAGAGAAGAC

3061 TGACCCTTGG TTTGCACACA GAACACCTAT GCCTAAAATA
CAAAATGTCT CCTCTAGTGA
3121 TTTGTTGATG CTCTTGCGAC AGAGTCCTAC TCCACATGGG
CTATCCTTAT CTGATCTCCA
3181 AGAAGCCAAA TATGAGACTT TTTCTGATGA TCCATCACCT
GGAGCAATAG ACAGTAATAA
3241 CAGCCTGTCT GAAATGACAC ACTTCAGGCC ACAGCTCCAT
CACAGTGGGG ACATGGTATT
3301 TACCCCTGAG TCAGGCCTCC AATTAAGATT AAATGAGAAA
CTGGGGACAA CTGCAGCAAC
3361 AGAGTTGAAG AAACTTGATT TCAAAGTTTC TAGTACATCA
AATAATCTGA TTTCAACAAT
3421 TCCATCAGAC AATTTGGCAG CAGGTACTGA TAATACAAGT
TCCTTAGGAC CCCCAAGTAT
3481 GCCAGTTCAT TATGATAGTC AATTAGATAC CACTCTATTT
GGCAAAAAGT CATCTCCCCT
3541 TACTGAGTCT GGTGGACCTC TGAGCTTGAG TGAAGAAAAT
AATGATTCAA AGTTGTTAGA
3601 ATCAGGTTTA ATGAATAGCC AAGAAAGTTC ATGGGGAAAA
AATGTATCGT CAACAGAGAG
3661 TGGTAGGTTA TTTAAAGGGA AAAGAGCTCA TGGACCTGCT
TTGTTGACTA AAGATAATGC
3721 CTTATTCAAA GTTAGCATCT CTTTGTTAAA GACAAACAAA
ACTTCCAATA ATTCAGCAAC
3781 TAATAGAAAG ACTCACATTG ATGGCCCATC ATTATTAATT
GAGAATAGTC CATCAGTCTG
3841 GCAAAATATA TTAGAAAGTG ACACTGAGTT TAAAAAAGTG
ACACCTTTGA TTCATGACAG
3901 AATGCTTATG GACAAAAATG CTACAGCTTT GAGGCTAAAT
CATATGTCAA ATAAAACTAC
3961 TTCATCAAAA AACATGGAAA TGGTCCAACA GAAAAAAGAG
GGCCCCATTC CACCAGATGC
4021 ACAAAATCCA GATATGTCGT TCTTTAAGAT GCTATTCTTG
CCAGAATCAG CAAGGTGGAT
4081 ACAAAGGACT CATGGAAAGA ACTCTCTGAA CTCTGGGCAA
GGCCCCAGTC CAAAGCAATT
4141 AGTATCCTTA GGACCAGAAA AATCTGTGGA AGGTCAGAAT
TTCTTGTCTG AGAAAAACAA
4201 AGTGGTAGTA GGAAAGGGTG AATTTACAAA GGACGTAGGA
CTCAAAGAGA TGGTTTTTCC
4261 AAGCAGCAGA AACCTATTTC TTACTAACTT GGATAATTTA
CATGAAAATA ATACACACAA
4321 TCAAGAAAAA AAAATTCAGG AAGAAATAGA AAAGAAGGAA
ACATTAATCC AAGAGAATGT
4381 AGTTTTGCCT CAGATACATA CAGTGACTGG CACTAAGAAT
TTCATGAAGA ACCTTTTCTT
4441 ACTGAGCACT AGGCAAAATG TAGAAGGTTC ATATGACGGG
GCATATGCTC CAGTACTTCA
4501 AGATTTTAGG TCATTAAATG ATTCAACAAA TAGAACAAAG
AAACACACAG CTCATTTCTC

4561 AAAAAAAGGG GAGGAAGAAA ACTTGGAAGG CTTGGGAAAT
CAAACCAAGC AAATTGTAGA
4621 GAAATATGCA TGCACCACAA GGATATCTCC TAATACAAGC
CAGCAGAATT TTGTCACGCA
4681 ACGTAGTAAG AGAGCTTTGA AACAATTCAG ACTCCCACTA
GAAGAAACAG AACTTGAAAA
4741 AAGGATAATT GTGGATGACA CCTCAACCCA GTGGTCCAAA
AACATGAAAC ATTTGACCCC
4801 GAGCACCCTC ACACAGATAG ACTACAATGA GAAGGAGAAA
GGGGCCATTA CTCAGTCTCC
4861 CTTATCAGAT TGCCTTACGA GGAGTCATAG CATCCCTCAA
GCAAATAGAT CTCCATTACC
4921 CATTGCAAAG GTATCATCAT TTCCATCTAT TAGACCTATA
TATCTGACCA GGGTCCTATT
4981 CCAAGACAAC TCTTCTCATC TTCCAGCAGC ATCTTATAGA
AAGAAAGATT CTGGGGTCCA
5041 AGAAAGCAGT CATTTCTTAC AAGGAGCCAA AAAAAATAAC
CTTTCTTTAG CCATTCTAAC
5101 CTTGGAGATG ACTGGTGATC AAAGAGAGGT TGGCTCCCTG
GGGACAAGTG CCACAAATTC
5161 AGTCACATAC AAGAAAGTTG AGAACACTGT TCTCCCGAAA
CCAGACTTGC CCAAAACATC
5221 TGGCAAAGTT GAATTGCTTC CAAAAGTTCA CATTTATCAG
AAGGACCTAT TCCCTACGGA
5281 AACTAGCAAT GGGTCTCCTG GCCATCTGGA TCTCGTGGAA
GGGAGCCTTC TTCAGGGAAC
5341 AGAGGGAGCG ATTAAGTGGA ATGAAGCAAA CAGACCTGGA
AAAGTTCCCT TTCTGAGAGT
5401 AGCAACAGAA AGCTCTGCAA AGACTCCCTC CAAGCTATTG
GATCCTCTTG CTTGGGATAA
5461 CCACTATGGT ACTCAGATAC CAAAAGAAGA GTGGAAATCC
CAAGAGAAGT CACCAGAAAA
5521 AACAGCTTTT AAGAAAAAGG ATACCATTTT GTCCCTGAAC
GCTTGTGAAA GCAATCATGC
5581 AATAGCAGCA ATAAATGAGG GACAAAATAA GCCCGAAATA
GAAGTCACCT GGGCAAAGCA
5641 AGGTAGGACT GAAAGGCTGT GCTCTCAAAA CCCACCAGTC
TTGAAACGCC ATCAACGGGA
5701 AATAACTCGT ACTACTCTTC AGTCAGATCA AGAGGAAATT
GACTATGATG ATACCATATC
5761 AGTTGAAATG AAGAAGGAAG ATTTTGACAT TTATGATGAG
GATGAAAATC AGAGCCCCCG
5821 CAGCTTTCAA AAGAAAACAC GACACTATTT TATTGCTGCA
GTGGAGAGGC TCTGGGATTA
5881 TGGGATGAGT AGCTCCCCAC ATGTTCTAAG AAACAGGGCT
CAGAGTGGCA GTGTCCCTCA
5941 GTTCAAGAAA GTTGTTTTCC AGGAATTTAC TGATGGCTCC
TTTACTCAGC CCTTATACCG
6001 TGGAGAACTA AATGAACATT TGGGACTCCT GGGGCCATAT
ATAAGAGCAG AAGTTGAAGA

6061 TAATATCATG GTAACTTTCA GAAATCAGGC CTCTCGTCCC
TATTCCTTCT ATTCTAGCCT
6121 TATTTCTTAT GAGGAAGATC AGAGGCAAGG AGCAGAACCT
AGAAAAAACT TTGTCAAGCC
6181 TAATGAAACC AAAACTTACT TTTGGAAAGT GCAACATCAT
ATGGCACCCA CTAAAGATGA
6241 GTTTGACTGC AAAGCCTGGG CTTATTTCTC TGATGTTGAC
CTGGAAAAAG ATGTGCACTC
6301 AGGCCTGATT GGACCCCTTC TGGTCTGCCA CACTAACACA
CTGAACCCTG CTCATGGGAG
6361 ACAAGTGACA GTACAGGAAT TTGCTCTGTT TTTCACCATC
TTTGATGAGA CCAAAAGCTG
6421 GTACTTCACT GAAAATATGG AAAGAAACTG CAGGGCTCCC
TGCAATATCC AGATGGAAGA
6481 TCCCACTTTT AAAGAGAATT ATCGCTTCCA TGCAATCAAT
GGCTACATAA TGGATACACT
6541 ACCTGGCTTA GTAATGGCTC AGGATCAAAG GATTCGATGG
TATCTGCTCA GCATGGGCAG
6601 CAATGAAAAC ATCCATTCTA TTCATTTCAG TGGACATGTG
TTCACTGTAC GAAAAAAAGA
6661 GGAGTATAAA ATGGCACTGT ACAATCTCTA TCCAGGTGTT
TTTGAGACAG TGGAAATGTT
6721 ACCATCCAAA GCTGGAATTT GGCGGGTGGA ATGCCTTATT
GGCGAGCATC TACATGCTGG
6781 GATGAGCACA CTTTTTCTGG TGTACAGCAA TAAGTGTCAG
ACTCCCCTGG GAATGGCTTC
6841 TGGACACATT AGAGATTTTC AGATTACAGC TTCAGGACAA
TATGGACAGT GGGCCCCAAA
6901 GCTGGCCAGA CTTCATTATT CCGGATCAAT CAATGCCTGG
AGCACCAAGG AGCCCTTTTC
6961 TTGGATCAAG GTGGATCTGT TGGCACCAAT GATTATTCAC
GGCATCAAGA CCCAGGGTGC
7021 CCGTCAGAAG TTCTCCAGCC TCTACATCTC TCAGTTTATC
ATCATGTATA GTCTTGATGG
7081 GAAGAAGTGG CAGACTTATC GAGGAAATTC CACTGGAACC
TTAATGGTCT TCTTTGGCAA
7141 TGTGGATTCA TCTGGGATAA AACACAATAT TTTTAACCCT
CCAATTATTG CTCGATACAT
7201 CCGTTTGCAC CCAACTCATT ATAGCATTCG CAGCACTCTT
CGCATGGAGT TGATGGGCTG
7261 TGATTTAAAT AGTTGCAGCA TGCCATTGGG AATGGAGAGT
AAAGCAATAT CAGATGCACA
7321 GATTACTGCT TCATCCTACT TTACCAATAT GTTTGCCACC
TGGTCTCCTT CAAAAGCTCG
7381 ACTTCACCTC CAAGGGAGGA GTAATGCCTG GAGACCTCAG
GTGAATAATC CAAAAGAGTG
7441 GCTGCAAGTG GACTTCCAGA AGACAATGAA AGTCACAGGA
GTAACTACTC AGGGAGTAAA
7501 ATCTCTGCTT ACCAGCATGT ATGTGAAGGA GTTCCTCATC
TCCAGCAGTC AAGATGGCCA

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7561  TCAGTGGACT CTCTTTTTTC AGAATGGCAA AGTAAAGGTT
   TTTCAGGGAA ATCAAGACTC
7621  CTTCACACCT GTGGTGAACT CTCTAGACCC ACCGTTACTG
   ACTCGCTACC TTCGAATTCA
7681  CCCCCAGAGT TGGGTGCACC AGATTGCCCT GAGGATGGAG
   GTTCTGGGCT GCGAGGCACA
7741  GGACCTCTAC
```

*The underlined nucleic acids encode a signal peptide.

[0171] FVIII polypeptides include full-length FVIII, full-length FVIII minus Met at the N-terminus, mature FVIII (minus the signal sequence), mature FVIII with an additional Met at the N-terminus, and/or FVIII with a full or partial deletion of the B domain. In certain embodiments, FVIII variants include B domain deletions, whether partial or full deletions.

[0172] The sequence of native mature human FVIII is presented as SEQ ID NO: 4. A native FVIII protein has the following formula: A1-a1-A2-a2-B-a3-A3-C1-C2, where A1, A2, and A3 are the structurally-related "A domains," B is the "B domain," C1 and C2 are the structurally-related "C domains," and a1, a2 and a3 are acidic spacer regions. Referring to the primary amino acid sequence position in SEQ ID NO:4, the A1 domain of human FVIII extends from Ala1 to about Arg336, the a1 spacer region extends from about Met337 to about Val374, the A2 domain extends from about Ala375 to about Tyr719, the a2 spacer region extends from about Glu720 to about Arg740, the B domain extends from about Ser741 to about Arg 1648, the a3 spacer region extends from about Glu1649 to about Arg1689, the A3 domain extends from about Ser1690 to about Leu2025, the C1 domain extends from about Gly2026 to about Asn2072, and the C2 domain extends from about Ser2073 to Tyr2332. Other than specific proteolytic cleavage sites, designation of the locations of the boundaries between the domains and regions of FVIII can vary in different literature references. The boundaries noted herein are therefore designated as approximate by use of the term "about."

[0173] The human FVIII gene was isolated and expressed in mammalian cells (Toole, J. J., *et al., Nature* 312:342-347 (1984); Gitschier, J., *et al., Nature* 312:326-330 (1984); Wood, W. I., *et al., Nature* 312:330-337 (1984); Vehar, G. A., *et al., Nature* 312:337-342 (1984); WO 87/04187; WO 88/08035; WO 88/03558; and U.S. Pat. No. 4,757,006). The FVIII amino acid sequence was

deduced from cDNA as shown in U.S. Pat. No. 4,965,199. In addition, partially or fully B-domain deleted FVIII is shown in U.S. Pat. Nos. 4,994,371 and 4,868,112. In some embodiments, the human FVIII B-domain is replaced with the human Factor V B-domain as shown in U.S. Pat. No. 5,004,803. The cDNA sequence encoding human Factor VIII and amino acid sequence are shown in SEQ ID NOs: 4 and 5, respectively, of US Application Publ. No. 2005/0100990.

[0174] The porcine FVIII sequence is published in Toole, J. J., *et al., Proc. Natl. Acad. Sci. USA* 83:5939-5942 (1986). Further, the complete porcine cDNA sequence obtained from PCR amplification of FVIII sequences from a pig spleen cDNA library has been reported in Healey, J. F., *et al., Blood* 88:4209-4214 (1996). Hybrid human/porcine FVIII having substitutions of all domains, all subunits, and specific amino acid sequences were disclosed in U.S. Pat. No. 5,364,771 by Lollar and Runge, and in WO 93/20093. More recently, the nucleotide and corresponding amino acid sequences of the A1 and A2 domains of porcine FVIII and a chimeric FVIII with porcine A1 and/or A2 domains substituted for the corresponding human domains were reported in WO 94/11503. U.S. Pat. No. 5,859,204, Lollar, J. S., also discloses the porcine cDNA and deduced amino acid sequences. U.S. Pat. No. 6,458,563 discloses a B-domain-deleted porcine FVIII.

[0175] U.S. Pat. No. 5,859,204 to Lollar, J. S. reports functional mutants of FVIII having reduced antigenicity and reduced immunoreactivity. U.S. Pat. No. 6,376,463 to Lollar, J. S. also reports mutants of FVIII having reduced immunoreactivity. US Appl. Publ. No. 2005/0100990 to Saenko *et al.* reports functional mutations in the A2 domain of FVIII.

[0176] In one embodiment, the FVIII (or FVIII portion of a chimeric protein) may be at least 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to a FVIII amino acid sequence of amino acids 1 to 1438 of SEQ ID NO: 6 or amino acids 1 to 2332 of SEQ ID NO: 4 (without a signal sequence) or a FVIII amino acid sequence of amino acids 1 to 19 of SEQ ID NO: 3 and 1 to 1438 of SEQ ID NO: 6 or amino acids 1 to 19 of SEQ ID NO: 3 and amino acids 1 to 2332 of SEQ ID NO: 4 (with a signal sequence), wherein the FVIII has a clotting activity, *e.g.*, activates Factor IX as a cofactor to convert Factor X to activated Factor X. The FVIII (or FVIII portion of a chimeric protein) may be identical to a

FVIII amino acid sequence of amino acids 1 to 1438 of SEQ ID NO: 6 or amino acids 1 to 2332 of SEQ ID NO: 4 (without a signal sequence). The FVIII may further comprise a signal sequence.

[0177] The "B-domain" of FVIII, as used herein, is the same as the B-domain known in the art that is defined by internal amino acid sequence identity and sites of proteolytic cleavage, *e.g.*, residues Ser741-Arg1648 of full-length human FVIII. The other human FVIII domains are defined by the following amino acid residues: A1, residues Ala1-Arg372; A2, residues Ser373-Arg740; A3, residues Ser1690-Asn2019; C1, residues Lys2020-Asn2172; C2, residues Ser2173-Tyr2332. The A3-C1-C2 sequence includes residues Ser1690-Tyr2332. The remaining sequence, residues Glu1649-Arg1689, is usually referred to as the a3 acidic region. The locations of the boundaries for all of the domains, including the B-domains, for porcine, mouse and canine FVIII are also known in the art. In one embodiment, the B domain of FVIII is deleted ("B-domain-deleted factor VIII" or "BDD FVIII"). An example of a BDD FVIII is REFACTO® (recombinant BDD FVIII), which has the same sequence as the Factor VIII portion of the sequence in Table 5. (BDD FVIII heavy chain is double underlined; B domain is italicized; and BDD FVIII light chain is in plain text). A nucleotide sequence encoding the amino acid sequence set forth in Table 5 (SEQ ID NO: 7) is shown in Table 6.

TABLE 5. Amino Acid Sequence of B-domain Deleted Factor VIII (BDD FVIII)

BDD FVIII (SEQ ID NO: 6)

ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSVVYKKTLFVEFT
DHLFNIAKPRPPWMGLLGPTIQAEVYDTVVITLKNMASHPVSLHAVGVSYWKASE
GAEYDDQTSQREKEDDKVFPGGSHTYVWQVLKENGPMASDPLCLTYSYLSHVDLV
KDLNSGLIGALLVCREGSLAKEKTQTLHKFILLFAVFDEGKSWHSETKNSLMQDR
DAASARAWPKMHTVNGYVNRSLPGLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHT
FLVRNHRQASLEISPITFLTAQTLLMDLGQFLLFCHISSHQHDGMEAYVKVDSCP
EEPQLRMKNNEEAEDYDDDLTDSEMDVVRFDDDNSPSFIQIRSVAKKHPKTWVHY
IAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGRKYKKVRFMAYTDETFKTRE
AIQHESGILGPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRPLYSRRLPKGV
KHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGLIGP
LLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAGVQLED
PEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKH
KMVYEDTLTLFPFSGETVFMSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNT
GDYYEDSYEDISAYLLSKNNAIEPR*SFSQNPPVLKRHQR*EITRTTLQSDQEEIDY
DDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAVERLWDYGMSSSPHVLR

NRAQSGSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVT
FRNQASRPYSFYSSLISYEEDQRQGAEPRKNFVKPNETKTYFWKVQHHMAPTKDE
FDCKAWAYFSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFD
ETKSWYFTENMERNCRAPCNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQR
IRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAG
IWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPK
LARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIM
YSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIR
STLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFATWSPSKARLHLQG
RSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQDGH
QWTLFFQNGKVKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVL
GCEAQDLY

TABLE 6. Nucleotide Sequence Encoding BDD FVIII (SEQ ID NO: 7)*

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661 A TGCAAATAGA
GCTCTCCACC TGCTTCTTTC
721 TGTGCCTTTT GCGATTCTGC TTTAGTGCCA CCAGAAGATA
CTACCTGGGT GCAGTGGAAC
781 TGTCATGGGA CTATATGCAA AGTGATCTCG GTGAGCTGCC
TGTGGACGCA AGATTTCCTC
841 CTAGAGTGCC AAAATCTTTT CCATTCAACA CCTCAGTCGT
GTACAAAAAG ACTCTGTTTG
901 TAGAATTCAC GGATCACCTT TTCAACATCG CTAAGCCAAG
GCCACCCTGG ATGGGTCTGC
961 TAGGTCCTAC CATCCAGGCT GAGGTTTATG ATACAGTGGT
CATTACACTT AAGAACATGG
1021 CTTCCCATCC TGTCAGTCTT CATGCTGTTG GTGTATCCTA
CTGGAAAGCT TCTGAGGGAG
1081 CTGAATATGA TGATCAGACC AGTCAAAGGG AGAAAGAAGA
TGATAAAGTC TTCCCTGGTG
1141 GAAGCCATAC ATATGTCTGG CAGGTCCTGA AAGAGAATGG
TCCAATGGCC TCTGACCCAC
1201 TGTGCCTTAC CTACTCATAT CTTTCTCATG TGGACCTGGT
AAAAGACTTG AATTCAGGCC
1261 TCATTGGAGC CCTACTAGTA TGTAGAGAAG GGAGTCTGGC
CAAGGAAAAG ACACAGACCT
1321 TGCACAAATT TATACTACTT TTTGCTGTAT TTGATGAAGG
GAAAAGTTGG CACTCAGAAA
1381 CAAAGAACTC CTTGATGCAG GATAGGGATG CTGCATCTGC
TCGGGCCTGG CCTAAAATGC
1441 ACACAGTCAA TGGTTATGTA AACAGGTCTC TGCCAGGTCT
GATTGGATGC CACAGGAAAT
1501 CAGTCTATTG GCATGTGATT GGAATGGGCA CCACTCCTGA
AGTGCACTCA ATATTCCTCG
1561 AAGGTCACAC ATTTCTTGTG AGGAACCATC GCCAGGCGTC
CTTGGAAATC TCGCCAATAA
1621 CTTTCCTTAC TGCTCAAACA CTCTTGATGG ACCTTGGACA
GTTTCTACTG TTTTGTCATA

1681 TCTCTTCCCA CCAACATGAT GGCATGGAAG CTTATGTCAA
AGTAGACAGC TGTCCAGAGG
1741 AACCCCAACT ACGAATGAAA AATAATGAAG AAGCGGAAGA
CTATGATGAT GATCTTACTG
1801 ATTCTGAAAT GGATGTGGTC AGGTTTGATG ATGACAACTC
TCCTTCCTTT ATCCAAATTC
1861 GCTCAGTTGC CAAGAAGCAT CCTAAAACTT GGGTACATTA
CATTGCTGCT GAAGAGGAGG
1921 ACTGGGACTA TGCTCCCTTA GTCCTCGCCC CCGATGACAG
AAGTTATAAA AGTCAATATT
1981 TGAACAATGG CCCTCAGCGG ATTGGTAGGA AGTACAAAAA
AGTCCGATTT ATGGCATACA
2041 CAGATGAAAC CTTTAAGACT CGTGAAGCTA TTCAGCATGA
ATCAGGAATC TTGGGACCTT
2101 TACTTTATGG GGAAGTTGGA GACACACTGT TGATTATATT
TAAGAATCAA GCAAGCAGAC
2161 CATATAACAT CTACCCTCAC GGAATCACTG ATGTCCGTCC
TTTGTATTCA AGGAGATTAC
2221 CAAAAGGTGT AAAACATTTG AAGGATTTTC CAATTCTGCC
AGGAGAAATA TTCAAATATA
2281 AATGGACAGT GACTGTAGAA GATGGGCCAA CTAAATCAGA
TCCTCGGTGC CTGACCCGCT
2341 ATTACTCTAG TTTCGTTAAT ATGGAGAGAG ATCTAGCTTC
AGGACTCATT GGCCCTCTCC
2401 TCATCTGCTA CAAAGAATCT GTAGATCAAA GAGGAAACCA
GATAATGTCA GACAAGAGGA
2461 ATGTCATCCT GTTTTCTGTA TTTGATGAGA ACCGAAGCTG
GTACCTCACA GAGAATATAC
2521 AACGCTTTCT CCCCAATCCA GCTGGAGTGC AGCTTGAGGA
TCCAGAGTTC CAAGCCTCCA
2581 ACATCATGCA CAGCATCAAT GGCTATGTTT TTGATAGTTT
GCAGTTGTCA GTTTGTTTGC
2641 ATGAGGTGGC ATACTGGTAC ATTCTAAGCA TTGGAGCACA
GACTGACTTC CTTTCTGTCT
2701 TCTTCTCTGG ATATACCTTC AAACACAAAA TGGTCTATGA
AGACACACTC ACCCTATTCC
2761 CATTCTCAGG AGAAACTGTC TTCATGTCGA TGGAAAACCC
AGGTCTATGG ATTCTGGGGT
2821 GCCACAACTC AGACTTTCGG AACAGAGGCA TGACCGCCTT
ACTGAAGGTT TCTAGTTGTG
2881 ACAAGAACAC TGGTGATTAT TACGAGGACA GTTATGAAGA
TATTTCAGCA TACTTGCTGA
2941 GTAAAAACAA TGCCATTGAA CCAAGAAGCT TCTCTCAAAA
CCCACCAGTC TTGAAACGCC
3001 ATCAACGGGA AATAACTCGT ACTACTCTTC AGTCAGATCA
AGAGGAAATT GACTATGATG
3061 ATACCATATC AGTTGAAATG AAGAAGGAAG ATTTTGACAT
TTATGATGAG GATGAAAATC
3121 AGAGCCCCCG CAGCTTTCAA AAGAAAACAC GACACTATTT
TATTGCTGCA GTGGAGAGGC

3181 TCTGGGATTA TGGGATGAGT AGCTCCCCAC ATGTTCTAAG
AAACAGGGCT CAGAGTGGCA
3241 GTGTCCCTCA GTTCAAGAAA GTTGTTTTCC AGGAATTTAC
TGATGGCTCC TTTACTCAGC
3301 CCTTATACCG TGGAGAACTA AATGAACATT TGGGACTCCT
GGGGCCATAT ATAAGAGCAG
3361 AAGTTGAAGA TAATATCATG GTAACTTTCA GAAATCAGGC
CTCTCGTCCC TATTCCTTCT
3421 ATTCTAGCCT TATTTCTTAT GAGGAAGATC AGAGGCAAGG
AGCAGAACCT AGAAAAAACT
3481 TTGTCAAGCC TAATGAAACC AAAACTTACT TTTGGAAAGT
GCAACATCAT ATGGCACCCA
3541 CTAAAGATGA GTTTGACTGC AAAGCCTGGG CTTATTTCTC
TGATGTTGAC CTGGAAAAAG
3601 ATGTGCACTC AGGCCTGATT GGACCCCTTC TGGTCTGCCA
CACTAACACA CTGAACCCTG
3661 CTCATGGGAG ACAAGTGACA GTACAGGAAT TTGCTCTGTT
TTTCACCATC TTTGATGAGA
3721 CCAAAAGCTG GTACTTCACT GAAAATATGG AAAGAAACTG
CAGGGCTCCC TGCAATATCC
3781 AGATGGAAGA TCCCACTTTT AAAGAGAATT ATCGCTTCCA
TGCAATCAAT GGCTACATAA
3841 TGGATACACT ACCTGGCTTA GTAATGGCTC AGGATCAAAG
GATTCGATGG TATCTGCTCA
3901 GCATGGGCAG CAATGAAAAC ATCCATTCTA TTCATTTCAG
TGGACATGTG TTCACTGTAC
3961 GAAAAAAAGA GGAGTATAAA ATGGCACTGT ACAATCTCTA
TCCAGGTGTT TTTGAGACAG
4021 TGGAAATGTT ACCATCCAAA GCTGGAATTT GGCGGGTGGA
ATGCCTTATT GGCGAGCATC
4081 TACATGCTGG GATGAGCACA CTTTTTCTGG TGTACAGCAA
TAAGTGTCAG ACTCCCCTGG
4141 GAATGGCTTC TGGACACATT AGAGATTTTC AGATTACAGC
TTCAGGACAA TATGGACAGT
4201 GGGCCCCAAA GCTGGCCAGA CTTCATTATT CCGGATCAAT
CAATGCCTGG AGCACCAAGG
4261 AGCCCTTTTC TTGGATCAAG GTGGATCTGT TGGCACCAAT
GATTATTCAC GGCATCAAGA
4321 CCCAGGGTGC CCGTCAGAAG TTCTCCAGCC TCTACATCTC
TCAGTTTATC ATCATGTATA
4381 GTCTTGATGG GAAGAAGTGG CAGACTTATC GAGGAAATTC
CACTGGAACC TTAATGGTCT
4441 TCTTTGGCAA TGTGGATTCA TCTGGGATAA AACACAATAT
TTTTAACCCT CCAATTATTG
4501 CTCGATACAT CCGTTTGCAC CCAACTCATT ATAGCATTCG
CAGCACTCTT CGCATGGAGT
4561 TGATGGGCTG TGATTTAAAT AGTTGCAGCA TGCCATTGGG
AATGGAGAGT AAAGCAATAT
4621 CAGATGCACA GATTACTGCT TCATCCTACT TTACCAATAT
GTTTGCCACC TGGTCTCCTT

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4681  CAAAAGCTCG ACTTCACCTC CAAGGGAGGA GTAATGCCTG
   GAGACCTCAG GTGAATAATC
4741  CAAAAGAGTG GCTGCAAGTG GACTTCCAGA AGACAATGAA
   AGTCACAGGA GTAACTACTC
4801  AGGGAGTAAA ATCTCTGCTT ACCAGCATGT ATGTGAAGGA
   GTTCCTCATC TCCAGCAGTC
4861  AAGATGGCCA TCAGTGGACT CTCTTTTTTC AGAATGGCAA
   AGTAAAGGTT TTTCAGGGAA
4921  ATCAAGACTC CTTCACACCT GTGGTGAACT CTCTAGACCC
   ACCGTTACTG ACTCGCTACC
4981  TTCGAATTCA CCCCCAGAGT TGGGTGCACC AGATTGCCCT
   GAGGATGGAG GTTCTGGGCT
5041  GCGAGGCACA GGACCTCTAC
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*The underlined nucleic acids encode a signal peptide.

[0178] A "B-domain-deleted FVIII" may have the full or partial deletions disclosed in U.S. Pat. Nos. 6,316,226, 6,346,513, 7,041,635, 5,789,203, 6,060,447, 5,595,886, 6,228,620, 5,972,885, 6,048,720, 5,543,502, 5,610,278, 5,171,844, 5,112,950, 4,868,112, and 6,458,563. In some embodiments, a B-domain-deleted FVIII sequence of the present invention comprises any one of the deletions disclosed at col. 4, line 4 to col. 5, line 28 and Examples 1-5 of U.S. Pat. No. 6,316,226 (also in US 6,346,513). In another embodiment, a B-domain deleted Factor VIII is the S743/Q1638 B-domain deleted Factor VIII (SQ BDD FVIII) (*e.g.*, Factor VIII having a deletion from amino acid 744 to amino acid 1637, *e.g.*, Factor VIII having amino acids 1-743 and amino acids 1638-2332 of SEQ ID NO: 4, *i.e.*, SEQ ID NO: 6). In some embodiments, a B-domain-deleted FVIII of the present invention has a deletion disclosed at col. 2, lines 26-51 and examples 5-8 of U.S. Patent No. 5,789,203 (also US 6,060,447, US 5,595,886, and US 6,228,620). In some embodiments, a B-domain-deleted Factor VIII has a deletion described in col. 1, lines 25 to col. 2, line 40 of US Patent No. 5,972,885; col. 6, lines 1-22 and example 1 of U.S. Patent no. 6,048,720; col. 2, lines 17-46 of U.S. Patent No. 5,543,502; col. 4, line 22 to col. 5, line 36 of U.S. Patent no. 5,171,844; col. 2, lines 55-68, figure 2, and example 1 of U.S. Patent No. 5,112,950; col. 2, line 2 to col. 19, line 21 and table 2 of U.S. Patent No. 4,868,112; col. 2, line 1 to col. 3, line 19, col. 3, line 40 to col. 4, line 67, col. 7, line 43 to col. 8, line 26, and col. 11, line 5 to col. 13, line 39 of U.S. Patent no. 7,041,635; or col. 4, lines 25-53, of U.S. Patent No. 6,458,563. In some embodiments, a B-domain-deleted FVIII has a deletion of most of the B domain, but still contains amino-terminal

sequences of the B domain that are essential for *in vivo* proteolytic processing of the primary translation product into two polypeptide chain, as disclosed in WO 91/09122. In some embodiments, a B-domain-deleted FVIII is constructed with a deletion of amino acids 747-1638, *i.e.*, virtually a complete deletion of the B domain. Hoeben R.C., *et al. J. Biol. Chem.* 265 (13): 7318-7323 (1990). A B-domain-deleted Factor VIII may also contain a deletion of amino acids 771–1666 or amino acids 868-1562 of FVIII. Meulien P., *et al. Protein Eng.* 2(4): 301-6 (1988). Additional B domain deletions that are part of the invention include: deletion of amino acids 982 through 1562 or 760 through 1639 (Toole *et al., Proc. Natl. Acad. Sci. U.S.A.* (1986) 83, 5939-5942)), 797 through 1562 (Eaton, *et al. Biochemistry* (1986) 25:8343-8347)), 741 through 1646 (Kaufman (PCT published application No. WO 87/04187)), 747-1560 (Sarver, *et al., DNA* (1987) 6:553-564)), 741 through 1648 (Pasek (PCT application No.88/00831)), or 816 through 1598 or 741 through 1648 (Lagner (Behring Inst. Mitt. (1988) No 82:16-25, EP 295597)). In other embodiments, BDD FVIII includes a FVIII polypeptide containing fragments of the B-domain that retain one or more N-linked glycosylation sites, *e.g.,* residues 757, 784, 828, 900, 963, or optionally 943, which correspond to the amino acid sequence of the full-length FVIII sequence. Examples of the B-domain fragments include 226 amino acids or 163 amino acids of the B-domain as disclosed in Miao, H.Z., *et al., Blood* 103(a): 3412-3419 (2004), Kasuda, A, *et al., J. Thromb. Haemost.* 6: 1352-1359 (2008), and Pipe, S.W., *et al., J. Thromb. Haemost.* 9: 2235-2242 (2011) (*i.e.,* the first 226 amino acids or 163 amino acids of the B domain are retained). In still other embodiments, BDD FVIII further comprises a point mutation at residue 309 (from Phe to Ser) to improve expression of the BDD FVIII protein. *See* Miao, H.Z., et al., Blood 103(a): 3412-3419 (2004). In still other embodiments, the BDD FVIII includes a FVIII polypeptide containing a portion of the B-domain, but not containing one or more furin cleavage sites (*e.g.,* Arg1313 and Arg 1648). *See* Pipe, S.W., *et al., J. Thromb. Haemost.* 9: 2235-2242 (2011). Each of the foregoing deletions may be made in any FVIII sequence.

[0179] In some embodiments, the FVIII has a partial B-domain. In some embodiments, the FVIII protein with a partial B-domain is FVIII198 (SEQ ID NO: 89). FVIII198 is a partial B-domain containing single chain FVIIIFc

molecule-226N6. 226 represents the N-terminus 226 amino acid of the FVIII B-domain, and N6 represents six N-glycosylation sites in the B-domain.

[0180] In one embodiment, FVIII is cleaved right after arginine at amino acid 1648 (in full-length Factor VIII or SEQ ID NO: 4), amino acid 754 (in the S743/Q1638 B-domain deleted Factor VIII or SEQ ID NO: 6), or the corresponding arginine residue (in other variants), thereby resulting in a heavy chain and a light chain. In another embodiment, FVIII comprises a heavy chain and a light chain, which are linked or associated by a metal ion-mediated non-covalent bond.

[0181] In other embodiments, FVIII is a single chain FVIII that has not been cleaved right after Arginine at amino acid 1648 (in full-length FVIII or SEQ ID NO: 4), amino acid 754 (in the S743/Q1638 B-domain-deleted FVIII or SEQ ID NO: 6), or the corresponding Arginine residue (in other variants). A single chain FVIII may comprise one or more amino acid substitutions. In one embodiment, the amino acid substitution is at a residue corresponding to residue 1648, residue 1645, or both of full-length mature Factor VIII polypeptide (SEQ ID NO: 4) or residue 754, residue 751, or both of SQ BDD Factor VIII (SEQ ID NO: 6). The amino acid substitution can be any amino acids other than arginine, *e.g.*, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, selenocysteine, serine, tyrosine, histidine, ornithine, pyrrolysine, or taurine.

[0182] FVIII can further be cleaved by thrombin and then activated as FVIIIa, serving as a cofactor for activated Factor IX (FIXa). And the activated FIX together with activated FVIII forms a Xase complex and converts Factor X to activated Factor X (FXa). For activation, FVIII is cleaved by thrombin after three Arginine residues, at amino acids 372, 740, and 1689 (corresponding to amino acids 372, 740, and 795 in the B-domain deleted FVIII sequence), the cleavage generating FVIIIa having the 50kDa A1, 43kDa A2, and 73kDa A3-C1-C2 chains. In one embodiment, the FVIII protein useful for the present invention is non-active FVIII. In another embodiment, the FVIII protein is an activated FVIII.

[0183] The protein having FVIII polypeptide linked to or associated with the VWF fragment can comprise a sequence at least 50%, 60%, 70%, 80%, 90%,

95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 4 or 6, wherein the sequence has the FVIII clotting activity, *e.g.*, activating Factor IX as a cofactor to convert Factor X to activated Factor X (FXa).

[0184] "Hybrid" or "chimeric" polypeptides and proteins, as used herein, includes a combination of a first polypeptide chain, *e.g.*, the VWF fragment, optionally fused to a first Ig constant region or a portion thereof, with a second polypeptide chain, *e.g.*, a FVIII protein linked to an XTEN sequence, optionally fused to a second Ig constant region or a portion thereof, thereby forming a heterodimer. In one embodiment, the first polypeptide and the second polypeptide in a hybrid are associated with each other via protein-protein interactions, such as charge-charge or hydrophobic interactions. In another embodiment, the first polypeptide and the second polypeptide in a hybrid are associated with each other via disulfide or other covalent bond(s). Hybrids are described, for example, in US 2004/101740 and US 2006/074199. The second polypeptide may be an identical copy of the first polypeptide or a non-identical polypeptide. In one embodiment, the first polypeptide is a FVIII protein(X)-Fc fusion protein, and the second polypeptide is a polypeptide comprising, consisting essentially of, or consisting of an Fc region, wherein the first polypeptide and the second polypeptide are associated with each other. In another embodiment, the first polypeptide comprises a VWF fragment-XTEN-Fc fusion protein, and the second polypeptide comprises FVIII-Fc fusion protein, making the hybrid a heterodimer. In other embodiments, the first polypeptide comprises a VWF fragment-Fc fusion protein, and the second polypeptide comprises FVIII(X)-Fc fusion protein, making the hybrid a heterodimer. In yet other embodiments, the first polypeptide comprises a VWF fragment-XTEN-Fc fusion protein, and the second polypeptide comprises FVIII(X)-Fc fusion protein. The first polypeptide and the second polypeptide can be associated through a covalent bond, *e.g.*, a disulfide bond, between the first Fc region and the second Fc region. The first polypeptide and the second polypeptide can further be associated with each other by binding between the VWF fragment and the FVIII protein.

[0185] A FVIII protein useful in the present invention can include FVIII having one or more additional XTEN sequences, which do not affect the FVIII coagulation activity. Such XTEN sequences can be fused to the C-terminus or N-

terminus of the FVIII protein or inserted between one or more of the two amino acid residues in the FVIII protein wherein the insertions do not affect the FVIII coagulation activity or FVIII function. In one embodiment, the insertions improve pharmacokinetic properties of the FVIII protein (*e.g.,* half-life). In another embodiment, the insertions can be multiple insertions, *e.g.,* more than two, three, four, five, six, seven, eight, nine, or ten insertions. Examples of the insertion sites include, but are not limited to, the sites listed in Tables 7, 8, 9, 10, 11, 12, 13, 14, 15 or any combinations thereof.

[0186] The FVIII protein linked to one or more XTEN sequences can be represented as FVIII(X), FVIII(X1), $FVIII_{(a\rightarrow b)}$-X-$FVIII_{(c\rightarrow d)}$, wherein $FVIII_{(a\rightarrow b)}$ comprises, consists essentially of, or consists of a first portion of a FVIII protein from amino acid residue "a" to amino acid residue "b"; X or X1 comprises, consists essentially of, or consists of one or more XTEN sequences, $FVIII_{(c\rightarrow d)}$ comprises, consists essentially of, or consists of a second portion of a FVIII protein from amino acid residue "c" to amino acid residue "d";
a is the N-terminal amino acid residue of the first portion of the FVIII protein,
b is the C-terminal amino acid residue of the first portion of the FVIII protein but is also the N-terminal amino acid residue of the two amino acids of an insertion site in which the XTEN sequence is inserted,
c is the N-terminal amino acid residue of the second portion of the FVIII protein but is also the C-terminal amino acid residue of the two amino acids of an insertion site in which the XTEN sequence is inserted, and
d is the C-terminal amino acid residue of the FVIII protein, and
wherein the first portion of the FVIII protein and the second portion of the FVIII protein are not identical to each other and are of sufficient length together such that the FVIII protein has a FVIII coagulation activity.

[0187] In one embodiment, the first portion of the FVIII protein and the second portion of the FVIII protein are fragments of SEQ ID NO: 4 [full length mature FVIII sequence] or SEQ ID NO: 6 [B-domain deleted FVIII], *e.g.,* N-terminal portion and C-terminal portion, respectively. In certain embodiments, the first portion of the FVIII protein comprises the A1 domain and the A2 domain of the FVIII protein. The second portion of the FVIII protein comprises the A3 domain, the C1 domain, and optionally the C2 domain. In yet other embodiments, the first

portion of the FVIII protein comprises the A1 domain and A2 domain, and the second portion of the FVIII protein comprises a portion of the B domain, the A3 domain, the C1 domain, and optionally the C2 domain. In still other embodiments, the first portion of the FVIII protein comprises the A1 domain, A2 domain, and a portion of the B domain of the FVIII protein, and the second portion of the FVIII protein comprises the A3 domain, the C1 domain, and optionally the C2 domain. In still other embodiments, the first portion of the FVIII protein comprises the A1 domain, A2 domain, and a first portion of the B domain of the FVIII protein. The second portion of the FVIII protein comprises a second portion of the B domain, the A3 domain, the C1 domain, and optionally the C2 domain. In some embodiments, the two amino acids ("b" and "c") can be any one or more of the amino acid residues insertion sites shown in Tables 7, 8, 9, 10, 11, 12, 13, 14, and 15. For example, "b" can be the amino acid residue immediately upstream of the site in which one or more XTEN sequences are inserted or linked, and "c" can be the amino acid residue immediately downstream of the site in which the one or more XTEN sequences are inserted or linked. In some embodiments, "a" is the first mature amino acid sequence of a FVIII protein, and "d" is the last amino acid sequence of a FVIII protein. For example, $FVIII_{(a \rightarrow b)}$ can be an amino acid sequence at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 1 to 745 of SEQ ID NO: 6 [B domain deleted FVIII amino acid sequence] or SEQ ID NO: 4 [full length FVIII] and $FVIII_{(c \rightarrow d)}$ can be amino acids 746 to 1438 of SEQ ID NO: 6 or amino acids 1641 to 2332 of SEQ ID NO: 4, respectively.

[0188] In some aspects, the insertion site in the FVIII protein is located in one or more domains of the FVIII protein, which is the N-terminus, the A1 domain, the A2 domain, the A3 domain, the B domain, the C1 domain, the C2 domain, the C-terminus, or two or more combinations thereof or between two domains of the FVIII protein, which are the A1 domain and a1 acidic region, and the a1 acidic region and A2 domain, the A2 domain and a2 acidic region, the a2 acidic region and B domain, the B domain and A3 domain, and the A3 domain and C1 domain, the C1 domain and C2 domain, or any combinations thereof. For example, the insertion sites in which the XTEN sequence can be inserted are selected from the group consisting of the N-terminus and A1 domain, the N-terminus and A2

domain, the N-terminus and A3 domain, the N-terminus and B domain, the N-terminus and C1 domain, the N-terminus and C2 domain, the N-terminus and the C-terminus, the A1 and A2 domains, the A1 and A3 domains, the A1 and B domains, the A1 and C1 domains, the A1 and C2 domains, the A1 domain and the C-terminus, the A2 and A3 domains, the A2 and B domains, the A2 and C1 domains, the A2 and C2 domains, the A2 domain and the C-terminus, the A3 and B domains, the A3 and C1 domains, the A3 and C2 domains, the A3 domain and the C-terminus, the B and C1 domains, the B and C2 domains, the B domain and the C-terminus, the C1 and C2 domains, the C1 and the C-terminus, the C2 domain, and the C-terminus, and two or more combinations thereof. Non-limiting examples of the insertion sites are listed in Tables 7, 8, 9, 10, 11, 12, 13, 14, and 15.

[0189] The FVIII protein, in which the XTEN sequence is inserted immediately downstream of one or more amino acids (*e.g.,* one or more XTEN insertion sites) in the FVIII protein or linked at the C-terminus or the N-terminus, retains the FVIII activity after linkage to or insertion by the XTEN sequence. The XTEN sequence can be inserted in the FVIII protein once or more than once, twice, three times, four times, five times, or six times such that the insertions do not affect the FVIII activity (*i.e.*, the FVIII protein still retains the coagulation property).

[0190] The FVIII protein useful in the present invention can be linked to one or more XTEN polypeptides at the N-terminus or C-terminus of the FVIII protein by an optional linker or inserted immediately downstream of one or more amino acids (*e.g.,* one or more XTEN insertion sites) in the FVIII protein by one or more optional linkers. In one embodiment, the two amino acid residues in which the XTEN sequence is inserted or the amino acid residue to which the XTEN sequence is linked correspond to the two or one amino acid residues of SEQ ID NO: 4 [full length mature FVIII] selected from the group consisting of the residues in Table 7, Table 8, Table 9, and Table 10 and any combinations thereof.

[0191] In other embodiments, at least one XTEN sequence is inserted in any one or more XTEN insertion sites disclosed herein or any combinations thereof. In one aspect, at least one XTEN sequence is inserted in one or more XTEN insertion sites disclosed in one or more amino acids disclosed in Table 7.

TABLE 7: Exemplary XTEN Insertion Sites

No.	XTEN Insertion Point*	Insertion Residue	FVIII BDD Downstream Sequence	FVIII Domain
1	0	(N-terminus)	ATR	A1
2	3	R	RYY	A1
3	17	M	QSD	A1
4	18	Q	SDL	A1
5	22	G	ELP	A1
6	24	L	PVD	A1
7	26	V	DAR	A1
8	28	A	RFP	A1
9	32	P	RVP	A1
10	38	F	PFN	A1
11	40	F	NTS	A1
12	41	N	TSV	A1
13	60	N	IAK	A1
14	61	I	AKP	A1
15	65	R	PPW	A1
16	81	Y	DTV	A1
17	111	G	AEY	A1
18	116	D	QTS	A1
19	119	S	QRE	A1
20	120	Q	REK	A1
21	128	V	FPG	A1
22	129	F	PGG	A1
23	130	P	GGS	A1
24	182	G	SLA	A1
25	185	A	KEK	A1
26	188	K	TQT	A1
27	205	G	KSW	A1
28	210	S	ETK	A1
29	211	E	TKN	A1
30	216	L	MQD	A1
31	220	R	DAA	A1
32	222	A	ASA	A1
33	223	A	SAR	A1
34	224	S	ARA	A1
35	230	K	MHT	A1
36	243	P	GLI	A1
37	244	G	LIG	A1
38	250	R	KSV	A1
39	318	D	GME	A1
40	333	P	QLR	A1
42	334	Q	LRM	A1
43	336	R	MKN	a1

No.	XTEN Insertion Point*	Insertion Residue	FVIII BDD Downstream Sequence	FVIII Domain
44	339	N	NEE	a1
45	345	D	YDD	a1
46	357	V	VRF	a1
47	367	S	FIQ	a1
48	370	S	RPY	a1
49	375	A	KKH	A2
50	376	K	KHP	A2
51	378	H	PKT	A2
52	399	V	LAP	A2
53	403	D	DRS	A2
54	405	R	SYK	A2
55	409	S	QYL	A2
56	416	P	QRI	A2
57	434	E	TFK	A2
58	438	T	REA	A2
59	441	A	IQH	A2
60	442	I	QHE	A2
61	463	I	IFK	A2
62	487	Y	SRR	A2
63	490	R	LPK	A2
64	492	P	KGV	A2
65	493	K	GVK	A2
66	494	G	VKH	A2
67	500	D	FPI	A2
68	506	G	EIF	A2
69	518	E	DGP	A2
70	556	K	ESV	A2
71	565	Q	IMS	A2
72	566	I	MSD	A2
73	598	P	AGV	A2
74	599	A	GVQ	A2
75	603	L	EDP	A2
76	616	S	ING	A2
77	686	G	LWI	A2
78	713	K	NTG	A2
79	719	Y	EDS	A2
80	730	L	LSK	A2
81	733	K	NNA	A2
82	745	N	PPV**	B
83	1640	P	PVL	B
84	1652	R	TTL	B
85	1656	Q	SDQ	A3
86	1685	N	QSP	A3
87	1711	M	SSS	A3
88	1713	S	SPH	A3

No.	XTEN Insertion Point*	Insertion Residue	FVIII BDD Downstream Sequence	FVIII Domain
89	1720	N	RAQ	A3
90	1724	S	GSV	A3
91	1725	G	SVP	A3
92	1726	S	VPQ	A3
93	1741	G	SFT	A3
94	1744	T	QPL	A3
95	1749	R	GEL	A3
96	1773	V	TFR	A3
97	1792	Y	EED	A3
98	1793	E	EDQ	A3
99	1796	Q	RQG	A3
100	1798	Q	GAE	A3
101	1799	G	AEP	A3
102	1802	P	RKN	A3
103	1803	R	KNF	A3
104	1807	V	KPN	A3
105	1808	K	PNE	A3
106	1827	K	DEF	A3
107	1844	E	KDV	A3
108	1861	N	TLN	A3
109	1863	L	NPA	A3
110	1896	E	RNC	A3
111	1900	R	APC	A3
112	1904	N	IQM	A3
113	1905	I	QME	A3
114	1910	P	TFK	A3
115	1920	A	ING	A3
116	1937	D	QRI	A3
117	1981	G	VFE	A3
118	2019	N	KCQ	A3
119	2020	K	CQT	C1
120	2044	G	QWA	C1
121	2068	F	SWI	C1
122	2073	V	DLL	C1
123	2090	R	QKF	C1
124	2092	K	FSS	C1
125	2093	F	SSL	C1
126	2111	K	WQT	C1
127	2115	Y	RGN	C1
128	2120	T	GTL	C1
129	2125	V	FFG	C1
130	2171	L	NSC	C1
131	2173	S	CSM	C2
132	2188	A	QIT	C2
133	2223	V	NNP	C2

No.	XTEN Insertion Point*	Insertion Residue	FVIII BDD Downstream Sequence	FVIII Domain
134	2224	N	NPK	C2
135	2227	K	EWL	C2
136	2268	G	HQW	C2
137	2277	N	GKV	C2
138	2278	G	KVK	C2
139	2290	F	TPV	C2
140	2332	Y	C terminus of FVIII	CT

* Indicates an insertion point for XTEN based on the amino acid number of mature full-length human FVIII, wherein the insertion could be either on the N- or C-terminal side of the indicated amino acid.

[0192] In some embodiments, one or more XTEN sequences are inserted within about six amino acids up or down from amino acids 32, 220, 224, 336, 339, 399, 416, 603, 1656, 1711, 1725, 1905, or 1910, corresponding to SEQ ID NO: 4 or any combinations thereof.

TABLE 8. Exemplary XTEN Insertion Ranges

No.	XTEN Insertion Point	Insertion Residue	FVIII BDD Downstream Sequence	FVIII Domain	Distance from insertion residue*
9	32	P	RVP	A1	-3, +6
31	220	R	DAA	A1	-
34	224	S	ARA	A1	+5
43	336	R	MKN	a1	-1, +6
44	339	N	NEE	a1	-4, +5
52	399	V	LAP	A2	-6, +3
56	416	P	QRI	A2	+6
75	603	L	EDP	A2	_6, +6
85	1656	Q	SDQ	B	-3, +6
87	1711	M	SSS	A3	-6, +1
91	1725	G	SVP	A3	+6
113	1905	I	QME	A3	+6
114	1910	P	TFK	A3	-5, +6

*Distance from insertion residue refers to the relative number of amino acids away from the N-terminus (negative numbers) or C-terminus (positive numbers) of the designated insertion residue (residue "0") where an insertion may be made. The designation "-x" refers to an insertion site which is x amino acids away on the N-terminal side of the designated insertion residue. Similarly, the designation "+x"

refers to an insertion site which is x amino acids away on the C-terminal side of the designated insertion residue.

For example, "-1, +2" indicates that the insertion is made at the N-terminus or C-terminus of amino acid residues denoted -1, 0, +1 or +2.

[0193] In other embodiments, one or more XTEN sequences are inserted immediately down stream of one or more amino acids corresponding to the full-length mature human FVIII selected from the group consisting of one or more insertion sites in Table 9.

TABLE 9. Exemplary XTEN Insertion Sites or Ranges

No.	XTEN Insertion Point Range*	First Insertion Residue	FVIII Domain
3	18-32	Q	A1
8	40	F	A1
18	211-224	E	A1
27	336-403	R	A1, A2
43	599	A	A2
47	745-1640	N	B
50	1656-1728	Q	B, a3, A3
57	1796-1804	R	A3
65	1900-1912	R	A3
81	2171-2332	L	C1, C2

* indicates range of insertion sites numbered relative to the amino acid number of mature human FVIII

[0194] In yet other embodiments, one or more XTENs are inserted in the B domain of FVIII. In one example, an XTEN is inserted between amino acids 740 and 1640 corresponding to SEQ ID NO: 4, wherein the FVIII sequence between amino acids 740 and 1640 is optionally not present. In another example, an XTEN is inserted between amino acids 741 and 1690 corresponding to SEQ ID NO: 4, wherein the FVIII sequence between amino acids 740 and 1690 is optionally not present. In other examples, an XTEN is inserted between amino acids 741 and 1648 corresponding to SEQ ID NO: 4, wherein the FVIII sequence between amino acids 741 and 1648 is optionally not present. In yet other examples, an XTEN is inserted between amino acids 743 and 1638 corresponding to SEQ ID NO: 4, wherein the FVIII sequence between amino acids 743 and 1638 is optionally not present. In still other examples, an XTEN is inserted between

amino acids 745 and 1656 corresponding to SEQ ID NO: 4, wherein the FVIII sequence between amino acids 745 and 1656 is optionally not present. In some examples, an XTEN is inserted between amino acids 745 and 1657 corresponding to SEQ ID NO: 4, wherein the FVIII sequence between amino acids 745 and 1657 is optionally not present. In certain examples, an XTEN is inserted between amino acids 745 and 1667 corresponding to SEQ ID NO: 4, wherein the FVIII sequence between amino acids 745 and 1667 is optionally not present. In still other examples, an XTEN is inserted between amino acids 745 and 1686 corresponding to SEQ ID NO: 4, wherein the FVIII sequence between amino acids 745 and 1686 is optionally not present. In some other examples, an XTEN is inserted between amino acids 747 and 1642 corresponding to SEQ ID NO: 4, wherein the FVIII sequence between amino acids 747 and 1642 is optionally not present. In still other examples, an XTEN is inserted between amino acids 751 and 1667 corresponding to SEQ ID NO: 4, wherein the FVIII sequence between amino acids 751 and 1667 is optionally not present.

[0195] In some embodiments, one or more XTENs are inserted in one or more amino acids immediately downstream of an amino acid of an insertion site selected from the group consisting of the amino acid residues in Table 10.

TABLE 10: FVIII XTEN insertion sites and construct designations

Construct Number	Domain	Upstream Residue No.*	Downstream Residue No.*	Upstream Sequence	Downstream Sequence
F8X-1	A1	3	4	ATR	RYY
F8X-2	A1	18	19	YMQ	SDL
F8X-3	A1	22	23	DLG	ELP
F8X-4	A1	26	27	LPV	DAR
F8X-5	A1	40	41	FPF	NTS
F8X-6	A1	60	61	LFN	IAK
F8X-7	A1	116	117	YDD	QTS
F8X-8	A1	130	131	VFP	GGS
F8X-9	A1	188	189	KEK	TQT
F8X-10	A1	216	217	NSL	MQD
F8X-11	A1	230	231	WPK	MHT
F8X-12	A1	333	334	EEP	QLR
F8X-13	A2	375	376	SVA	KKH
F8X-14	A2	403	404	APD	DRS
F8X-15	A2	442	443	EAI	QHE
F8X-16	A2	490	491	RRL	PKG
F8X-17	A2	518	519	TVE	DGP
F8X-18	A2	599	600	NPA	GVQ
F8X-19	A2	713	714	CDK	NTG
F8X-20	BD	745	746	SQN	PPV
F8X-21	BD	745	746	SQN	PPV
F8X-22	BD**	745	746	SQN	PPV
F8X-23	A3	1720	1721	APT	KDE
F8X-24	A3	1796	1797	EDQ	RQG
F8X-25	A3	1802	1803	AEP	RKN
F8X-26	A3	1827	1828	PTK	DEF
F8X-27	A3	1861	1862	HTN	TLN
F8X-28	A3	1896	1897	NME	RNC
F8X-29	A3	1900	1901	NCR	APC
F8X-30	A3	1904	1905	PCN	IQM
F8X-31	A3	1937	1938	AQD	QRI
F8X-32	C1	2019	2020	YSN	KCQ
F8X-33	C1	2068	2069	EPF	SWI
F8X-34	C1	2111	2112	GKK	WQT
F8X-35	C1	2120	2121	NST	GTL
F8X-36	C2	2171	2172	CDL	NSC
F8X-37	C2	2188	2189	SDA	QIT
F8X-38	C2	2227	2228	NPK	EWL
F8X-39	C2	2277	2278	FQN	GKV
F8X-40	CT	2332	NA	DLY	NA
F8X-41	CT	2332	NA	DLY	NA
F8X-42	A1	3	4	ATR	ATR
pSD0001	A2	403	404		
pSD0002	A2	599	600		

pSD0021	N-term	0	1		
pSD0022	A1	32	33		
pSD0023	A1	65	66		
pSD0024	A1	81	82		
pSD0025	A1	119	120		
pSD0026	A1	211	212		
pSD0027	A1	220	221		
pSD0028	A1	224	225		
pSD0029	A1	336	337		
pSD0030	A1	339	340		
pSD0031	A2	378	379		
pSD0032	A2	399	400		
pSD0033	A2	409	410		
pSD0034	A2	416	417		
pSD0035	A2	487	488		
pSD0036	A2	494	495		
pSD0037	A2	500	501		
pSD0038	A2	603	604		
pSD0039	A3	1656	1657		
pSD0040	A3	1711	1712		
pSD0041	A3	1725	1726		
pSD0042	A3	1749	1750		
pSD0043	A3	1905	1906		
pSD0044	A3	1910	1911		
pDS0062	A3	1900	1901		

* Indicates the amino acid number of the mature FVIII protein

[0196] In one embodiment, the one or more XTEN insertion sites are located within one or more surface-exposed, flexible loop structure of the FVIII protein (*e.g.,* a permissive loop). For example, at least one XTEN sequence can be inserted in each FVIII "A" domain comprising at least two "permissive loops" into which at least one XTEN polypeptide can be inserted without eliminating procoagulant activity of the recombinant protein, or the ability of the recombinant proteins to be expressed in vivo or *in vitro* in a host cell. The permissive loops are regions that allow insertion of at least one XTEN sequence with, among other attributes, high surface or solvent exposure and high conformational flexibility. The A1 domain comprises a permissive loop-1 (A1-1) region and a permissive loop-2 (A1-2) region, the A2 domain comprises a permissive loop-1 (A2-1) region and a permissive loop-2 (A2-2) region, the A3 domain comprises a permissive loop-1 (A3-1) region and a permissive loop-2 (A3-2) region.

[0197] In one aspect, a first permissive loop in the FVIII A1 domain (A1-1) is located between beta strand 1 and beta strand 2, and a second permissive loop in the FVIII A2 domain (A1-2) is located between beta strand 11 and beta strand 12. A first permissive loop in the FVIII A2 domain (A2-1) is located between beta strand 22 and beta strand 23, and a second permissive loop in the FVIII A2 domain (A2-2) is located between beta strand 32 and beta strand 33. A first permissive loop in the FVIII A3 domain (A3-1) is located between beta strand 38 and beta strand 39, and a second permissive loop in the FVIII A3 (A3-2) is located between beta strand 45 and beta strand 46. In certain aspects, the surface-exposed, flexible loop structure comprising A1-1 corresponds to a region in native mature human FVIII from about amino acid 15 to about amino acid 45 of SEQ IDNO: 4, *e.g.,* from about amino acid 18 to about amino acid 41 of SEQ ID NO: 4. In other aspects, the surface-exposed, flexible loop structure comprising A1-2 corresponds to a region in native mature human FVIII from about amino acid 201 to about amino acid 232 of SEQ ID NO: 4, *e.g.,* from about amino acid 218 to about amino acid 229 of SEQ ID NO: 4. In yet other aspects, the surface-exposed, flexible loop structure comprising A2-1 corresponds to a region in native mature human FVIII from about amino acid 395 to about amino acid 421 of SEQ ID NO: 4, *e.g.* from about amino acid 397 to about amino acid 418 of SEQ ID NO: 4. In still other embodiments, the surface-exposed, flexible loop structure comprising A2-2 corresponds to a region in native mature human FVIII from about amino acid 577 to about amino acid 635 of SEQ ID NO: 4, *e.g.,* from about amino acid 595 to about amino acid 607 of SEQ ID NO: 4. In certain aspects the surface-exposed, flexible loop structure comprising A3-1 corresponds to a region in native mature human FVIII from about amino acid 1705 to about amino acid 1732 of SEQ ID NO: 4, *e.g.,* from about amino acid 1711 to about amino acid 1725 of SEQ ID NO: 4. In yet other aspects, the surface-exposed, flexible loop structure comprising A3-2 corresponds to a region in native mature human FVIII from about amino acid 1884 to about amino acid 1917 of SEQ ID NO: 4, *e.g.,* from about amino acid 1899 to about amino acid 1911 of SEQ ID NO: 4.

[0198] In another embodiment, the one or more amino acids in which at least one XTEN sequence is inserted is located within a3 domain, *e.g.,* amino acids 1649 to 1689, corresponding to full-length mature FVIII polypeptide. In a particular

embodiment, an XTEN sequence is inserted between amino acids 1656 and 1657 of SEQ ID NO: 4 (full-length mature FVIII). In a specific embodiment, a FVIII protein comprising an XTEN sequence inserted immediately downstream of amino acid 1656 corresponding to SEQ ID NO: 4 further comprises a deletion from amino acid 745 to amino acid 1656 corresponding to SEQ ID NO: 4.

[0199] In some embodiments, the one or more insertion sites for one or more XTEN insertions are immediately downstream of one or more amino acids selected from the group consisting of:

(1) amino acid 3, (2) amino acid 18, (3) amino acid 22,
(4) amino acid 26, (5) amino acid 32, (6) amino acid 40,
(7) amino acid 60, (8) amino acid 65, (9) amino acid 81,
(10) amino acid 116, (11) amino acid 119, (12) amino acid 130,
(13) amino acid 188, (14) amino acid 211, (15) amino acid 216,
(16) amino acid 220, (17) amino acid 224, (18) amino acid 230,
(19) amino acid 333, (20) amino acid 336, (21) amino acid 339,
(22) amino acid 375, (23) amino acid 399, (24) amino acid 403,
(25) amino acid 409, (26) amino acid 416, (26) amino acid 442,
(28) amino acid 487, (29) amino acid 490, (30) amino acid 494,
(31) amino acid 500, (32) amino acid 518, (33) amino acid 599,
(34) amino acid 603, (35) amino acid 713, (36) amino acid 745,
(37) amino acid 1656, (38) amino acid 1711, (39) amino acid 1720,
(40) amino acid 1725, (41) amino acid 1749, (42) amino acid 1796,
(43) amino acid 1802, (44) amino acid 1827, (45) amino acid 1861,
(46) amino acid 1896, (47) amino acid 1900, (48) amino acid 1904,
(49) amino acid 1905, (50) amino acid 1910, (51) amino acid 1937,
(52) amino acid 2019, (53) amino acid 2068, (54) amino acid 2111,

(55) amino acid 2120, (56) amino acid 2171, (57) amino acid 2188,

(58) amino acid 2227, (59) amino acid 2277, and

(60) two or more combinations thereof.

[0200] In one embodiment, a FVIII protein useful for the invention comprises two XTEN sequences, a first XTEN sequence inserted into a first XTEN insertion site and a second XTEN inserted into a second XTEN insertion site. Non-limiting examples of the first XTEN insertion site and the second XTEN insertion site are listed in Table 11.

TABLE 11. Exemplary Insertion Sites for Two XTENs

Insertion 1		Insertion 2	
Insertion Site	Domain	Insertion Site	Domain
745	B	2332	CT
26	A1	403	A2
40	A1	403	A2
18	A1	403	A2
26	A1	599	A2
40	A1	599	A2
18	A1	599	A2
1720	A3	1900	A3
1725	A3	1900	A3
1711	A3	1905	A3
1720	A3	1905	A3
1725	A3	1905	A3
1656	A3	26	A1
1656	A3	18	A1
1656	A3	40	A1
1656	A3	399	A2
1656	A3	403	A2
1656	A3	1725	A3
1656	A3	1720	A3
1900	A3	18	A1
1900	A3	26	A1
1900	A3	40	A1
1905	A3	18	A1
1905	A3	40	A1
1905	A3	26	A1
1910	A3	26	A1
18	A1	399	A2
26	A1	399	A2
40	A1	399	A2
18	A1	403	A2

Insertion 1		Insertion 2	
Insertion Site	Domain	Insertion Site	Domain
1656	A3	1900	A3
1656	A3	1905	A3
1711	A3	40	A1
1711	A3	26	A1
1720	A3	26	A1
1720	A3	40	A1
1720	A3	18	A1
1725	A3	26	A1
1725	A3	40	A1
1725	A3	18	A1
1720	A3	403	A2
1720	A3	399	A2
1711	A3	403	A2
1720	A3	403	A2
1725	A3	403	A2
1725	A3	399	A2
1711	A3	403	A2
1900	A3	399	A2
1900	A3	403	A2
1905	A3	403	A2
1905	A3	399	A2
1910	A3	403	A2

[0201] The two XTENs inserted or linked to the FVIII protein can be identical or different. In some embodiments, a FVIII protein useful for the invention comprises two XTEN sequences inserted in the FVIII protein, a first XTEN sequence inserted immediately downstream of amino acid 745 corresponding to SEQ ID NO: 4, and a second XTEN sequence inserted immediately downstream of amino acid 2332 corresponding to SEQ ID NO: 4 (the C-terminus). In other embodiments, the first XTEN sequence is inserted immediately downstream of amino acid 18, 26, 40, 1656, or 1720 corresponding to SEQ ID NO: 4, and a second XTEN sequence inserted immediately downstream of amino acid 403 corresponding to SEQ ID NO: 4. In yet other embodiments, the first XTEN sequence is inserted immediately downstream of amino acid 18, 26, or 40 corresponding to SEQ ID NO: 4, and a second XTEN sequence inserted immediately downstream of amino acid 599 corresponding to SEQ ID NO: 4. In still other embodiments, the first XTEN sequence is inserted immediately downstream of amino acid 1656 corresponding to SEQ ID NO: 4, and a second XTEN sequence inserted immediately downstream of amino acid 18, 26, 40, 399,

403, 1725, 1720, 1900, 1905, or 2332 corresponding to SEQ ID NO: 4. In certain embodiments, the first XTEN sequence is inserted immediately downstream of amino acid 1900 corresponding to SEQ ID NO: 4, and a second XTEN sequence inserted immediately downstream of amino acid 18, 26, or 40 corresponding to SEQ ID NO: 4. In some embodiments, the first XTEN sequence is inserted immediately downstream of amino acid 18, 26, or 40 corresponding to SEQ ID NO: 4, and a second XTEN sequence inserted immediately downstream of amino acid 399 corresponding to SEQ ID NO: 4. In other embodiments, the first XTEN sequence is inserted immediately downstream of amino acid 1720 corresponding to SEQ ID NO: 4, and a second XTEN sequence inserted immediately downstream of amino acid 18, 26, or 40 corresponding to SEQ ID NO: 4. In still other embodiments, the first XTEN sequence is inserted immediately downstream of amino acid 1720 corresponding to SEQ ID NO: 4, and a second XTEN sequence inserted immediately downstream of amino acid 18 corresponding to SEQ ID NO: 4. In a particular embodiment, the FVIII protein comprising two XTEN sequences, a first XTEN sequence inserted immediately downstream of amino acid 745 corresponding to SEQ ID NO: 4 and a second XTEN sequence inserted immediately downstream of amino acid 2332 corresponding to SEQ ID NO: 4, wherein the FVIII protein further has a deletion from amino acid 745 corresponding to SEQ ID NO: 4 to amino acid 1685 corresponding to SEQ ID NO: 4, a mutation or substitution at amino acid 1680 corresponding to SEQ ID NO: 4, *e.g.,* Y1680F, a mutation or substitution at amino acid 1648 corresponding to SEQ ID NO: 4, *e.g.,* R1648A, or at least two mutations or substitutions at amino acid 1648 corresponding to SEQ ID NO: 4, *e.g.,* R1648A, and amino acid 1680 corresponding to SEQ ID NO: 4, *e.g.,* Y1680F. In a specific embodiment, the FVIII protein comprises two XTEN sequences, a first XTEN inserted immediately downstream of amino acid 1656 corresponding to SEQ ID NO: 4 and a second XTEN sequence inserted immediately downstream of amino acid 2332 of SEQ ID NO: 4, wherein the FVIII protein further has a deletion from amino acid 745 to amino acid 1656 corresponding to SEQ ID NO: 4.

[0202] In certain embodiments, a FVIII protein comprises three XTEN sequences, a first XTEN sequence inserted into a first XTEN insertion site, a second XTEN sequence inserted into a second XTEN sequence, and a third XTEN sequence

inserted into a third XTEN insertion site. The first, second, or third XTEN sequences can be identical or different. The first, second, and third insertion sites can be selected from the group of any one of the insertion sites disclosed herein. In some embodiments, the FVIII protein comprising three XTEN sequences can further comprise a mutation or substitution, *e.g.,* amino acid 1648 corresponding to SEQ ID NO: 4, *e.g.,* R1648A. For example, non-limiting examples of the first, second, and third XTEN insertion sites are listed in Table 12.

TABLE 12. Exemplary Insertion Sites for Three XTENs

Insertion 1		Insertion 2		Insertion 3	
Insertion Site	Domain	Insertion Site	Domain	Insertion Site	Domain
26	A1	403	A2	1656	A3
26	A1	403	A2	1720	A3
26	A1	403	A2	1900	A3
26	A1	1656	A3	1720	A3
26	A1	1656	A3	1900	A3
26	A1	1720	A3	1900	A3
403	A2	1656	A3	1720	A3
403	A2	1656	A3	1900	A3
403	A2	1720	A3	1900	A3
1656	A3	1720	A3	1900	A3
745	B	1900		2332	CT
18	A1	745	B	2332	CT
26	A1	745	B	2332	CT
40	A1	745	B	2332	CT
18	A1	745	B	2332	CT
40	A1	745	B	2332	CT
403	A2	745	B	2332	CT
399	A2	745	B	2332	CT
1725	A3	745	B	2332	CT
1720	A3	745	B	2332	CT
1711	A3	745	B	2332	CT
1900	A3	745	B	2332	CT
1905	A3	745	B	2332	CT
1910	A3	745	B	2332	CT

[0203] In some embodiments, a FVIII protein comprises three XTEN sequences, a first XTEN sequence inserted immediately downstream of amino acid 26 corresponding to SEQ ID NO: 4, a second XTEN sequence inserted downstream of amino acid 403 corresponding to SEQ ID NO: 4, and a third XTEN sequence inserted downstream of amino acid 1656, 1720, or 1900 corresponding to SEQ ID

NO: 4. In other embodiments, the first XTEN sequence is inserted immediately downstream of amino acid 26 corresponding to SEQ ID NO: 4, a second XTEN sequence is inserted downstream of amino acid 1656 corresponding to SEQ ID NO: 4, and a third XTEN sequence is inserted downstream of amino acid 1720 or 1900 corresponding to SEQ ID NO: 4. In yet other embodiments, the first XTEN sequence is inserted immediately downstream of amino acid 26 corresponding to SEQ ID NO: 4, a second XTEN sequence is inserted downstream of amino acid 1720 corresponding to SEQ ID NO: 4, and a third XTEN sequence is inserted downstream of amino acid 1900 corresponding to SEQ ID NO: 4. In still other embodiments, the first XTEN sequence is inserted immediately downstream of amino acid 403 corresponding to SEQ ID NO: 4, a second XTEN sequence is inserted downstream of amino acid 1656 corresponding to SEQ ID NO: 4, and a third XTEN sequence is inserted downstream of amino acid 1720 or 1900 corresponding to SEQ ID NO: 4. In other embodiments, the first XTEN sequence is inserted immediately downstream of amino acid 403 or 1656 corresponding to SEQ ID NO: 4, a second XTEN sequence is inserted downstream of amino acid 1720 corresponding to SEQ ID NO: 4, and a third XTEN sequence is inserted downstream of amino acid 1900 corresponding to SEQ ID NO: 4. In other embodiments, the first XTEN sequence is inserted immediately downstream of amino acid 18, 26, 40, 399, 403, 1711, 1720, 1725, 1900, 1905, or 1910 corresponding to SEQ ID NO: 4, a second XTEN sequence is inserted downstream of amino acid 745 corresponding to SEQ ID NO: 4, and a third XTEN sequence is inserted downstream of amino acid 2332 corresponding to SEQ ID NO: 4.

[0204] In other embodiments, a FVIII protein in the invention comprises four XTEN sequences, a first XTEN sequence inserted into a first insertion site, a second XTEN sequence inserted into a second insertion site, a third XTEN sequence inserted into a third insertion site, and a fourth XTEN sequence inserted into a fourth insertion site. The first, second, third, and fourth XTEN sequences can be identical, different, or combinations thereof. In some embodiments, the FVIII protein comprising four XTEN sequences can further comprise a mutation or substitution, *e.g.,* amino acid 1648 corresponding to SEQ ID NO: 4, *e.g.,*

R1648A. Non-limiting examples of the first, second, third, and fourth XTEN insertion sites are listed in Table 13.

TABLE 13. Exemplary Insertion Sites for Four XTENs

Insertion 1		Insertion 2		Insertion 3		Insertion 4	
Insertion Site	Domain	Insertion Site	Domain	Insertion Site	Domain	Insertion Site	Domain
26	A1	403	A2	1656	a3	1720	A3
26	A1	403	A2	1656	a3	1900	A3
26	A1	403	A2	1720	A3	1900	A3
26	A1	1656	a3	1720	A3	1900	A3
403	A2	1656	a3	1720	A3	1900	A3
0040	A1	0403	A2	745	B	2332	CT
0040	A1	0403	A2	745	B	2332	CT
0018	A1	0409	A2	745	B	2332	CT
0040	A1	0409	A2	745	B	2332	CT
0040	A1	0409	A2	745	B	2332	CT
0018	A1	0409	A2	745	B	2332	CT
0040	A1	1720	A3	745	B	2332	CT
0026	A1	1720	A3	745	B	2332	CT
0018	A1	1720	A3	745	B	2332	CT
0018	A1	1720	A3	745	B	2332	CT
0018	A1	1720	A3	745	B	2332	CT
0026	A1	1720	A3	745	B	2332	CT
0018	A1	1720	A3	745	B	2332	CT
0018	A1	1900	A3	745	B	2332	CT
0018	A1	1900	A3	745	B	2332	CT
0026	A1	1900	A3	745	B	2332	CT
0040	A1	1900	A3	745	B	2332	CT
0040	A1	1905	A3	745	B	2332	CT
0018	A1	1905	A3	745	B	2332	CT
0040	A1	1905	A3	745	B	2332	CT
0026	A1	1905	A3	745	B	2332	CT
0018	A1	1905	A3	745	B	2332	CT
0018	A1	1905	A3	745	B	2332	CT
0018	A1	1910	A3	745	B	2332	CT
0018	A1	1910	A3	745	B	2332	CT
0040	A1	1910	A3	745	B	2332	CT
0026	A1	1910	A3	745	B	2332	CT
0018	A1	1910	A3	745	B	2332	CT
0026	A1	1910	A3	745	B	2332	CT
0040	A1	1910	A3	745	B	2332	CT
0018	A1	1910	A3	745	B	2332	CT
0409	A2	1720	A3	745	B	2332	CT
0403	A2	1720	A3	745	B	2332	CT
0409	A2	1720	A3	745	B	2332	CT
0403	A2	1720	A3	745	B	2332	CT

0403	A2	1720	A3	745	B	2332	CT
0403	A2	1900	A3	745	B	2332	CT
0403	A2	1900	A3	745	B	2332	CT
0409	A2	1900	A3	745	B	2332	CT
0403	A2	1900	A3	745	B	2332	CT
0403	A2	1900	A3	745	B	2332	CT
0409	A2	1900	A3	745	B	2332	CT
0409	A2	1905	A3	745	B	2332	CT
0403	A2	1905	A3	745	B	2332	CT
0403	A2	1905	A3	745	B	2332	CT
0403	A2	1905	A3	745	B	2332	CT
0409	A2	1905	A3	745	B	2332	CT
0403	A2	1905	A3	745	B	2332	CT
0409	A2	1910	A3	745	B	2332	CT
0403	A2	1910	A3	745	B	2332	CT
0403	A2	1910	A3	745	B	2332	CT
0403	A2	1910	A3	745	B	2332	CT
0403	A2	1910	A3	745	B	2332	CT
1720	A3	1900	A3	745	B	2332	CT
1720	A3	1905	A3	745	B	2332	CT
1720	A3	1910	A3	745	B	2332	CT
1720	A3	1910	A3	745	B	2332	CT
0403	A2	1656	a3	1720	A3	2332	CT
0403	A2	1656	a3	1900	A3	2332	CT
0403	A2	1720	A3	1900	A3	2332	CT
1656	a3	1720	A3	1900	A3	2332	CT
0018	A1	0403	A2	1656	a3	2332	CT
0018	A1	0403	A2	1720	A3	2332	CT
0018	A1	0403	A2	1900	A3	2332	CT
0018	A1	1656	a3	1720	A3	2332	CT
0018	A1	1656	a3	1900	A3	2332	CT
0018	A1	1720	A3	1900	A3	2332	CT
0018	A1	0403	A2	0745	B	2332	CT
0018	A1	0745	B	1720	A3	2332	CT
0018	A1	0745	B	1900	A3	2332	CT
0403	A2	0745	B	1720	A3	2332	CT
0403	A2	0745	B	1900	A3	2332	CT
0745	B	1720	A3	1900	A3	2332	CT
0188	A1	1900	A3	0745	B	2332	CT
0599		1900	A3	0745	B	2332	CT
2068		1900	A3	0745	B	2332	CT
2171		1900	A3	0745	B	2332	CT
2227		1900	A3	0745	B	2332	CT
2277		1900	A3	0745	B	2332	CT

[0205] In some embodiments, a FVIII protein comprises five XTEN sequences, a first XTEN sequence inserted into a first insertion site, a second XTEN sequence

inserted into a second insertion site, a third XTEN sequence inserted into a third XTEN insertion site, a fourth XTEN sequence inserted into a fourth XTEN insertion site, and a fifth XTEN sequence inserted into a fifth XTEN insertion site. The first, second, third, fourth, of fifth XTEN sequences can be identical, different, or combinations thereof. Non-limiting examples of the first, second, third, fourth, and fifth insertion sites are listed in Table 14.

TABLE 14. Exemplary Insertion Sites for Five XTENs

XTEN Insertion 1	XTEN insertion 2	XTEN Insertion 3	XTEN Insertion 4	XTEN Insertion 5
0403	1656	1720	1900	2332
0018	0403	1656	1720	2332
0018	0403	1656	1900	2332
0018	0403	1720	1900	2332
0018	1656	1720	1900	2332
0018	0403	0745	1720	2332
0018	0403	0745	1900	2332
0018	0745	1720	1900	2332
0403	0745	1720	1900	2332

[0206] In certain embodiments, a FVIII protein comprises six XTEN sequences, a first XTEN sequence inserted into a first XTEN insertion site, a second XTEN sequence inserted into a second XTEN insertion site, a third XTEN sequence inserted into a third XTEN insertion site, a fourth XTEN sequence inserted into a fourth XTEN insertion site, a fifth XTEN sequence inserted into a fifth XTEN insertion site, and a sixth XTEN sequence inserted into a sixth XTEN insertion site. The first, second, third, fourth, fifth, or sixth XTEN sequences can be identical, different, or combinations thereof. Examples of the six XTEN insertion sites include, but are not limited to the insertion sites listed in Table 15.

TABLE 15. Exemplary XTEN Insertion Sites for Six XTENs

XTEN Insertion 1	XTEN insertion 2	XTEN Insertion 3	XTEN Insertion 4	XTEN Insertion 5	XTEN Insertion 6
0018	0403	1656	1720	1900	2332
0018	0403	0745	1720	1900	2332

[0207] In a particular example, a first XTEN is inserted between amino acids 26 and 27 corresponding to SEQ ID NO: 4, and a second XTEN is inserted between

amino acids 1720 and 1721 corresponding to SEQ ID NO: 4 (full-length mature FVIII). In another example, a first XTEN is inserted between amino acids 403 and 404 corresponding to SEQ ID NO: 4, and a second XTEN is inserted between amino acids 1720 and 1721 corresponding to SEQ ID NO: 4. In some examples, a first XTEN is inserted between amino acids 1656 and 1657 corresponding to SEQ ID NO: 4, and a second XTEN is inserted between amino acids 1720 and 1721 corresponding to SEQ ID NO: 4. In other examples, a first XTEN is inserted between amino acids 26 and 27 corresponding to SEQ ID NO: 4, a second XTEN is inserted between amino acids 1656 and 1657 corresponding to SEQ ID NO: 4, and a third XTEN is inserted between amino acids 1720 and 1721 corresponding to SEQ ID NO: 4. In yet other embodiments, a first XTEN is inserted between amino acids 403 and 404 corresponding to SEQ ID NO: 4, a second XTEN is inserted between amino acids 1656 and 1657 corresponding to SEQ ID NO: 4, and a third XTEN is inserted between amino acids 1720 and 1721 corresponding to SEQ ID NO: 4. In still other embodiments, a first XTEN is inserted between amino acids 403 and 404 corresponding to SEQ ID NO: 4, a second XTEN is inserted between amino acids 1656 and 1657 corresponding to SEQ ID NO: 4, and a third XTEN is inserted between amino acids 1720 and 1721 corresponding to SEQ ID NO: 4. In certain embodiments, a first XTEN is inserted between amino acids 26 and 27 corresponding to SEQ ID NO: 4, a second XTEN is inserted between amino acids 1720 and 1721 corresponding to SEQ ID NO: 4, and a third XTEN is inserted between amino acids 1900 and 1901 corresponding to SEQ ID NO: 4. In some embodiments, a first XTEN is inserted between amino acids 26 and 27 corresponding to SEQ ID NO: 4, a second XTEN is inserted between amino acids 1656 and 1657 corresponding to SEQ ID NO: 4, a third XTEN is inserted between amino acids 1720 and 1721 corresponding to SEQ ID NO: 4, and a fourth XTEN is inserted between 1900 and 1901 corresponding to SEQ ID NO: 4.

[0208] In a particular embodiment, an XTEN sequence is inserted between amino acids 745 and 746 of a full-length Factor VIII or the corresponding insertion site of the B-domain deleted Factor VIII.

[0209] In some embodiments, a chimeric protein of the invention comprises two polypeptide sequences, a first polypeptide sequence comprising an amino acid

sequence at least about 80%, 90%, 95%, or 100% identical to a sequence selected from FVIII-161 (SEQ ID NO: 101), FVIII-169 (SEQ ID NO: 103), FVIII-170 (SEQ ID NO: 102), FVIII-173 (SEQ ID NO: 104); FVIII-195 (SEQ ID NO: 105); FVIII-196 (SEQ ID NO: 106), FVIII-199 (SEQ ID NO: 107), FVIII-201 (SEQ ID NO: 108); FVIII-203 (SEQ ID NO: 109), FVIII-204 (SEQ ID NO: 110), FVIII-205 (SEQ ID NO: 111), FVIII-266 (SEQ ID NO:112), FVIII-267 (SEQ ID NO: 113), FVIII-268 (SEQ ID NO: 114), FVIII-269 (SEQ ID NO: 115), FVIII-271 (SEQ ID NO: 116), or FVIII-272 (SEQ ID NO: 117) and a second polypeptide sequence comprising an amino acid sequence at least about 80%, 90%, 95%, or 100% identical to a sequence selected from VWF031 (SEQ ID NO: 118), VWF034 (SEQ ID NO: 119), or VWF-036 (SEQ ID NO: 120).

D) Ig Constant Region or a portion thereof

[0210] The VWF fragment or the FVIII protein linked to an XTEN sequence in the present invention can further comprise an Ig constant region or a portion thereof. The Ig constant region or a portion thereof can improve pharmacokinetic or pharmacodynamic properties of the VWF fragment or the FVIII protein in combination with the XTEN sequence. In certain embodiments, the Ig constant region or a portion thereof extends a half-life of a molecule fused to the Ig constant region or a portion thereof.

[0211] An Ig constant region is comprised of domains denoted CH (constant heavy) domains (CH1, CH2, etc.). Depending on the isotype, (*i.e.* IgG, IgM, IgA, IgD, or IgE), the constant region can be comprised of three or four CH domains. Some isotypes (*e.g.* IgG) constant regions also contain a hinge region. *See* Janeway *et al.* 2001, *Immunobiology*, Garland Publishing, N.Y., N.Y.

[0212] An Ig constant region or a portion thereof for producing the chimeric protein of the present invention may be obtained from a number of different sources. In some embodiments, an Ig constant region or a portion thereof is derived from a human Ig. It is understood, however, that the Ig constant region or a portion thereof may be derived from an Ig of another mammalian species, including for example, a rodent (*e.g.* a mouse, rat, rabbit, guinea pig) or non-human primate (*e.g.* chimpanzee, macaque) species. Moreover, the Ig constant region or a portion thereof may be derived from any Ig class, including IgM, IgG,

IgD, IgA, and IgE, and any Ig isotype, including IgGl, IgG2, IgG3, and IgG4. In one embodiment, the human isotype IgG1 is used.

[0213] A variety of the Ig constant region gene sequences (*e.g.,* human constant region gene sequences) are available in the form of publicly accessible deposits. Constant region domains sequence can be selected having a particular effector function (or lacking a particular effector function) or with a particular modification to reduce immunogenicity. Many sequences of antibodies and antibody-encoding genes have been published and suitable Ig constant region sequences (*e.g.,* hinge, CH2, and/or CH3 sequences, or portions thereof) can be derived from these sequences using art recognized techniques. The genetic material obtained using any of the foregoing methods may then be altered or synthesized to obtain polypeptides of the present invention. It will further be appreciated that the scope of this invention encompasses alleles, variants and mutations of constant region DNA sequences.

[0214] The sequences of the Ig constant region or a portion thereof can be cloned, *e.g.,* using the polymerase chain reaction and primers which are selected to amplify the domain of interest. To clone a sequence of the Ig constant region or a portion thereof from an antibody, mRNA can be isolated from hybridoma, spleen, or lymph cells, reverse transcribed into DNA, and antibody genes amplified by PCR. PCR amplification methods are described in detail in U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; 4,965,188; and in, *e.g.,* "PCR Protocols: A Guide to Methods and Applications" Innis et al. eds., Academic Press, San Diego, CA (1990); Ho et al. 1989. Gene 77:51; Horton et al. 1993. Methods Enzymol. 217:270). PCR may be initiated by consensus constant region primers or by more specific primers based on the published heavy and light chain DNA and amino acid sequences. As discussed above, PCR also may be used to isolate DNA clones encoding the antibody light and heavy chains. In this case the libraries may be screened by consensus primers or larger homologous probes, such as mouse constant region probes. Numerous primer sets suitable for amplification of antibody genes are known in the art (*e.g.,* 5' primers based on the N-terminal sequence of purified antibodies (Benhar and Pastan. 1994. Protein Engineering 7:1509); rapid amplification of cDNA ends (Ruberti, F. et al. 1994. J. Immunol. Methods 173:33); antibody leader sequences (Larrick et al. 1989 Biochem.

Biophys. Res. Commun. 160:1250). The cloning of antibody sequences is further described in Newman et al., U.S. Pat. No. 5,658,570, filed January 25, 1995, which is incorporated by reference herein.

[0215] An Ig constant region used herein can include all domains and the hinge region or portions thereof. In one embodiment, the Ig constant region or a portion thereof comprises CH2 domain, CH3 domain, and a hinge region, *i.e.,* an Fc region or an FcRn binding partner.

[0216] As used herein, the term "Fc region" is defined as the portion of a polypeptide which corresponds to the Fc region of native Ig, *i.e.,* as formed by the dimeric association of the respective Fc domains of its two heavy chains. A native Fc region forms a homodimer with another Fc region. In contrast, the term "genetically-fused Fc region" or "single-chain Fc region" (scFc region), as used herein, refers to a synthetic dimeric Fc region comprised of Fc domains genetically linked within a single polypeptide chain (*i.e.,* encoded in a single contiguous genetic sequence).

[0217] In one embodiment, the "Fc region" refers to the portion of a single Ig heavy chain beginning in the hinge region just upstream of the papain cleavage site (*i.e.* residue 216 in IgG, taking the first residue of heavy chain constant region to be 114) and ending at the C-terminus of the antibody. Accordingly, a complete Fc domain comprises at least a hinge domain, a CH2 domain, and a CH3 domain.

[0218] The Fc region of an Ig constant region, depending on the Ig isotype can include the CH2, CH3, and CH4 domains, as well as the hinge region. Chimeric proteins comprising an Fc region of an Ig bestow several desirable properties on a chimeric protein including increased stability, increased serum half-life (see Capon *et al.,* 1989, *Nature* 337:525) as well as binding to Fc receptors such as the neonatal Fc receptor (FcRn) (U.S. Pat. Nos. 6,086,875, 6,485,726, 6,030,613; WO 03/077834; US2003-0235536A1), which are incorporated herein by reference in their entireties.

[0219] An Ig constant region or a portion thereof can be an FcRn binding partner. FcRn is active in adult epithelial tissues and expressed in the lumen of the intestines, pulmonary airways, nasal surfaces, vaginal surfaces, colon and rectal surfaces (U.S. Pat. No. 6,485,726). An FcRn binding partner is a portion of an Ig that binds to FcRn.

[0220] The FcRn receptor has been isolated from several mammalian species including humans. The sequences of the human FcRn, monkey FcRn, rat FcRn, and mouse FcRn are known (Story et al. 1994, J. Exp. Med. 180:2377). The FcRn receptor binds IgG (but not other Ig classes such as IgA, IgM, IgD, and IgE) at relatively low pH, actively transports the IgG transcellularly in a luminal to serosal direction, and then releases the IgG at relatively higher pH found in the interstitial fluids. It is expressed in adult epithelial tissue (U.S. Pat. Nos. 6,485,726, 6,030,613, 6,086,875; WO 03/077834; US2003-0235536A1) including lung and intestinal epithelium (Israel et al. 1997, Immunology 92:69) renal proximal tubular epithelium (Kobayashi et al. 2002, Am. J. Physiol. Renal Physiol. 282:F358) as well as nasal epithelium, vaginal surfaces, and biliary tree surfaces.

[0221] FcRn binding partners useful in the present invention encompass molecules that can be specifically bound by the FcRn receptor including whole IgG, the Fc fragment of IgG, and other fragments that include the complete binding region of the FcRn receptor. The region of the Fc portion of IgG that binds to the FcRn receptor has been described based on X-ray crystallography (Burmeister et al. 1994, Nature 372:379). The major contact area of the Fc with the FcRn is near the junction of the CH2 and CH3 domains. Fc-FcRn contacts are all within a single Ig heavy chain. The FcRn binding partners include whole IgG, the Fc fragment of IgG, and other fragments of IgG that include the complete binding region of FcRn. The major contact sites include amino acid residues 248, 250-257, 272, 285, 288, 290-291, 308-311, and 314 of the CH2 domain and amino acid residues 385-387, 428, and 433-436 of the CH3 domain. References made to amino acid numbering of Igs or Ig fragments, or regions, are all based on Kabat et al. 1991, Sequences of Proteins of Immunological Interest, U.S. Department of Public Health, Bethesda, Md.

[0222] Fc regions or FcRn binding partners bound to FcRn can be effectively shuttled across epithelial barriers by FcRn, thus providing a non-invasive means to systemically administer a desired therapeutic molecule. Additionally, fusion proteins comprising an Fc region or an FcRn binding partner are endocytosed by cells expressing the FcRn. But instead of being marked for degradation, these fusion proteins are recycled out into circulation again, thus increasing the *in vivo*

half-life of these proteins. In certain embodiments, the portions of Ig constant regions are an Fc region or an FcRn binding partner that typically associates, via disulfide bonds and other non-specific interactions, with another Fc region or another FcRn binding partner to form dimers and higher order multimers.

[0223] Two FcRn receptors can bind a single Fc molecule. Crystallographic data suggest that each FcRn molecule binds a single polypeptide of the Fc homodimer. In one embodiment, linking the FcRn binding partner, *e.g.,* an Fc fragment of an IgG, to a biologically active molecule provides a means of delivering the biologically active molecule orally, buccally, sublingually, rectally, vaginally, as an aerosol administered nasally or via a pulmonary route, or via an ocular route. In another embodiment, the chimeric protein can be administered invasively, *e.g.,* subcutaneously, intravenously.

[0224] An FcRn binding partner region is a molecule or a portion thereof that can be specifically bound by the FcRn receptor with consequent active transport by the FcRn receptor of the Fc region. Specifically bound refers to two molecules forming a complex that is relatively stable under physiologic conditions. Specific binding is characterized by a high affinity and a low to moderate capacity as distinguished from nonspecific binding which usually has a low affinity with a moderate to high capacity. Typically, binding is considered specific when the affinity constant KA is higher than 10^6 M^{-1}, or higher than 10^8 M^{-1}. If necessary, non-specific binding can be reduced without substantially affecting specific binding by varying the binding conditions. The appropriate binding conditions such as concentration of the molecules, ionic strength of the solution, temperature, time allowed for binding, concentration of a blocking agent (*e.g.* serum albumin, milk casein), etc., may be optimized by a skilled artisan using routine techniques.

[0225] In certain embodiments, a chimeric protein of the invention comprises one or more truncated Fc regions that are nonetheless sufficient to confer Fc receptor (FcR) binding properties to the Fc region. For example, the portion of an Fc region that binds to FcRn (*i.e.,* the FcRn binding portion) comprises from about amino acids 282-438 of IgG1, EU numbering (with the primary contact sites being amino acids 248, 250-257, 272, 285, 288, 290-291, 308-311, and 314 of the CH2 domain and amino acid residues 385-387, 428, and 433-436 of the CH3 domain. Thus, an Fc region of the invention may comprise or consist of an FcRn binding

portion. FcRn binding portions may be derived from heavy chains of any isotype, including IgGl, IgG2, IgG3 and IgG4. In one embodiment, an FcRn binding portion from an antibody of the human isotype IgG1 is used. In another embodiment, an FcRn binding portion from an antibody of the human isotype IgG4 is used.

[0226] In another embodiment, the "Fc region" includes an amino acid sequence of an Fc domain or derived from an Fc domain. In certain embodiments, an Fc region comprises at least one of: a hinge (*e.g.,* upper, middle, and/or lower hinge region) domain (about amino acids 216-230 of an antibody Fc region according to EU numbering), a CH2 domain (about amino acids 231-340 of an antibody Fc region according to EU numbering), a CH3 domain (about amino acids 341-438 of an antibody Fc region according to EU numbering), a CH4 domain, or a variant, portion, or fragment thereof. In other embodiments, an Fc region comprises a complete Fc domain (*i.e.,* a hinge domain, a CH2 domain, and a CH3 domain). In some embodiments, an Fc region comprises, consists essentially of, or consists of a hinge domain (or a portion thereof) fused to a CH3 domain (or a portion thereof), a hinge domain (or a portion thereof) fused to a CH2 domain (or a portion thereof), a CH2 domain (or a portion thereof) fused to a CH3 domain (or a portion thereof), a CH2 domain (or a portion thereof) fused to both a hinge domain (or a portion thereof) and a CH3 domain (or a portion thereof). In still other embodiments, an Fc region lacks at least a portion of a CH2 domain (*e.g.,* all or part of a CH2 domain). In a particular embodiment, an Fc region comprises or consists of amino acids corresponding to EU numbers 221 to 447.

[0227] The Fc regions denoted as F, F1, or F2 herein may be obtained from a number of different sources. In one embodiment, an Fc region of the polypeptide is derived from a human Ig. It is understood, however, that an Fc region may be derived from an Ig of another mammalian species, including for example, a rodent (*e.g.* a mouse, rat, rabbit, or guinea pig) or non-human primate (*e.g.* chimpanzee, macaque) species. Moreover, the polypeptide of the Fc domains or portions thereof may be derived from any Ig class, including IgM, IgG, IgD, IgA and IgE, and any Ig isotype, including IgGl, IgG2, IgG3 and IgG4. In another embodiment, the human isotype IgG1 is used.

[0228] In certain embodiments, the Fc variant confers a change in at least one effector function imparted by an Fc region comprising said wild-type Fc domain (*e.g.,* an improvement or reduction in the ability of the Fc region to bind to Fc receptors (*e.g.* FcγRI, FcγRII, or FcγRIII) or complement proteins (*e.g.* C1q), or to trigger antibody-dependent cytotoxicity (ADCC), phagocytosis, or complement-dependent cytotoxicity (CDCC)). In other embodiments, the Fc variant provides an engineered cysteine residue.

[0229] The Fc regions of the invention may employ art-recognized Fc variants which are known to impart a change (*e.g.,* an enhancement or reduction) in effector function and/or FcR or FcRn binding. Specifically, a binding molecule of the invention may include, for example, a change (*e.g.,* a substitution) at one or more of the amino acid positions disclosed in International PCT Publications WO88/07089A1, WO96/14339A1, WO98/05787A1, WO98/23289A1, WO99/51642A1, WO99/58572A1, WO00/09560A2, WO00/32767A1, WO00/42072A2, WO02/44215A2, WO02/060919A2, WO03/074569A2, WO04/016750A2, WO04/029207A2, WO04/035752A2, WO04/063351A2, WO04/074455A2, WO04/099249A2, WO05/040217A2, WO04/044859, WO05/070963A1, WO05/077981A2, WO05/092925A2, WO05/123780A2, WO06/019447A1, WO06/047350A2, and WO06/085967A2; US Patent Publication Nos. US2007/0231329, US2007/0231329, US2007/0237765, US2007/0237766, US2007/0237767, US2007/0243188, US20070248603, US20070286859, US20080057056 ; or US Patents 5,648,260; 5,739,277; 5,834,250; 5,869,046; 6,096,871; 6,121,022; 6,194,551; 6,242,195; 6,277,375; 6,528,624; 6,538,124; 6,737,056; 6,821,505; 6,998,253; 7,083,784; 7,404,956, and 7,317,091, each of which is incorporated by reference herein. In one embodiment, the specific change (*e.g.,* the specific substitution of one or more amino acids disclosed in the art) may be made at one or more of the disclosed amino acid positions. In another embodiment, a different change at one or more of the disclosed amino acid positions (*e.g.,* the different substitution of one or more amino acid position disclosed in the art) may be made.

[0230] The Fc region or FcRn binding partner of IgG can be modified according to well recognized procedures such as site directed mutagenesis and the like to yield modified IgG or Fc fragments or portions thereof that will be bound by

FcRn. Such modifications include modifications remote from the FcRn contact sites as well as modifications within the contact sites that preserve or even enhance binding to the FcRn. For example, the following single amino acid residues in human IgG1 Fc (Fc γ1) can be substituted without significant loss of Fc binding affinity for FcRn: P238A, S239A, K246A, K248A, D249A, M252A, T256A, E258A, T260A, D265A, S267A, H268A, E269A, D270A, E272A, L274A, N276A, Y278A, D280A, V282A, E283A, H285A, N286A, T289A, K290A, R292A, E293A, E294A, Q295A, Y296F, N297A, S298A, Y300F, R301A, V303A, V305A, T307A, L309A, Q311A, D312A, N315A, K317A, E318A, K320A, K322A, S324A, K326A, A327Q, P329A, A330Q, P331A, E333A, K334A, T335A, S337A, K338A, K340A, Q342A, R344A, E345A, Q347A, R355A, E356A, M358A, T359A, K360A, N361A, Q362A, Y373A, S375A, D376A, A378Q, E380A, E382A, S383A, N384A, Q386A, E388A, N389A, N390A, Y391F, K392A, L398A, S400A, D401A, D413A, K414A, R416A, Q418A, Q419A, N421A, V422A, S424A, E430A, N434A, T437A, Q438A, K439A, S440A, S444A, and K447A, where for example P238A represents wild type proline substituted by alanine at position number 238. As an example, a specific embodiment incorporates the N297A mutation, removing a highly conserved N-glycosylation site. In addition to alanine other amino acids may be substituted for the wild type amino acids at the positions specified above. Mutations may be introduced singly into Fc giving rise to more than one hundred Fc regions distinct from the native Fc. Additionally, combinations of two, three, or more of these individual mutations may be introduced together, giving rise to hundreds more Fc regions. Moreover, one of the Fc region of a construct of the invention may be mutated and the other Fc region of the construct not mutated at all, or they both may be mutated but with different mutations.

[0231] Certain of the above mutations may confer new functionality upon the Fc region or FcRn binding partner. For example, one embodiment incorporates N297A, removing a highly conserved N-glycosylation site. The effect of this mutation is to reduce immunogenicity, thereby enhancing circulating half-life of the Fc region, and to render the Fc region incapable of binding to FcγRI, FcγRIIA, FcγRIIB, and FcγRIIIA, without compromising affinity for FcRn (Routledge et al. 1995, Transplantation 60:847; Friend et al. 1999, Transplantation 68:1632; Shields

et al. 1995, J. Biol. Chem. 276:6591). As a further example of new functionality arising from mutations described above affinity for FcRn may be increased beyond that of wild type in some instances. This increased affinity may reflect an increased "on" rate, a decreased "off" rate or both an increased "on" rate and a decreased "off" rate. Examples of mutations believed to impart an increased affinity for FcRn include, but not limited to, T256A, T307A, E380A, and N434A (Shields et al. 2001, J. Biol. Chem. 276:6591).

[0232] Additionally, at least three human Fc gamma receptors appear to recognize a binding site on IgG within the lower hinge region, generally amino acids 234-237. Therefore, another example of new functionality and potential decreased immunogenicity may arise from mutations of this region, as for example by replacing amino acids 233-236 of human IgG1 "ELLG" to the corresponding sequence from IgG2 "PVA" (with one amino acid deletion). It has been shown that FcγRI, FcγRII, and FcγRIII, which mediate various effector functions will not bind to IgG1 when such mutations have been introduced. Ward and Ghetie 1995, Therapeutic Immunology 2:77 and Armour et al. 1999, Eur. J. Immunol. 29:2613.

[0233] In one embodiment, the Ig constant region or a portion thereof, e.g, an Fc region, is a polypeptide including the sequence PKNSSMISNTP (SEQ ID NO: 52) and optionally further including a sequence selected from HQSLGTQ (SEQ ID NO: 53), HQNLSDGK (SEQ ID NO: 54), HQNISDGK (SEQ ID NO: 55), or VISSHLGQ (SEQ ID NO: 56) (U.S. Pat. No. 5,739,277).

[0234] In another embodiment, the immunoglobulin constant region or a portion thereof comprises an amino acid sequence in the hinge region or a portion thereof that forms one or more disulfide bonds with another immunoglobulin constant region or a portion thereof. The disulfide bond by the immunoglobulin constant region or a portion thereof places the first polypeptide comprising FVIII and the second polypeptide comprising the VWF fragment together so that endogenous VWF does not replace the VWF fragment and does not bind to the FVIII. Therefore, the disulfide bond between the first immunoglobulin constant region or a portion thereof and a second immunoglobulin constant region or a portion thereof prevents interaction between endogenous VWF and the FVIII protein. This inhibition of interaction between the VWF and the FVIII protein allows the half-life of the FVIII protein to go beyond the two fold limit. The hinge region or

a portion thereof can further be linked to one or more domains of CH1, CH2, CH3, a fragment thereof, and any combinations thereof. In a particular embodiment, the immunoglobulin constant region or a portion thereof is a hinge region and CH2.

[0235] In certain embodiments, the Ig constant region or a portion thereof is hemi-glycosylated. For example, the chimeric protein comprising two Fc regions or FcRn binding partners may contain a first, glycosylated, Fc region (*e.g.,* a glycosylated CH2 region) or FcRn binding partner and a second, aglycosylated, Fc region (*e.g.,* an aglycosylated CH2 region) or FcRn binding partner. In one embodiment, a linker may be interposed between the glycosylated and aglycosylated Fc regions. In another embodiment, the Fc region or FcRn binding partner is fully glycosylated, *i.e.,* all of the Fc regions are glycosylated. In other embodiments, the Fc region may be aglycosylated, *i.e.,* none of the Fc moieties are glycosylated.

[0236] In certain embodiments, a chimeric protein of the invention comprises an amino acid substitution to an Ig constant region or a portion thereof (*e.g.,* Fc variants), which alters the antigen-independent effector functions of the Ig constant region, in particular the circulating half-life of the protein.

[0237] Such proteins exhibit either increased or decreased binding to FcRn when compared to proteins lacking these substitutions and, therefore, have an increased or decreased half-life in serum, respectively. Fc variants with improved affinity for FcRn are anticipated to have longer serum half-lives, and such molecules have useful applications in methods of treating mammals where long half-life of the administered polypeptide is desired, *e.g.,* to treat a chronic disease or disorder (*see, e.g.*, US Patents 7,348,004, 7,404,956, and 7,862,820). In contrast, Fc variants with decreased FcRn binding affinity are expected to have shorter half-lives, and such molecules are also useful, for example, for administration to a mammal where a shortened circulation time may be advantageous, *e.g.* for in vivo diagnostic imaging or in situations where the starting polypeptide has toxic side effects when present in the circulation for prolonged periods. Fc variants with decreased FcRn binding affinity are also less likely to cross the placenta and, thus, are also useful in the treatment of diseases or disorders in pregnant women. In addition, other applications in which reduced FcRn binding affinity may be

desired include those applications in which localization the brain, kidney, and/or liver is desired. In one exemplary embodiment, the chimeric protein of the invention exhibit reduced transport across the epithelium of kidney glomeruli from the vasculature. In another embodiment, the chimeric protein of the invention exhibit reduced transport across the blood brain barrier (BBB) from the brain, into the vascular space. In one embodiment, a protein with altered FcRn binding comprises at least one Fc region or FcRn binding partner (e.g, one or two Fc regions or FcRn binding partners) having one or more amino acid substitutions within the "FcRn binding loop" of an Ig constant region. The FcRn binding loop is comprised of amino acid residues 280-299 (according to EU numbering) of a wild-type, full-length, Fc region. In other embodiments, an Ig constant region or a portion thereof in a chimeric protein of the invention having altered FcRn binding affinity comprises at least one Fc region or FcRn binding partner having one or more amino acid substitutions within the 15 Å FcRn "contact zone." As used herein, the term 15 Å FcRn "contact zone" includes residues at the following positions of a wild-type, full-length Fc moiety: 243-261, 275-280, 282-293, 302-319, 336- 348, 367, 369, 372-389, 391, 393, 408, 424, 425-440 (EU numbering). In other embodiments, a Ig constant region or a portion thereof of the invention having altered FcRn binding affinity comprises at least one Fc region or FcRn binding partner having one or more amino acid substitutions at an amino acid position corresponding to any one of the following EU positions: 256, 277-281, 283-288, 303-309, 313, 338, 342, 376, 381, 384, 385, 387, 434 (*e.g.,* N434A or N434K), and 438. Exemplary amino acid substitutions which altered FcRn binding activity are disclosed in International PCT Publication No. WO05/047327 which is incorporated by reference herein.

[0238] An Fc region or FcRn binding partner used in the invention may also comprise an art recognized amino acid substitution which alters the glycosylation of the chimeric protein. For example, the Fc region or FcRn binding partner of the chimeric protein linked to a VWF fragment or a FVIII protein may comprise an Fc region having a mutation leading to reduced glycosylation (*e.g.*, N- or O-linked glycosylation) or may comprise an altered glycoform of the wild-type Fc moiety (*e.g.*, a low fucose or fucose-free glycan).

[0239] In one embodiment, an unprocessed chimeric protein of the invention may comprise a genetically fused Fc region (*i.e.,* scFc region) having two or more of its constituent Ig constant region or a portion thereof independently selected from the Ig constant region or a portion thereof described herein. In one embodiment, the Fc regions of a dimeric Fc region are the same. In another embodiment, at least two of the Fc regions are different. For example, the Fc regions or FcRn binding partners of the proteins of the invention comprise the same number of amino acid residues or they may differ in length by one or more amino acid residues (*e.g.,* by about 5 amino acid residues (*e.g.,* 1, 2, 3, 4, or 5 amino acid residues), about 10 residues, about 15 residues, about 20 residues, about 30 residues, about 40 residues, or about 50 residues). In yet other embodiments, the Fc regions or FcRn binding partners of the protein of the invention may differ in sequence at one or more amino acid positions. For example, at least two of the Fc regions or FcRn binding partners may differ at about 5 amino acid positions (*e.g.,* 1, 2, 3, 4, or 5 amino acid positions), about 10 positions, about 15 positions, about 20 positions, about 30 positions, about 40 positions, or about 50 positions).

E) Linkers

[0240] The chimeric protein of the present invention further comprises one or more linkers. One type of the linkers is a cleavable linker, which can be cleaved by various proteases when administered to a subject in vivo, *e.g.,* at a site of coagulation. In one embodiment, the cleavable linker allows cleavage of moiety, *e.g.,* a VWF fragment, from the chimeric protein at the site of the coagulation cascade, thus allowing activated FVIII (FVIIIa) to have its FVIIIa activity. Another type of the linkers is a processable linker, which contains an intracellular cleavage site and thus can be cleaved by an intracellular processing enzyme in a host cell, allowing convenient expression of a polypeptide and formation of a chimeric protein.

[0241] One or more linkers can be present between any two proteins in the chimeric protein. In one embodiment, a chimeric protein comprises (i) a VWF fragment, (ii) an XTEN sequence, and (iii) a FVIII protein, wherein the VWF fragment is linked to the XTEN sequence by a linker, *e.g.,* a cleavable linker, and the XTEN sequence is further linked to the FVIII protein (*i.e.,* V-L-X-FVIII). In another embodiment, a chimeric protein comprises (i) a VWF fragment, (ii) an

XTEN sequence, and (iii) a FVIII protein, wherein the VWF fragment is linked to the XTEN sequence, and the XTEN sequence is linked to the FVIII protein by a linker, *e.g.,* a cleavable linker (*i.e.,* V-X-L-FVIII).

[0242] In certain embodiments, a chimeric protein comprises (i) a VWF fragment, (ii) an XTEN sequence, (iii) a first Ig constant region or a portion thereof (*e.g.,* a first Fc region), (iv) a FVIII protein, and (v) a second Ig constant region or a portion thereof (*e.g.,* a second Fc region), wherein the VWF fragment is linked to the XTEN sequence by an optional linker, *e.g.,* a cleavable linker. The XTEN sequence can be further linked to the first Ig constant region or a portion thereof by a linker, *e.g.,* a cleavable linker. The FVIII protein (with or without an XTEN sequence) can also be linked to the second Ig constant region or a portion thereof by an optional linker, *e.g.* a cleavable linker. In certain embodiments, the chimeric protein further comprises one or more linkers, *e.g.,* processable linkers, between the first Ig constant region or a portion thereof (*e.g.,* first Fc region) and the second Ig constant region or a portion thereof (*e.g.,* second Fc region), between the VWF fragment and the second Ig constant region or a portion thereof, or between the FVIII protein and the first Ig constant region or a portion thereof (*e.g.,* first Fc region).

[0243] In some embodiments, the present invention includes a chimeric protein comprising (i) a FVIII protein, (ii) an XTEN sequence, (iii) a first Ig constant region or a portion thereof, and (iv) a second Ig constant region or a portion thereof, wherein the first Ig constant region or a portion thereof and the second Ig constant region or a portion thereof are linked by a processable linker.

[0244] The linker useful in the present invention can comprise any organic molecule. In one embodiment, the linker comprises a polymer, *e.g.*, polyethylene glycol (PEG) or hydroxyethyl starch (HES). In another embodiment, the linker comprises an amino acids sequence. The linker can comprise at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 300, 400 ,500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, or 2000 amino acids. The linker can comprise 1-5 amino acids, 1-10 amino acids, 1-20 amino acids, 10-50 amino acids, 50-100 amino acids, 100-200 amino acids, 200-300 amino acids, 300-400 amino acids, 400-500 amino acids, 500-600 amino acids, 600-700 amino acids, 700-800 amino acids, 800-900 amino acids, or 900-1000

amino acids. In one embodiment, the linker comprises an XTEN sequence. Additional examples of XTEN can be used according to the present invention and are disclosed in US Patent Publication Nos. 2010/0239554 A1, 2010/0323956 A1, 2011/0046060 A1, 2011/0046061 A1, 2011/0077199 A1, or 2011/0172146 A1, or International Patent Publication Nos. WO 2010091122 A1, WO 2010144502 A2, WO 2010144508 A1, WO 2011028228 A1, WO 2011028229 A1, or WO 2011028344 A2. In another embodiment, the linker is a PAS sequence.

[0245] The linker useful in the present invention can comprise any organic molecule. In one embodiment, the linker is a polymer, *e.g.,* polyethylene glycol (PEG) or hydroxyethyl starch (HES). In another embodiment, the linker is an amino acid sequence. The linker can comprise at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, or 2000 amino acids. The linker can comprise 1-5 amino acids, 1-10 amino acids, 1-20 amino acids, 10-50 amino acids, 50-100 amino acids, 100-200 amino acids, 200-300 amino acids, 300-400 amino acids, 400-500 amino acids, 500-600 amino acids, 600-700 amino acids, 700-800 amino acids, 800-900 amino acids, or 900-1000 amino acids.

[0246] Examples of linkers are well known in the art. In one embodiment, the linker comprises the sequence G_n. The linker can comprise the sequence $(GA)_n$. The linker can comprise the sequence $(GGS)_n$. In other embodiments, the linker comprises $(GGGS)_n$ (SEQ ID NO: 57). In still other embodiments, the linker comprises the sequence $(GGS)_n(GGGGS)_n$ (SEQ ID NO: 58). In these instances, n may be an integer from 1-100. In other instances, n may be an integer from 1-20, *i.e.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20. Examples of linkers include, but are not limited to, GGG, SGGSGGS (SEQ ID NO: 59), GGSGGSGGSGGSGGG (SEQ ID NO: 60), GGSGGSGGGGSGGGGS (SEQ ID NO: 61), GGSGGSGGSGGSGGSGGS (SEQ ID NO: 62), or GGGGSGGGGSGGGGS (SEQ ID NO: 63). The linker does not eliminate or diminish the VWF fragment activity or the clotting activity of Factor VIII. Optionally, the linker enhances the VWF fragment activity or the clotting activity of Factor VIII protein, *e.g.*, by further diminishing the effects of steric hindrance and making the VWF fragment or Factor VIII portion more accessible to its target binding site.

[0247] In one embodiment, the linker useful for the chimeric protein is 15-25 amino acids long. In another embodiment, the linker useful for the chimeric protein is 15-20 amino acids long. In some embodiments, the linker for the chimeric protein is 10-25 amino acids long. In other embodiments, the linker for the chimeric protein is 15 amino acids long. In still other embodiments, the linker for the chimeric protein is $(GGGGS)_n$ (SEQ ID NO: 64) where G represents glycine, S represents serine and n is an integer from 1-20.

F) Cleavage Sites

[0248] The linker may also incorporate a moiety capable of being cleaved either chemically (*e.g.,* hydrolysis of an ester bond), enzymatically (*i.e.,* incorporation of a protease cleavage sequence), or photolytically (*e.g.*, a chromophore such as 3-amino-3-(2-nitrophenyl) proprionic acid (ANP)) in order to release one molecule from another.

[0249] In one embodiment, the linker is a cleavable linker. The cleavable linkers can comprise one or more cleavage sites at the N-terminus or C-terminus or both. In another embodiment, the cleavable linker consists essentially of or consists of one or more cleavable sites. In other embodiments, the cleavable linker comprises heterologous amino acid linker sequences described herein or polymers and one or more cleavable sites.

[0250] In certain embodiments, a cleavable linker comprises one or more cleavage sites that can be cleaved in a host cell (*i.e.,* intracellular processing sites). Non limiting examples of the cleavage site include RRRR (SEQ ID NO: 9), RKRRKR (SEQ ID NO: 10), and RRRRS (SEQ ID NO: 11).

[0251] In other embodiments, a cleavable linker comprises one or more cleavage sites that are cleaved by a protease after a chimeric protein comprising the cleavable linker is administered to a subject. In one embodiment, the cleavage site is cleaved by a protease selected from the group consisting of factor XIa, factor XIIa, kallikrein, factor VIIa, factor IXa, factor Xa, factor IIa (thrombin), Elastase-2, MMP-12, MMP-13, MMP-17, and MMP-20. In another embodiment, the cleavage site is selected from the group consisting of a FXIa cleavage site (*e.g.,* KLTR↓AET (SEQ ID NO: 65)), a FXIa cleavage site (e.g, DFTR↓VVG (SEQ ID NO: 66)), a FXIIa cleavage site (*e.g.,* TMTR↓IVGG (SEQ ID NO: 67)), a Kallikrein cleavage site (*e.g.,* SPFR↓STGG (SEQ ID NO: 68)), a FVIIa cleavage

site (*e.g.,* LQVR↓IVGG (SEQ ID NO: 69)), a FIXa cleavage site (*e.g.,* PLGR↓IVGG (SEQ ID NO: 70)), a FXa cleavage site (*e.g.,* IEGR↓TVGG (SEQ ID NO: 71)), a FIIa (thrombin) cleavage site (e.g, LTPR↓SLLV (SEQ ID NO: 72)), a Elastase-2 cleavage site (e.g, LGPV↓SGVP (SEQ ID NO: 73)), a Granzyme-B cleavage (e.g, VAGD↓SLEE (SEQ ID NO: 74)), a MMP-12 cleavage site (*e.g.,* GPAG↓LGGA (SEQ ID NO: 75)), a MMP-13 cleavage site (*e.g.,* GPAG↓LRGA (SEQ ID NO: 76)), a MMP-17 cleavage site (*e.g.,* APLG↓LRLR (SEQ ID NO: 77)), a MMP-20 cleavage site (*e.g.,* PALP↓LVAQ (SEQ ID NO: 78)), a TEV cleavage site (*e.g.,* ENLYFQ↓G (SEQ ID NO: 79)), a Enterokinase cleavage site (*e.g.,* DDDK↓IVGG (SEQ ID NO: 80)), a Protease 3C (PRESCISSION™) cleavage site (*e.g.,* LEVLFQ↓GP (SEQ ID NO: 81)), and a Sortase A cleavage site (*e.g.,* LPKT↓GSES) (SEQ ID NO: 82). In certain embodiments, the FXIa cleavage sites include, but are not limited to, *e.g.,* TQSFNDFTR (SEQ ID NO: 83) and SVSQTSKLTR (SEQ ID NO: 84). Non-limiting exemplary thrombin cleavage sites include, *e.g.,* DFLAEGGGVR (SEQ ID NO: 85), TTKIKPR (SEQ ID NO: 86), or LVPRG (SEQ ID NO: 87), and a sequence comprising, consisting essentially of, or consisting of ALRPR (SEQ ID NO: 17) (*e.g.,* ALRPRVVGGA (SEQ ID NO: 88)).

[0252] In a specific embodiment, the cleavage site is TLDPRSFLLRNPNDKYEPFWEDEEK (SEQ ID NO: 8).

POLYNUCLEOTIDES, VECTORS, AND HOST CELLS

[0253] Also provided in the invention is a polynucleotide encoding (a) a VWF fragment linked to an XTEN sequence and a FVIII protein, (b) a FVIII protein linked to an XTEN sequence and Fc, or (c) a FVIII protein linked to an XTEN sequence and a VWF fragment described herein. When a chimeric protein is a single polypeptide chain (*e.g.,* F2-L2-X-V-L1-F1-FVIII, wherein FVIII comprises a FVIII protein, F1 comprises a first Ig constant region or a portion thereof, *e.g.,* a first Fc region, L1 comprises a first linker, V comprises a VWF fragment, X comprises an XTEN sequence, L2 comprises a second linker, and F2 comprises a second Ig constant region or a portion thereof, *e.g.,* a second Fc region), the invention is drawn to a single polynucleotide chain encoding the single polypeptide chain. When the chimeric protein comprises a first and a second polypeptide chains (F2-L2-X-V:FVIII-F1), the first polypeptide chain comprising

a VWF fragment linked to a XTEN sequence, which is further linked to a first Ig constant region or a portion thereof (*e.g.*, a first Fc region) by a cleavable linker (*e.g.,* F2-L2-X-V) and the second polypeptide chain comprising a FVIII protein and a second Ig constant region or a portion thereof (*e.g.*, a second Fc region) (e.g, FVIII-F1), wherein the first polypeptide chain and the second polypeptide chain are associated with each other, a polynucleotide can comprise the first nucleotide sequence and the second nucleotide sequence. In one embodiment, the first polypeptide chain and the second polypeptide chain can be encoded by a single polynucleotide chain. In another embodiment, the first polypeptide chain and the second polypeptide chain are encoded by two different polynucleotides, *i.e.,* a first nucleotide sequence and a second nucleotide sequence. In another embodiment, the first nucleotide sequence and the second nucleotide sequence are on two different polynucleotides (*e.g.*, different vectors). In certain embodiments, the present invention is directed to a set of polynucleotides comprising a first nucleotide chain and a second nucleotide chain, wherein the first nucleotide chain encodes the VWF fragment of the chimeric protein and the second nucleotide chain encodes the FVIII protein. In some embodiments, a chimeric protein comprising two polypeptide chains or three polypeptide chains can be encoded by a single polynucleotide chain, and then processed into two or three (or more) polypeptide chains. In yet other embodiments, a chimeric protein comprising these polypeptide chains can be encoded by two or three polynucleotide chains.

[0254] In other embodiments, the set of the polynucleotides further comprises an additional nucleotide chain (*e.g.*, a second nucleotide chain when the chimeric polypeptide is encoded by a single polynucleotide chain or a third nucleotide chain when the chimeric protein is encoded by two polynucleotide chains) which encodes a protein convertase. The protein convertase can be selected from the group consisting of proprotein convertase subtilisin/kexin type 5 (PCSK5 or PC5), proprotein convertase subtilisin/kexin type 7 (PCSK7 or PC5), a yeast Kex 2, proprotein convertase subtilisin/kexin type 3 (PACE or PCSK3), and two or more combinations thereof. In some embodiments, the protein convertase is PACE, PC5, or PC7. In a specific embodiment, the protein convertase is PC5 or PC7. See International Application no. PCT/US2011/043568.

[0255] As used herein, an expression vector refers to any nucleic acid construct which contains the necessary elements for the transcription and translation of an inserted coding sequence, or in the case of an RNA viral vector, the necessary elements for replication and translation, when introduced into an appropriate host cell. Expression vectors can include plasmids, phagemids, viruses, and derivatives thereof.

[0256] Expression vectors of the invention will include polynucleotides encoding the chimeric protein described herein. In one embodiment, one or more of the coding sequences for the VWF fragment and XTEN, the FVIII protein and XTEN, or both are operably linked to an expression control sequence. As used herein, two nucleic acid sequences are operably linked when they are covalently linked in such a way as to permit each component nucleic acid sequence to retain its functionality. A coding sequence and a gene expression control sequence are said to be operably linked when they are covalently linked in such a way as to place the expression or transcription and/or translation of the coding sequence under the influence or control of the gene expression control sequence. Two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to a coding nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that coding nucleic acid sequence such that the resulting transcript is translated into the desired protein or polypeptide.

[0257] A gene expression control sequence as used herein is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the coding nucleic acid to which it is operably linked. The gene expression control sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine

phosphoribosyl transferase (HPRT), adenosine deaminase, pyruvate kinase, beta-actin promoter, and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the cytomegalovirus (CMV), simian virus (*e.g.*, SV40), papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of Moloney leukemia virus, and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

[0258] In general, the gene expression control sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined coding nucleic acid. The gene expression sequences optionally include enhancer sequences or upstream activator sequences as desired.

[0259] Viral vectors include, but are not limited to, nucleic acid sequences from the following viruses: retrovirus, such as Moloney murine leukemia virus, Harvey murine sarcoma virus, murine mammary tumor virus, and Rous sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyomaviruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors well-known in the art. Certain viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (*i.e.*, capable of

directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., Gene Transfer and Expression, A Laboratory Manual, W.H. Freeman Co., New York (1990) and Murry, E. J., Methods in Molecular Biology, Vol. 7, Humana Press, Inc., Cliffton, N.J. (1991).

[0260] In one embodiment, the virus is an adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication-deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hematopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

[0261] Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well-known to those of skill in the art. See, *e.g.*, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been found to be particularly advantageous for delivering genes to cells *in vivo* because of their inability to replicate within and integrate into a host genome. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operably encoded within the plasmid. Some

commonly used plasmids available from commercial suppliers include pBR322, pUC18, pUC19, various pcDNA plasmids, pRC/CMV, various pCMV plasmids, pSV40, and pBlueScript. Additional examples of specific plasmids include pcDNA3.1, catalog number V79020; pcDNA3.1/hygro, catalog number V87020; pcDNA4/myc-His, catalog number V86320; and pBudCE4.1, catalog number V53220, all from Invitrogen (Carlsbad, CA.). Other plasmids are well-known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using standard molecular biology techniques to remove and/or add specific fragments of DNA.

[0262] In one insect expression system that may be used to produce the proteins of the invention, *Autographa californica* nuclear polyhidrosis virus (AcNPV) is used as a vector to express the foreign genes. The virus grows in *Spodoptera frugiperda* cells. A coding sequence may be cloned into non-essential regions (for example, the polyhedron gene) of the virus and placed under control of an ACNPV promoter (for example, the polyhedron promoter). Successful insertion of a coding sequence will result in inactivation of the polyhedron gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedron gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed. (see, *e.g.*, Smith *et al.* (1983) *J Virol* 46:584; U.S. Pat. No. 4,215,051). Further examples of this expression system may be found in Ausubel *et al.*, eds. (1989) Current Protocols in Molecular Biology, Vol. 2, Greene Publish. Assoc. & Wiley Interscience.

[0263] Another system which can be used to express the proteins of the invention is the glutamine synthetase gene expression system, also referred to as the "GS expression system" (Lonza Biologics PLC, Berkshire UK). This expression system is described in detail in U.S. Pat. No. 5,981,216.

[0264] In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, a coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region

E1 or E3) will result in a recombinant virus that is viable and capable of expressing peptide in infected hosts. See, *e.g.*, Logan & Shenk (1984) *Proc Natl Acad Sci USA* 81:3655). Alternatively, the vaccinia 7.5 K promoter may be used. See, *e.g.*, Mackett *et al.* (1982) *Proc Natl Acad Sci USA* 79:7415; Mackett *et al.* (1984) *J Virol* 49:857; Panicali *et al.* (1982) *Proc Natl Acad Sci USA* 79:4927.

[0265] To increase efficiency of production, the polynucleotides can be designed to encode multiple units of the protein of the invention separated by enzymatic cleavage sites. The resulting polypeptide can be cleaved (*e.g.*, by treatment with the appropriate enzyme) in order to recover the polypeptide units. This can increase the yield of polypeptides driven by a single promoter. When used in appropriate viral expression systems, the translation of each polypeptide encoded by the mRNA is directed internally in the transcript; *e.g.*, by an internal ribosome entry site, IRES. Thus, the polycistronic construct directs the transcription of a single, large polycistronic mRNA which, in turn, directs the translation of multiple, individual polypeptides. This approach eliminates the production and enzymatic processing of polyproteins and may significantly increase the yield of polypeptides driven by a single promoter.

[0266] Vectors used in transformation will usually contain a selectable marker used to identify transformants. In bacterial systems, this can include an antibiotic resistance gene such as ampicillin or kanamycin. Selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. One amplifiable selectable marker is the dihydrofolate reductase (DHFR) gene. Simonsen C C *et al.* (1983) *Proc Natl Acad Sci USA* 80:2495-9. Selectable markers are reviewed by Thilly (1986) Mammalian Cell Technology, Butterworth Publishers, Stoneham, Mass., and the choice of selectable markers is well within the level of ordinary skill in the art.

[0267] Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, U.S. Pat. No. 4,713,339).

[0268] The expression vectors can encode for tags that permit easy purification of the recombinantly produced protein. Examples include, but are not limited to, vector pUR278 (Ruther *et al.* (1983) *EMBO J* 2:1791), in which coding sequences for the protein to be expressed may be ligated into the vector in frame with the lac z coding region so that a tagged fusion protein is produced; pGEX vectors may be used to express proteins of the invention with a glutathione S-transferase (GST) tag. These proteins are usually soluble and can easily be purified from cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The vectors include cleavage sites (thrombin or Factor Xa protease or PRESCISSION PROTEASE™ (Pharmacia, Peapack, N.J.)) for easy removal of the tag after purification.

[0269] The expression vector or vectors are then transfected or co-transfected into a suitable target cell, which will express the polypeptides. Transfection techniques known in the art include, but are not limited to, calcium phosphate precipitation (Wigler *et al.* (1978) *Cell* 14:725), electroporation (Neumann *et al.* (1982) *EMBO J* 1:841), and liposome-based reagents. A variety of host-expression vector systems may be utilized to express the proteins described herein including both prokaryotic and eukaryotic cells. These include, but are not limited to, microorganisms such as bacteria (*e.g.*, *E. coli*) transformed with recombinant bacteriophage DNA or plasmid DNA expression vectors containing an appropriate coding sequence; yeast or filamentous fungi transformed with recombinant yeast or fungi expression vectors containing an appropriate coding sequence; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing an appropriate coding sequence; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus or tobacco mosaic virus) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing an appropriate coding sequence; or animal cell systems, including mammalian cells (*e.g.*, HEK 293, CHO, Cos, HeLa, HKB11, and BHK cells).

[0270] In one embodiment, the host cell is a eukaryotic cell. As used herein, a eukaryotic cell refers to any animal or plant cell having a definitive nucleus. Eukaryotic cells of animals include cells of vertebrates, *e.g.*, mammals, and cells of invertebrates, *e.g.*, insects. Eukaryotic cells of plants specifically can include,

without limitation, yeast cells. A eukaryotic cell is distinct from a prokaryotic cell, *e.g.*, bacteria.

[0271] In certain embodiments, the eukaryotic cell is a mammalian cell. A mammalian cell is any cell derived from a mammal. Mammalian cells specifically include, but are not limited to, mammalian cell lines. In one embodiment, the mammalian cell is a human cell. In another embodiment, the mammalian cell is a HEK 293 cell, which is a human embryonic kidney cell line. HEK 293 cells are available as CRL-1533 from American Type Culture Collection, Manassas, VA, and as 293-H cells, Catalog No. 11631-017 or 293-F cells, Catalog No. 11625-019 from Invitrogen (Carlsbad, Calif.). In some embodiments, the mammalian cell is a PER.C6® cell, which is a human cell line derived from retina. PER.C6® cells are available from Crucell (Leiden, The Netherlands). In other embodiments, the mammalian cell is a Chinese hamster ovary (CHO) cell. CHO cells are available from American Type Culture Collection, Manassas, VA. (*e.g.*, CHO-K1; CCL-61). In still other embodiments, the mammalian cell is a baby hamster kidney (BHK) cell. BHK cells are available from American Type Culture Collection, Manassas, Va. (*e.g.*, CRL-1632). In some embodiments, the mammalian cell is a HKB11 cell, which is a hybrid cell line of a HEK293 cell and a human B cell line. Mei *et al., Mol. Biotechnol.* 34(2): 165-78 (2006).

[0272] In one embodiment, a plasmid including a FVIII(X)-Fc fusion coding sequence, a VWF fragment-L-Fc fusion coding sequence, or both and a selectable marker, *e.g.*, zeocin resistance, are transfected into HEK 293 cells, for production of a chimeric protein.

[0273] In another embodiment, a plasmid including a FVIII-Fc fusion coding sequence, a VWF fragment-XTEN-L-Fc fusion coding sequence, or both and a selectable marker, *e.g.*, zeocin resistance, are transfected into HEK 293 cells, for production of a chimeric protein.

[0274] In other embodiments, a plasmid including a FVIII(X)-Fc fusion coding sequence, a Fc coding sequence, or both and a selectable marker, *e.g.*, zeocin resistance, are transfected into HEK 293 cells, for production of a chimeric protein.

[0275] In some embodiments, a first plasmid including a FVIII(X)-Fc fusion coding sequence and a first selectable marker, *e.g.*, a zeocin resistance gene, and a

second plasmid including an Fc coding sequence or a VWF fragment-L-Fc coding sequence and a second selectable marker, *e.g.*, a neomycin resistance gene, and a third plasmid including a protein convertase coding sequence and a third selectable marker, *e.g.*, a hygromycin resistance gene, are cotransfected into HEK 293 cells, for production of the chimeric protein. The first and second plasmids can be introduced in equal amounts (*i.e.*, 1:1 molar ratio), or they can be introduced in unequal amounts.

[0276] In still other embodiments, a first plasmid including a FVIII-Fc fusion coding sequence and a first selectable marker, *e.g.*, a zeocin resistance gene, and a second plasmid including a VWF fragment-XTEN-L-Fc coding sequence and a second selectable marker, *e.g.*, a neomycin resistance gene, and a third plasmid including a protein convertase coding sequence and a third selectable marker, *e.g.*, a hygromycin resistance gene, are cotransfected into HEK 293 cells, for production of the chimeric protein. The first and second plasmids can be introduced in equal amounts (*i.e.*, 1:1 molar ratio), or they can be introduced in unequal amounts.

[0277] In yet other embodiments, a first plasmid including a FVIII(X)-Fc fusion coding sequence and a first selectable marker, *e.g.*, a zeocin resistance gene, and a second plasmid including a VWF fragment-XTEN-L-Fc coding sequence and a second selectable marker, *e.g.*, a neomycin resistance gene, and a third plasmid including a protein convertase coding sequence and a third selectable marker, *e.g.*, a hygromycin resistance gene, are cotransfected into HEK 293 cells, for production of the chimeric protein. The first and second plasmids can be introduced in equal amounts (*i.e.*, 1:1 molar ratio), or they can be introduced in unequal amounts.

[0278] In certain embodiments, a first plasmid, including a chimeric protein encoding FVIII (with or without XTEN)-F1-L1-V-XTEN-L2-F2 coding sequence and a first selectable marker, *e.g.*, a zeocin resistance gene, and a second plasmid including a protein convertase coding sequence and a second selectable marker, *e.g.*, a hygromycin resistance gene, are cotransfected into HEK 293 cells, for production of the chimeric protein. The promoters for the FVIII(X)-Fc coding sequence and the VWF-XTEN-Fc coding sequence can be different or they can be the same.

[0279] In still other embodiments, transfected cells are stably transfected. These cells can be selected and maintained as a stable cell line, using conventional techniques known to those of skill in the art.

[0280] Host cells containing DNA constructs of the protein are grown in an appropriate growth medium. As used herein, the term "appropriate growth medium" means a medium containing nutrients required for the growth of cells. Nutrients required for cell growth may include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals, and growth factors. Optionally, the media can contain one or more selection factors. Optionally the media can contain bovine calf serum or fetal calf serum (FCS). In one embodiment, the media contains substantially no IgG. The growth medium will generally select for cells containing the DNA construct by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker on the DNA construct or co-transfected with the DNA construct. Cultured mammalian cells are generally grown in commercially available serum-containing or serum-free media (*e.g.*, MEM, DMEM, DMEM/F12). In one embodiment, the medium is CD293 (Invitrogen, Carlsbad, CA.). In another embodiment, the medium is CD17 (Invitrogen, Carlsbad, CA.). Selection of a medium appropriate for the particular cell line used is within the level of those ordinary skilled in the art.

[0281] In order to co-express the two polypeptide chains of the chimeric protein, the host cells are cultured under conditions that allow expression of both chains. As used herein, culturing refers to maintaining living cells *in vitro* for at least a definite time. Maintaining can, but need not include, an increase in population of living cells. For example, cells maintained in culture can be static in population, but still viable and capable of producing a desired product, *e.g.*, a recombinant protein or recombinant fusion protein. Suitable conditions for culturing eukaryotic cells are well known in the art and include appropriate selection of culture media, media supplements, temperature, pH, oxygen saturation, and the like. For commercial purposes, culturing can include the use of any of various types of scale-up systems including shaker flasks, roller bottles, hollow fiber bioreactors, stirred-tank bioreactors, airlift bioreactors, Wave bioreactors, and others.

[0282] The cell culture conditions are also selected to allow association of the VWF fragment with the FVIII protein. Conditions that allow expression of the VWF fragment and/or the FVIII protein may include the presence of a source of vitamin K. For example, in one embodiment, stably transfected HEK 293 cells are cultured in CD293 media (Invitrogen, Carlsbad, CA) or OptiCHO media (Invitrogen, Carlsbad, CA) supplemented with 4 mM glutamine.

[0283] In one aspect, the present invention is directed to a method of expressing, making, or producing the chimeric protein of the invention comprising a) transfecting a host cell comprising a polynucleotide encoding the chimeric protein and b) culturing the host cell in a culture medium under a condition suitable for expressing the chimeric protein, wherein the chimeric protein is expressed.

[0284] In further embodiments, the protein product containing the VWF fragment linked to an XTEN sequence or the FVIII protein linked to an XTEN sequence is secreted into the media. Media is separated from the cells, concentrated, filtered, and then passed over two or three affinity columns, *e.g.*, a protein A column and one or two anion exchange columns.

[0285] In certain aspects, the present invention relates to the chimeric protein produced by the methods described herein.

[0286] *In vitro* production allows scale-up to give large amounts of the desired altered polypeptides of the invention. Techniques for mammalian cell cultivation under tissue culture conditions are known in the art and include homogeneous suspension culture, *e.g.* in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, *e.g.* in hollow fibers, microcapsules, on agarose microbeads or ceramic cartridges. If necessary and/or desired, the solutions of polypeptides can be purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, hydrophobic interaction chromatography (HIC, chromatography over DEAE-cellulose or affinity chromatography.

PHARMACEUTICAL COMPOSITION

[0287] Compositions containing the chimeric protein of the present invention may contain a suitable pharmaceutically acceptable carrier. For example, they may contain excipients and/or auxiliaries that facilitate processing of the active compounds into preparations designed for delivery to the site of action.

[0288] The pharmaceutical composition can be formulated for parenteral administration (*i.e.* intravenous, subcutaneous, or intramuscular) by bolus injection. Formulations for injection can be presented in unit dosage form, *e.g.*, in ampoules or in multidose containers with an added preservative. The compositions can take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient can be in powder form for constitution with a suitable vehicle, *e.g.*, pyrogen free water.

[0289] Suitable formulations for parenteral administration also include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, including, for example, sodium carboxymethyl cellulose, sorbitol and dextran. Optionally, the suspension may also contain stabilizers. Liposomes also can be used to encapsulate the molecules of the invention for delivery into cells or interstitial spaces. Exemplary pharmaceutically acceptable carriers are physiologically compatible solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like. In some embodiments, the composition comprises isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride. In other embodiments, the compositions comprise pharmaceutically acceptable substances such as wetting agents or minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the active ingredients.

[0290] Compositions of the invention may be in a variety of forms, including, for example, liquid (*e.g.*, injectable and infusible solutions), dispersions, suspensions, semi-solid and solid dosage forms. The preferred form depends on the mode of administration and therapeutic application.

[0291] The composition can be formulated as a solution, micro emulsion, dispersion, liposome, or other ordered structure suitable to high drug

concentration. Sterile injectable solutions can be prepared by incorporating the active ingredient in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active ingredient into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

[0292] The active ingredient can be formulated with a controlled-release formulation or device. Examples of such formulations and devices include implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, for example, ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for the preparation of such formulations and devices are known in the art. See *e.g.*, Sustained and Controlled Release Drug Delivery Systems, J. R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

[0293] Injectable depot formulations can be made by forming microencapsulated matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the polymer employed, the rate of drug release can be controlled. Other exemplary biodegradable polymers are polyorthoesters and polyanhydrides. Depot injectable formulations also can be prepared by entrapping the drug in liposomes or microemulsions.

[0294] Supplementary active compounds can be incorporated into the compositions. In one embodiment, the chimeric protein of the invention is formulated with another clotting factor, or a variant, fragment, analogue, or derivative thereof. For example, the clotting factor includes, but is not limited to,

factor V, factor VII, factor VIII, factor IX, factor X, factor XI, factor XII, factor XIII, prothrombin, fibrinogen, von Willebrand factor or recombinant soluble tissue factor (rsTF) or activated forms of any of the preceding. The clotting factor of hemostatic agent can also include anti-fibrinolytic drugs, *e.g.*, epsilon-amino-caproic acid, tranexamic acid.

[0295] Dosage regimens may be adjusted to provide the optimum desired response. For example, a single bolus may be administered, several divided doses may be administered over time, or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. See, *e.g.*, Remington's Pharmaceutical Sciences (Mack Pub. Co., Easton, Pa. 1980).

[0296] In addition to the active compound, the liquid dosage form may contain inert ingredients such as water, ethyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils, glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols, and fatty acid esters of sorbitan.

[0297] Non-limiting examples of suitable pharmaceutical carriers are also described in Remington's Pharmaceutical Sciences by E. W. Martin. Some examples of excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, and the like. The composition can also contain pH buffering reagents, and wetting or emulsifying agents.

[0298] For oral administration, the pharmaceutical composition can take the form of tablets or capsules prepared by conventional means. The composition can also be prepared as a liquid for example a syrup or a suspension. The liquid can include suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats), emulsifying agents (lecithin or acacia), non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol, or fractionated vegetable oils), and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations can also include flavoring, coloring and sweetening agents.

Alternatively, the composition can be presented as a dry product for constitution with water or another suitable vehicle.

[0299] For buccal administration, the composition may take the form of tablets or lozenges according to conventional protocols.

[0300] For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of a nebulized aerosol with or without excipients or in the form of an aerosol spray from a pressurized pack or nebulizer, with optionally a propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoromethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit can be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator can be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[0301] The pharmaceutical composition can also be formulated for rectal administration as a suppository or retention enema, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

[0302] In one embodiment, a pharmaceutical composition comprises a chimeric protein, the polynucleotide encoding the chimeric protein, the vector comprising the polynucleotide, or the host cell comprising the vector, and a pharmaceutically acceptable carrier. The FVIII protein in a chimeric protein has extended half-life compared to wild type FVIII protein or the corresponding FVIII protein without the VWF fragment. In one embodiment, wherein the half-life of the FVIII protein is extended at least about 1.5 times, at least about 2 times, at least about 2.5 times, at least about 3 times, at least about 4 times, at least about 5 times, at least about 6 times, at least about 7 times, at least about 8 times, at least about 9 times, at least about 10 times, at least about 11 times, or at least about 12 times longer than wild type FVIII. In another embodiment, the half-life of Factor VIII is at least about 17 hours, at least about 18 hours, at least about 19 hours, at least about 20 hours, at least about 21 hours, at least about 22 hours, at least about 23 hours, at least about 24 hours, at least about 25 hours, at least about 26 hours, at least about 27 hours, at least about 28 hours, at least about 29 hours, at least about 30 hours, at least about 31 hours, at least about 32 hours, at least about 33 hours, at least about 34 hours, at least about 35 hours, at least about 36 hours, at least about 48 hours, at

least about 60 hours, at least about 72 hours, at least about 84 hours, at least about 96 hours, or at least about 108 hours.

[0303] In some embodiments, the composition is administered by a route selected from the group consisting of topical administration, intraocular administration, parenteral administration, intrathecal administration, subdural administration and oral administration. The parenteral administration can be intravenous or subcutaneous administration.

[0304] In other embodiments, the composition is used to treat a bleeding disease or condition in a subject in need thereof. The bleeding disease or condition is selected from the group consisting of a bleeding coagulation disorder, hemarthrosis, muscle bleed, oral bleed, hemorrhage, hemorrhage into muscles, oral hemorrhage, trauma, trauma capitis, gastrointestinal bleeding, intracranial hemorrhage, intra-abdominal hemorrhage, intrathoracic hemorrhage, bone fracture, central nervous system bleeding, bleeding in the retropharyngeal space, bleeding in the retroperitoneal space, bleeding in the illiopsoas sheath and any combinations thereof. In still other embodiments, the subject is scheduled to undergo a surgery. In yet other embodiments, the treatment is prophylactic or on-demand.

GENE THERAPY

[0305] A chimeric protein thereof of the invention can be produced in vivo in a mammal, *e.g.*, a human patient, using a gene therapy approach to treatment of a bleeding disease or disorder selected from the group consisting of a bleeding coagulation disorder, hemarthrosis, muscle bleed, oral bleed, hemorrhage, hemorrhage into muscles, oral hemorrhage, trauma, trauma capitis, gastrointestinal bleeding, intracranial hemorrhage, intra-abdominal hemorrhage, intrathoracic hemorrhage, bone fracture, central nervous system bleeding, bleeding in the retropharyngeal space, bleeding in the retroperitoneal space, and bleeding in the illiopsoas sheath would be therapeutically beneficial. In one embodiment, the bleeding disease or disorder is hemophilia. In another embodiment, the bleeding disease or disorder is hemophilia A. This involves administration of a suitable chimeric protein-encoding nucleic acid operably linked to suitable expression control sequences. In certain embodiment, these sequences are incorporated into a viral vector. Suitable viral vectors for such gene

therapy include adenoviral vectors, lentiviral vectors, baculoviral vectors, Epstein Barr viral vectors, papovaviral vectors, vaccinia viral vectors, herpes simplex viral vectors, and adeno associated virus (AAV) vectors. The viral vector can be a replication-defective viral vector. In other embodiments, an adenoviral vector has a deletion in its E1 gene or E3 gene. When an adenoviral vector is used, the mammal may not be exposed to a nucleic acid encoding a selectable marker gene. In other embodiments, the sequences are incorporated into a non-viral vector known to those skilled in the art.

METHODS OF USING CHIMERIC PROTEIN

[0306] The present invention is directed to a method of using a chimeric protein described herein to prevent or inhibit endogenous VWF binding to a FVIII protein. The present invention is also directed to a method of using a chimeric protein having a FVIII protein linked to XTEN and an Ig constant region or a portion thereof.

[0307] One aspect of the present invention is directed to preventing or inhibiting FVIII interaction with endogenous VWF by blocking or shielding the VWF binding site on the FVIII from endogenous VWF and at the same time extending half-life of the FVIII protein using an XTEN sequence in combination with an Ig constant region or a portion thereof, which can also be a half-life extender. In one embodiment, the invention is directed to a method of constructing a FVIII protein having half-life longer than wild-type FVIII. In one embodiment, an XTEN sequence inhibits or prevents interaction of a FVIII protein in a chimeric protein with endogenous VWF. In another embodiment, an Ig constant region or a portion thereof inhibits or prevents interaction of the FVIII protein with endogenous VWF. The chimeric protein useful in the method includes any one or more chimeric protein described herein.

[0308] Another aspect of the invention includes a method of administering to a subject in need thereof a chimeric protein comprising a FVIII protein having half-life longer than wild-type FVIII, wherein the method comprises administering the chimeric protein described herein to the subject.

[0309] In one embodiment, the invention is directed to a method of using an XTEN sequence and an Ig constant region or a portion thereof to extend a half-life of a FVIII protein and a VWF fragment to prevent or inhibit endogenous VWF

interaction with a FVIII protein. A FVIII protein linked to an XTEN sequence (*e.g.,* FVIII(X)) and then bound to or associated with a VWF fragment is shielded or protected from the clearance pathway of VWF and thus has reduced clearance compared to the FVIII protein not bound to the VWF fragment. The shielded FVIII protein thus has maximum extension of a half-life compared to a FVIII protein not bound to or associated with the XTEN sequence and the VWF fragment. In certain embodiments, the FVIII protein associated with or protected by a VWF fragment and linked to an XTEN sequence is not cleared by a VWF clearance receptor. In other embodiments, the FVIII protein associated with or protected by a VWF fragment and linked to an XTEN sequence is cleared from the system slower than the FVIII protein that is not associated with or protected by the VWF fragment and linked to the XTEN sequence.

[0310] In one aspect, the chimeric protein comprising the FVIII protein linked to an XTEN sequence or the FVIII protein bound to or associated with a VWF fragment linked to XTEN has reduced clearance from circulation as the VWF fragment does not contain a VWF clearance receptor binding site. The VWF fragment prevents or inhibits clearance of FVIII bound to or associated with the VWF fragment from the system through the VWF clearance pathway. The VWF fragments useful for the present invention can also provide at least one or more VWF-like FVIII protection properties that are provided by endogenous VWF. In certain embodiments, the VWF fragment or the XTEN sequence can also mask one or more FVIII clearance receptor binding site, thereby preventing clearance of FVIII by its own clearance pathway.

[0311] In some embodiments, the prevention or inhibition of a FVIII protein binding to endogenous VWF by the VWF fragment or the XTEN sequence can be *in vitro* or in vivo.

[0312] Also provided is a method of increasing the half-life of a FVIII protein comprising administering the chimeric protein described herein to a subject in need thereof. The half-life of non-activated FVIII bound to or associated with full-length VWF is about 12 to 14 hours in plasma. In VWD type 3, wherein there is almost no VWF in circulation, the half-life of FVIII is only about six hours, leading to symptoms of mild to moderate hemophilia A in such patients due to decreased concentrations of FVIII. The half-life of the FVIII protein linked to or

associated with the VWF fragment or the XTEN sequence of the present invention can increase at least about 1.5 times, 1.6 times, 1.7 times, 1.8 times, 1.9 times, 2.0 times, 2.1 times, 2.2 times, 2.3 times, 2.4 times, 2.6 times, 2.7. times, 2.8 times, 2.9 times, 3.0 times, 3.1 times, 3.2 times, 3.3 times, 3.4 times, 3.5 times, 3.6 times, 3.7 times, 3.8 times, 3.9 times, or 4.0 times higher than the half-life of the non-activated FVIII bound to or associated with full-length VWF.

[0313] In one embodiment, the half-life of the FVIII protein linked to or associated with the VWF fragment or linked to an Ig constant region or a portion thereof in the chimeric protein comprising an XTEN sequence increases at least about 2 times, 2.5 times, 3.0 times, 3.5 times, 4.0 times, 4.5 times, 5.0 times, 5.5 times, 6.0 times, 7 times, 8 times, 9 times, or 10 times higher than the half-life of the non-activated FVIII bound to or associated with full-length VWF. In another embodiment, the half-life of the FVIII protein linked to or associated with the VWF fragment or an Ig constant region or a portion thereof in the chimeric protein comprising an XTEN sequence increases about 2 to about 5 times, about 3 to about 10 times, about 5 to about 15 times, about 10 to about 20 times, about 15 to about 25 times, about 20 to about 30 times, about 25 to about 35 times, about 30 to about 40 times, about 35 to about 45 times higher than the half-life of the non-activated FVIII bound to or associated with full-length VWF or wild type FVIII. In a specific embodiment, the half-life of the FVIII protein linked to or associated with the VWF fragment or linked to an Ig constant region in the chimeric protein comprising an XTEN sequence increases at least about 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 times higher than the half-life of the wild type FVIII in a FVIII and VWF double knockout mouse.

[0314] In some embodiments, the half-life of the chimeric protein comprising the VWF fragment fused to a first Ig constant region or a portion thereof, *e.g.,* a first Fc region and an XTEN sequence, and a FVIII protein linked to an XTEN sequence and a second Ig constant region or a portion thereof, *e.g.,* a second Fc region, is longer than the half-life of a FVIII associated with endogenous VWF. In other embodiments, the half-life of the chimeric protein is at least about 1.5 times, 2 times, 2.5 times, 3.5 times, 3.6 times, 3.7 times, 3.8 times, 3.9 times, 4.0 times, 4.5 times, or 5.0 times the half-life of wild type FVIII or a FVIII protein associated with endogenous VWF.

[0315] In some embodiments, as a result of the invention the half-life of the FVIII protein is extended compared to a FVIII protein without the VWF fragment or wild-type FVIII. The half-life of the chimeric protein of the invention is at least about 1.5 times, at least about 2 times, at least about 2.5 times, at least about 3 times, at least about 4 times, at least about 5 times, at least about 6 times, at least about 7 times, at least about 8 times, at least about 9 times, at least about 10 times, at least about 11 times, or at least about 12 times longer than the half-life of a FVIII protein without the VWF fragment or wild-type FVIII. In one embodiment, the half-life of FVIII is about 1.5-fold to about 20-fold, about 1.5 fold to about 15 fold, or about 1.5 fold to about 10 fold longer than the half-life of wild-type FVIII. In another embodiment, the half-life of the FVIII is extended about 2-fold to about 10-fold, about 2-fold to about 9-fold, about 2-fold to about 8-fold, about 2-fold to about 7-fold, about 2-fold to about 6-fold, about 2-fold to about 5-fold, about 2-fold to about 4-fold, about 2-fold to about 3-fold, about 2.5-fold to about 10-fold, about 2.5-fold to about 9-fold, about 2.5-fold to about 8-fold, about 2.5-fold to about 7-fold, about 2.5-fold to about 6-fold, about 2.5-fold to about 5-fold, about 2.5-fold to about 4-fold, about 2.5-fold to about 3-fold, about 3-fold to about 10-fold, about 3-fold to about 9-fold, about 3-fold to about 8-fold, about 3-fold to about 7-fold, about 3-fold to about 6-fold, about 3-fold to about 5-fold, about 3-fold to about 4-fold, about 4-fold to about 6 fold, about 5-fold to about 7-fold, or about 6-fold to about 8 fold as compared to wild-type FVIII or a FVIII protein without the VWF fragment. In other embodiments, the half-life of the chimeric protein of the invention is at least about 17 hours, at least about 18 hours, at least about 19 hours, at least about 20 hours, at least about 21 hours, at least about 22 hours, at least about 23 hours, at least about 24 hours, at least about 25 hours, at least about 26 hours, at least about 27 hours, at least about 28 hours, at least about 29 hours, at least about 30 hours, at least about 31 hours, at least about 32 hours, at least about 33 hours, at least about 34 hours, at least about 35 hours, at least about 36 hours, at least about 48 hours, at least about 60 hours, at least about 72 hours, at least about 84 hours, at least about 96 hours, or at least about 108 hours. In still other embodiments, the half-life of the chimeric protein of the invention is about 15 hours to about two weeks, about 16 hours to about one week, about 17 hours to about one week, about 18 hours to about one week, about 19 hours to

about one week, about 20 hours to about one week, about 21 hours to about one week, about 22 hours to about one week, about 23 hours to about one week, about 24 hours to about one week, about 36 hours to about one week, about 48 hours to about one week, about 60 hours to about one week, about 24 hours to about six days, about 24 hours to about five days, about 24 hours to about four days, about 24 hours to about three days, or about 24 hours to about two days.

[0316] In some embodiments, the average half-life of the chimeric protein of the invention per subject is about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours (1 day), about 25 hours, about 26 hours, about 27 hours, about 28 hours, about 29 hours, about 30 hours, about 31 hours, about 32 hours, about 33 hours, about 34 hours, about 35 hours, about 36 hours, about 40 hours, about 44 hours, about 48 hours (2 days), about 54 hours, about 60 hours, about 72 hours (3 days), about 84 hours, about 96 hours (4 days), about 108 hours, about 120 hours (5 days), about six days, about seven days (one week), about eight days, about nine days, about 10 days, about 11 days, about 12 days, about 13 days, or about 14 days.

[0317] In addition, the invention provides a method of treating or preventing a bleeding disease or disorder comprising administering an effective amount of a chimeric protein. In one embodiment, the bleeding disease or disorder is selected from the group consisting of a bleeding coagulation disorder, hemarthrosis, muscle bleed, oral bleed, hemorrhage, hemorrhage into muscles, oral hemorrhage, trauma, trauma capitis, gastrointestinal bleeding, intracranial hemorrhage, intra-abdominal hemorrhage, intrathoracic hemorrhage, bone fracture, central nervous system bleeding, bleeding in the retropharyngeal space, bleeding in the retroperitoneal space, and bleeding in the illiopsoas sheath. In a specific embodiment, the bleeding disease or disorder is hemophilia A.

[0318] The chimeric protein comprising an XTEN sequence and an Ig constant region or a portion thereof in combination with a VWF fragment described herein, that prevents or inhibits interaction of the FVIII protein with endogenous VWF prepared by the invention, has many uses as will be recognized by one skilled in the art, including, but not limited to methods of treating a subject having a hemostatic disorder and methods of treating a subject in need of a general

hemostatic agent. In one embodiment, the invention relates to a method of treating a subject having a hemostatic disorder comprising administering a therapeutically effective amount of the chimeric protein.

[0319] The FVIII protein portion in the chimeric protein treats or prevents a hemostatic disorder by serving as a cofactor to Factor IX on a negatively charged phospholipid surface, thereby forming a Xase complex. The binding of activated coagulation factors to a phospholipid surface localizes this process to sites of vascular damage. On a phospholipid surface, Factor VIIIa increases the maximum velocity of Factor X activation by Factor IXa, by approximately 200,000-fold, leading to the large second burst of thrombin generation.

[0320] The chimeric protein of the invention can be used to treat any hemostatic disorder. The hemostatic disorders that may be treated by administration of the chimeric protein of the invention include, but are not limited to, hemophilia A, as well as deficiencies or structural abnormalities relating to Factor VIII. In one embodiment, the hemostatic disorder is hemophilia A.

[0321] The chimeric protein of the invention can be used prophylactically to treat a subject with a hemostatic disorder. The chimeric protein of the invention can be used to treat an acute bleeding episode in a subject with a hemostatic disorder. In another embodiment, the hemostatic disorder can be the result of a defective clotting factor, *e.g.,* von Willebrand's factor. In one embodiment, the hemostatic disorder is an inherited disorder. In another embodiment, the hemostatic disorder is an acquired disorder. The acquired disorder can result from an underlying secondary disease or condition. The unrelated condition can be, as an example, but not as a limitation, cancer, an auto-immune disease, or pregnancy. The acquired disorder can result from old age or from medication to treat an underlying secondary disorder (*e.g.* cancer chemotherapy).

[0322] The invention also relates to methods of treating a subject that does not have a congenital hemostatic disorder, but has a secondary disease or condition resulting in acquisition of a hemostatic disorder, *e.g.,* due to development of an anti-FVIII antibody or a surgery. The invention thus relates to a method of treating a subject in need of a general hemostatic agent comprising administering a therapeutically effective amount of the chimeric protein prepared by the present methods.

[0323] The present invention is also related to methods of reducing immunogenicity of FVIII or inducing less immunogenicity against FVIII comprising administering an effective amount of the chimeric proteins described herein, or the polynucleotides encoding the same.

[0324] In one embodiment, the subject in need of a general hemostatic agent is undergoing, or is about to undergo, surgery. The chimeric protein of the invention can be administered prior to, during, or after surgery as a prophylactic regimen. The chimeric protein of the invention can be administered prior to, during, or after surgery to control an acute bleeding episode..

[0325] The chimeric protein of the invention can be used to treat a subject having an acute bleeding episode who does not have a hemostatic disorder. The acute bleeding episode can result from severe trauma, *e.g.,* surgery, an automobile accident, wound, laceration gun shot, or any other traumatic event resulting in uncontrolled bleeding. Non limiting examples of bleeding episodes include a bleeding coagulation disorder, hemarthrosis, muscle bleed, oral bleed, hemorrhage, hemorrhage into muscles, oral hemorrhage, trauma, trauma capitis, gastrointestinal bleeding, intracranial hemorrhage, intra-abdominal hemorrhage, intrathoracic hemorrhage, bone fracture, central nervous system bleeding, bleeding in the retropharyngeal space, bleeding in the retroperitoneal space, bleeding in the illiopsoas sheath, and any combinations thereof.

[0326] In prophylactic applications, one or more compositions containing the chimeric protein of the invention or a cocktail thereof are administered to a patient not already in the disease state to enhance the patient's resistance or reduce symptoms associated with a disease or disorder. Such an amount is defined to be a "prophylactic effective dose." In therapeutic applications, a relatively high dosage (*e.g.*, from about 1 to 400 mg/kg of polypeptide per dose, with dosages of from 5 to 25 mg being more commonly used for radioimmuno conjugates and higher doses for cytotoxin-drug modified polypeptides) at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, the patient can be administered a prophylactic regime.

[0327] In some embodiments, a chimeric protein or a composition of the invention is used for on-demand treatment, which includes treatment for a bleeding episode,

hemarthrosis, muscle bleed, oral bleed, hemorrhage, hemorrhage into muscles, oral hemorrhage, trauma, trauma capitis (head trauma), gastrointestinal bleeding, intracranial hemorrhage, intra-abdominal hemorrhage, intrathoracic hemorrhage, bone fracture, central nervous system bleeding, bleeding in the retropharyngeal space, bleeding in the retroperitoneal space, or bleeding in the illiopsoas sheath. The subject may be in need of surgical prophylaxis, peri-operative management, or treatment for surgery. Such surgeries include, *e.g.,* minor surgery, major surgery, tooth extraction, tonsillectomy, inguinal herniotomy, synovectomy, total knee replacement, craniotomy, osteosynthesis, trauma surgery, intracranial surgery, intra-abdominal surgery, intrathoracic surgery, or joint replacement surgery.

[0328] In one embodiment, the chimeric protein of the present invention is administered intravenously, subcutaneously, intramuscularly, or via any mucosal surface, *e.g.*, orally, sublingually, buccally, nasally, rectally, vaginally or via pulmonary route. The chimeric protein comprising a VWF fragment and a FVIII protein of the present invention can be implanted within or linked to a biopolymer solid support that allows for the slow release of the chimeric protein to the site of bleeding or implanted into bandage/dressing. The dose of the chimeric protein will vary depending on the subject and upon the particular route of administration used. Dosages can range from 0.1 to 100,000 μg/kg body weight. In one embodiment, the dosing range is 0.1-1,000 μg/kg. In another embodiment, the dosing range is 0.1-500 μg/kg. The protein can be administered continuously or at specific timed intervals. *In vitro* assays may be employed to determine optimal dose ranges and/or schedules for administration. *In vitro* assays that measure clotting factor activity are known in the art, *e.g.*, STA-CLOT VIIa-rTF clotting assay or ROTEM clotting assay. Additionally, effective doses may be extrapolated from dose-response curves obtained from animal models, *e.g.*, a hemophiliac dog (Mount *et al.* 2002, *Blood* 99(8):2670).

[0329] Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention. All patents, publications, and articles referred to herein are expressly and specifically incorporated herein by reference.

Examples

[0330] Throughout the examples, the following materials and methods were used unless otherwise stated.

Materials and Methods

[0331] In general, the practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, biophysics, molecular biology, recombinant DNA technology, immunology (especially, *e.g.*, antibody technology), and standard techniques in electrophoresis. See, *e.g.*, Sambrook, Fritsch and Maniatis, Molecular Cloning: Cold Spring Harbor Laboratory Press (1989); Antibody Engineering Protocols (Methods in Molecular Biology), 510, Paul, S., Humana Pr (1996); Antibody Engineering: A Practical Approach (Practical Approach Series, 169), McCafferty, Ed., Irl Pr (1996); Antibodies: A Laboratory Manual, Harlow *et al.*, CS.H.L. Press, Pub. (1999); and Current Protocols in Molecular Biology, eds. Ausubel *et al.*, John Wiley & Sons (1992).

Example 1: Cloning different VWF domains (Figure 1)

(a) Cloning of pSYN-VWF-002

[0332] pSYN-VWF-002 contains nucleotide sequences encoding a VWF fragment, which are amino acids 1-477 of SEQ ID NO: 100. [VWF-D'D3 protein sequence] Amino acid numbering represents the mature VWF sequence without propeptide and corresponds to amino acids 764-1240 of SEQ ID NO: 2. pSYN-VWF-002 construct has the FVIII signal peptide at N-terminus, which allows proper secretion of the synthesized protein and followed by a 6xHis tag at C-terminus, which is used for protein purification. It was synthesized by using following primer combinations:

ESC48- Fwd - VWF-D'D3 with VIII signal and BsiW1 site

TCGCGACGTACGGCCGCCACCATGCAAATAGAGCTCTCCACCTGCTTCTTTCTGT GCCTTTTGCGATTCTGCTTTAGCCTATCCTGTCGGCCCCCCATG (SEQ ID NO: 90)

ESC51- Rev- VWF D'D3 (1-477 amino acid) with 6His and Not 1 site

TGACCTCGAGCGGCCGCTCAGTGGTGATGGTGATGATGCGGCTCCTGGCAGGCTT CACAGGTGAGGTTGACAAC (SEQ ID NO: 91)

[0333] A 50 µl PCR reaction was carried out with ESC 48/ESC 51 primer combinations and full length VWF plasmid as the template, using the 2 step PCR

amplification cycle: 94 °C 2minutes; 21 cycles of (96 °C 30 seconds, 68 °C 2 minute). The 1460bp band was gel purified with a Gel Extraction kit (Qiagen, Valencia, Calif.) and cloned into the BsiWI and Not1 restriction sites of pcDNA 4 to generate pSYN-VWF 002.

(b) Cloning of pSYN-VWF- 010 and 013

[0334] pSYN-VWF-010 was constructed using pSYN-VWF-008 and pSYN-VWF-002. pSYN-VWF-008 contains the full-length VWF sequence in pcDNA 3.1 (amino acids 1-2813 of SEQ ID NO: 2), it includes 763 amino acid propeptide (*i.e.*, D1D2 domains) followed by remaining 2050 amino acids sequence of mature VWF. The FVIII signal peptide in pSYN-VWF-002 was replaced with D1D2 domains from pSYN-VWF-008, the resulting construct is pSYN-VWF-010. pSYN-VWF- 008 has a BamH1 site at Arg907 and Not1 site at the end of coding region (after stop codon). pSYN- VWF- 008 and 002 were digested with BamH1 and Not1 restriction enzymes. Inserts from pSYN- VWF-002 (1026 bp) were ligated into bamH1/Not1 digested pSYN-VWF- 008 (8242bp) to obtain pSYN-VWF -010 (D1D2D'D3: amino acid 1-1240 of SEQ ID NO: 2), a 6xHis tag was also added at the C-terminus. In transformed cells pSYN-VWF-010 is synthesized with propeptide but due to intracellular processing the secreted products do not contain any propeptide (D1D2). Protein from VWF-010 exists as dimer.

[0335] pSYN-VWF-010 was used to generate pSYN-VWF-013 which has two point mutations at C336A and C379A corresponding to SEQ ID NO: 100 (amino acid numbering represents mature VWF sequence without D1D2 domain-VWF sequence 2). These mutations are predicted to prevent dimerization of VWF D'D3 domain.

(c) Cloning of pSYN-VWF-025 and pSYN-VWF-029

[0336] pSYN-VWF-025 contains wild type D1D2D'D3 sequences of full-length VWF in pLIVE vector, and pSYN-VWF-029 contains D1D2D'D3 sequence with C336A and C379A mutation. For cloning pSYN-VWF-025, the following primer combination was used:

ESC 89-fwd with Nhe1site= CTCACTATAGGGAGACCCAAGCTGGCTAGCCG (SEQ ID NO: 92)

ESC 91-rev with Sal1=

CTGGATCCCGGGAGTCGACTCGTCAGTGGTGATGGTGATGATG (SEQ ID NO: 93)

[0337] A 50 µl PCR reaction was carried out with ESC 89/ESC91 primer combinations and either pSYN-VWF 010 (for pSYN-VWF-025) or pSYN-VWF 013 (for pSYN-VWF-029) plasmid as the template using the 3 step PCR amplification cycle: 94 °C 2minutes; 21 cycles of (96 °C -30 seconds, 55 °C-30 second, 68 °C-4 minutes). The expected sized band (~3800bp) was gel purified with a Gel Extraction kit (Qiagen, Valencia, Calif.) and cloned into the Nhe1and Sal1 restriction sites of pLIVE-Mirus vector (Invitrogen, Carlsbad, Calif.) to generate pSYN-VWF 025 and 029.

(d) Cloning pSYN-VWF-031

[0338] pSYN-VWF-031 is a D1D2D'D3(C336A/C379A) -Fc construct which has a 48 amino acid long thrombin cleavable linker (8x GGGGS (SEQ ID NO 94) + thrombin site) in between the VWF D1D2D'D3(C336A/C379A) and the Fc sequences. To make this construct, VWF-Fc region was amplified from construct pSYN-FVIII-064 (refer FVIII-VWF construct below). pSYN-FVIII-VWF was digested with Xba1 and Nhe1. Resulting insert region of 4165bp, containing the VWF fragment and Fc region was used as a template for amplifying the VWF and Fc region by primer combinations LW 22/LW23.

LW 22-FWD-VWF-D'D3 with FVIII signal sequence and BsiW1 site

GCGCCGGCCGTACGATGCAAATAGAGCTCTCCACCTGCTTCTTTCTGTGCCTTTT GCGATTCTGCTTTAGCCTATCCTGTCGGCCCCCCATG (SEQ ID NO: 95)

LW 23-Rev- Fc with stop codon and Not1 site

TCATCAATGTATCTTATCATGTCTGAATTCGCGGCCGCTCATTTACC (SEQ ID NO:96)

[0339] The PCR product obtained from LW22/LW23 amplification (~2300bp) was cloned in BsiW1/Not1 digested pSYN-VWF-002 to obtain pSYN-VWF-014 intermediate. pSYN-VWF-014 contains FVIII signal peptide-D'D3-20 amino acid thrombin cleavable linker followed by the Fc region.

[0340] To generate the D1D2D'D3-Fc construct, the D1D2D'D3 region was amplified from pSYN-VWF-013 using primer combination LW24/LW27 by standard PCR method.

LW24- Fwd- VWF D1D2D'D3 cloning oligo with BsiW1 site

GCGCCGGCCGTACGATGATTCCTGCCAGATTTGCCGGGGTG (SEQ ID NO:97)

LW27-Rev-VWF D'D3 oligo with EcoRV

CCACCGCCAGATATCGGCTCCTGGCAGGCTTCACAGGTGAG (SEQ ID NO:98)

[0341] The PCR product obtained from LW22/LW23 amplification (~3750bp) was cloned in BsiW1/EcoRV digested pSYN-VWF-014 to obtain pSYN-VWF-015 intermediate. The linker length between the VWF fragment and Fc region was changed to obtain pSYN-VWF-031.

VWF-D1D2D'D3 protein sequence 1 (SEQ ID NO: 99)

```
  1   MIPARFAGVL LALALILPGT LCAEGTRGRS STARCSLFGS
DFVNTFDGSM
 51   YSFAGYCSYL LAGGCQKRSF SIIGDFQNGK RVSLSVYLGE
  FFDIHLFVNG
101   TVTQGDQRVS MPYASKGLYL ETEAGYYKLS GEAYGFVARI
  DGSGNFQVLL
151   SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL TSDPYDFANS
  WALSSGEQWC
201   ERASPPSSSC NISSGEMQKG LWEQCQLLKS TSVFARCHPL
  VDPEPFVALC
251   EKTLCECAGG LECACPALLE YARTCAQEGM VLYGWTDHSA
  CSPVCPAGME
301   YRQCVSPCAR TCQSLHINEM CQERCVDGCS CPEGQLLDEG
  LCVESTECPC
351   VHSGKRYPPG TSLSRDCNTC ICRNSQWICS NEECPGECLV
  TGQSHFKSFD
401   NRYFTFSGIC QYLLARDCQD HSFSIVIETV QCADDRDAVC
  TRSVTVRLPG
451   LHNSLVKLKH GAGVAMDGQD IQLPLLKGDL RIQHTVTASV
  RLSYGEDLQM
501   DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN QGDDFLTPSG
  LAEPRVEDFG
551   NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS EEACAVLTSP
  TFEACHRAVS
601   PLPYLRNCRY DVCSCSDGRE CLCGALASYA AACAGRGVRV
  AWREPGRCEL
651   NCPKGQVYLQ CGTPCNLTCR SLSYPDEECN EACLEGCFCP
  PGLYMDERGD
701   CVPKAQCPCY YDGEIFQPED IFSDHHTMCY CEDGFMHCTM
  SGVPGSLLPD
751   AVLSSPLSHR SKRSLSCRPP MVKLVCPADN LRAEGLECTK
  TCQNYDLECM
801   SMGCVSGCLC PPGMVRHENR CVALERCPCF HQGKEYAPGE
  TVKIGCNTCV
851   CRDRKWNCTD HVCDATCSTI GMAHYLTFDG LKYLFPGECQ
  YVLVQDYCGS
901   NPGTFRILVG NKGCSHPSVK CKKRVTILVE GGEIELFDGE
  VNVKRPMKDE
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951 THFEVVESGR YIILLLGKAL SVVWDRHLSI SVVLKQTYQE
KVCGLCGNFD
1001 GIQNNDLTSS NLQVEEDPVD FGNSWKVSSQ CADTRKVPLD
SSPATCHNNI
1051 MKQTMVDSSC RILTSDVFQD CNKLVDPEPY LDVCIYDTCS
CESIGDCACF
1101 CDTIAAYAHV CAQHGKVVTW RTATLCPQSC EERNLRENGY
ECEWRYNSCA
1151 PACQVTCQHP EPLACPVQCV EGCHAHCPPG KILDELLQTC
VDPEDCPVCE
1201 VAGRRFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP*

VWF-D'D3 protein sequence 2 (SEQ ID NO: 100)
1 SLSCRPPMVK LVCPADNLRA EGLECTKTCQ NYDLECMSMG
CVSGCLCPPG
51 MVRHENRCVA LERCPCFHQG KEYAPGETVK IGCNTCVCRD
RKWNCTDHVC
101 DATCSTIGMA HYLTFDGLKY LFPGECQYVL VQDYCGSNPG
TFRILVGNKG
151 CSHPSVKCKK RVTILVEGGE IELFDGEVNV KRPMKDETHF
EVVESGRYII
201 LLLGKALSVV WDRHLSISVV LKQTYQEKVC GLCGNFDGIQ
NNDLTSSNLQ
251 VEEDPVDFGN SWKVSSQCAD TRKVPLDSSP ATCHNNIMKQ
TMVDSSCRIL
301 TSDVFQDCNK LVDPEPYLDV CIYDTCSCES IGDCACFCDT
IAAYAHVCAQ
351 HGKVVTWRTA TLCPQSCEER NLRENGYECE WRYNSCAPAC
QVTCQHPEPL
401 ACPVQCVEGC HAHCPPGKIL DELLQTCVDP EDCPVCEVAG
RRFASGKKVT
451 LNPSDPEHCQ ICHCDVVNLT CEACQEP

Example 2: Effects of D'D3 and XTEN fusion on FVIII half-life extension

[0342] To evaluate D'D3 FVIII half-life extension potential on rFVIII-XTEN fusion protein, a VWF D'D3 dimer was introduced into FVIII-VWF DKO mice by hydrodynamic injection of its corresponding DNA construct VWF-025 (Example 1). After D'D3 has reached the steady state expression (day5 post injection), a single dose of rFVIII-XTEN was administered by IV injection at 200 IU/kg dose. Blood samples were collected up to 120hrs post rFVIII-XTEN dosing. Plasma FVIII activity was analyzed by a FVIII chromogenic assay. The D'D3 expression level was measured by VWF ELISA, and rFVIIIFc PK profile was analyzed using WinNonlin program.

[0343] The study results were shown in Figure 2, and the PK parameter of rFVIII-XTEN with/without D'D3 in circulation was listed in Table 16. The D'D3 dimer further extended rFIII-XTEN $t_{1/2}$ from 3.4hr to 17.8hr, a 5 fold increase. In

addition to half-life, 5 fold of increase on MRT, 3.6 fold increases on AUC, 3.8 fold decreases on clearance were also observed.

[0344] We have observed a synergistic effect of D'D3 fragment and XTEN technology, a serial of FVIII/VWF/XTEN constructs will be evaluated for their FVIII half-life extension potential in Hemophilic animals.

TABLE 16: rFVIII-XTEN PK parameter with/without D'D3 in blood circulation

Treatment	5min Recovery (%)	$t_{1/2}$ (hr)	MRT (hr)	Cl (mL/hr/kg)	Vss (mL/kg)	AUC_D (hr*kg*mIU /mL/mIU)
rFVIIIXTEN VWF-025	80	17.8	19.3	3.5	67.4	0.29
rFVIIIXTEN	74	3.4	3.8	13.1	63.68	0.08
Improvement fold	1.1	5.2	5.1	3.8	0.9	3.6

Protein purification of FVIII-XTEN

[0345] An AE288 XTEN was inserted at the C-terminus of BDD-FVIII for this study. To purify this protein, a tangential flow filtration (TFF) step was used first to buffer exchange the conditioned media. Products in the filtrate were then captured using a strong anion exchange chromatography, and then further purified using affinity chromatography. Purity of the molecule was acceptable by HPLC-SEC and was further confirmed by western blotting. The specific activity of the molecule was comparable to B-domain deleted FVIII, as measured by aPTT assay and ELISA.

FVIII chromogenic assay

[0346] The FVIII activity was measured using the COATEST SP FVIII kit from DiaPharma (lot# N089019) and all incubations were performed on a 37°C plate heater with shaking.

[0347] The range of rFVIII standard was from 100 mIU/mL to 0.78 mIU/mL. A pooled normal human plasma assay control and plasma samples (diluted with 1X Coatest buffer) were added into Immulon 2HB 96-well plates in duplicate (25 μL/well). Freshly prepared IXa/FX/Phospholipid mix (50 μL), 25 μL of 25mM CaCl2, and 50 μL of FXa substrate were added sequentially into each well with 5

minutes incubation between each addition. After incubating with the substrate, 25 μL of 20% Acetic Acid was added to terminate the color reaction, and the absorbance of OD405 was measured with a SpectraMAX plus (Molecular Devices) instrument. Data were analyzed with SoftMax Pro software (version 5.2). The Lowest Level of Quantification (LLOQ) is 7.8 mIU/mL.

VWF ELISA:

[0348] Goat anti-human VWF antibody (Affinity purified, affinity biological, GAVWF-AP) was used as the capture antibody at 0.5ug/well and VWF-EIA-D (Affinity Biologicals, VWF-EIA-D, 1:100 dilution) was used as the detecting antibody for the VWF ELISA. ELISA assay was performed following the standard ELISA procedure, TMB was used as the HRP substrate, PBST/1.5% BSA/0.5M NaCl buffer was used as blocking and binding buffer. The assay standard range is 100ng to 0.78ng, and assay's lowest limit of quantification (LLOQ) is 7.8ng/mL.

Example 3: Plasmid construction of XTEN containing FVIII/VWF constructs

(a) Cloning of pSYN-FVIII-161 (Figure 3)

[0349] The FVIII-161 plasmid comprises a single chain Fc (scFc) scaffold with enzyme cleavage sites which are processed during synthesis in a cell. The construct has a FVIII binding domain of full-length VWF (D'D3).

[0350] Plasmid (pSYN-FVIII-161) was designed for the expression FVIII-Fc and VWF-Fc heterodimer, where the D'D3 domains to bind FVIII and prevents FVIII interaction with phospholipids and activated protein C. Protein from pSYN-FVIII-161 is expressed in the cell as a single polypeptide where the C-terminus of the FVIII-Fc subunit is linked to the N-terminus of the VWF D'D3-Fc subunit by a 6x (GGGGS) polypeptide linker (SEQ ID NO: 64). In addition, RRRRS (SEQ ID NO: 11) and RKRRKR (SEQ ID NO: 10) sequences were inserted at the 5' and 3' end of the polypeptide linker, respectively, for intracellular cleavage by proprotein convertases following the last Arg at each sequence. Hence, the cells can express a double chain FVIII-Fc/D'D3-Fc heterodimer where the FVIII-Fc chain has a RRRRS sequence (SEQ ID NO: 11) at the C-terminus, but the remainder of the linker sequence has been removed. An AE288 XTEN fragment immediately followed by IS{5X(GGGGS)}LVPRGSGG (SEQ ID NO: 122) polypeptide (contains thrombin cleavage site) is introduced in between the VWF domains and

the Fc region to facilitate release of the VWF fragment from FVIII once the FVIII-VWF hetero-dimeric protein is activated by thrombin allowing interaction of FVIII with other clotting factors.

[0351] pSYN-FVIII-161 (SEQ ID NO: 101).protein sequence (FVIII sequence amino acid position 1-1457; underlined region represents Fc region; curvy underline represents cleavable linker in between first Fc and VWF fragment; double underlined region represents VWF fragment; bold region represents cleavable linker in between VWF fragment and Fc.

```
  1  MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQSDL
 GELPVDARFP
 51  PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL
 LGPTIQAEVY
101  DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR
 EKEDDKVFPG
151  GSHTYVWQVL KENGPMASDP LCLTYSYLSH VDLVKDLNSG
 LIGALLVCRE
201  GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
 AASARAWPKM
251  HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL
 EGHTFLVRNH
301  RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME
 AYVKVDSCPE
351  EPQLRMKNNE EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI
 RSVAKKHPKT
401  WVHYIAAEEE DWDYAPLVLA PDDRSYKSQY LNNGPQRIGR
 KYKKVRFMAY
451  TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR
 PYNIYPHGIT
501  DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
 TKSDPRCLTR
551  YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR
 NVILFSVFDE
601  NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV
 FDSLQLSVCL
651  HEVAYWYILS IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF
 PFSGETVFMS
701  MENPGLWILG CHNSDFRNRG MTALLKVSSC DKNTGDYYED
 SYEDISAYLL
751  SKNNAIEPRS FSQNPPVLKR HQREITRTTL QSDQEEIDYD
 DTISVEMKKE
801  DFDIYDEDEN QSPRSFQKKT RHYFIAAVER LWDYGMSSSP
 HVLRNRAQSG
851  SVPQFKKVVF QEFTDGSFTQ PLYRGELNEH LGLLGPYIRA
 EVEDNIMVTF
901  RNQASRPYSF YSSLISYEED QRQGAEPRKN FVKPNETKTY
 FWKVQHHMAP
```

951 TKDEFDCKAW AYFSDVDLEK DVHSGLIGPL LVCHTNTLNP
AHGRQVTVQE
1001 FALFFTIFDE TKSWYFTENM ERNCRAPCNI QMEDPTFKEN
YRFHAINGYI
1051 MDTLPGLVMA QDQRIRWYLL SMGSNENIHS IHFSGHVFTV
RKKEEYKMAL
1101 YNLYPGVFET VEMLPSKAGI WRVECLIGEH LHAGMSTLFL
VYSNKCQTPL
1151 GMASGHIRDF QITASGQYGQ WAPKLARLHY SGSINAWSTK
EPFSWIKVDL
1201 LAPMIIHGIK TQGARQKFSS LYISQFIIMY SLDGKKWQTY
RGNSTGTLMV
1251 FFGNVDSSGI KHNIFNPPII ARYIRLHPTH YSIRSTLRME
LMGCDLNSCS
1301 MPLGMESKAI SDAQITASSY FTNMFATWSP SKARLHLQGR
SNAWRPQVNN
1351 PKEWLQVDFQ KTMKVTGVTT QGVKSLLTSM YVKEFLISSS
QDGHQWTLFF
1401 QNGKVKVFQG NQDSFTPVVN SLDPPLLTRY LRIHPQSWVH
QIALRMEVLG
1451 CEAQDLYDKT HTCPPCPAPE LLGGPSVFLF PPKPKDTLMI
SRTPEVTCVV
1501 VDVSHEDPEV KFNWYVDGVE VHNAKTKPRE EQYNSTYRVV
SVLTVLHQDW
1551 LNGKEYKCKV SNKALPAPIE KTISKAKGQP REPQVYTLPP
SRDELTKNQV
1601 SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSDGS
FFLYSKLTVD
1651 KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPGKRRRRSG
GGGSGGGGSG
1701 GGGSGGGGSG GGGSGGGGSR KRRKRSLSCR PPMVKLVCPA
DNLRAEGLEC
1751 TKTCQNYDLE CMSMGCVSGC LCPPGMVRHE NRCVALERCP
CFHQGKEYAP
1801 GETVKIGCNT CVCRDRKWNC TDHVCDATCS TIGMAHYLTF
DGLKYLFPGE
1851 CQYVLVQDYC GSNPGTFRIL VGNKGCSHPS VKCKKRVTIL
VEGGEIELFD
1901 GEVNVKRPMK DETHFEVVES GRYIILLLGK ALSVVWDRHL
SISVVLKQTY
1951 QEKVCGLCGN FDGIQNNDLT SSNLQVEEDP VDFGNSWKVS
SQCADTRKVP
2001 LDSSPATCHN NIMKQTMVDS SCRILTSDVF QDCNKLVDPE
PYLDVCIYDT
2051 CSCESIGDCA AFCDTIAAYA HVCAQHGKVV TWRTATLCPQ
SCEERNLREN
2101 GYEAEWRYNS CAPACQVTCQ HPEPLACPVQ CVEGCHAHCP
PGKILDELLQ
2151 TCVDPEDCPV CEVAGRRFAS GKKVTLNPSD PEHCQICHCD
VVNLTCEACQ

2201 **EPISGTSESA TPESGPGSEP ATSGSETPGT SESATPESGP**
GSEPATSGSE
2251 **TPGTSESATP ESGPGTSTEP SEGSAPGSPA GSPTSTEEGT**
SESATPESGP
2301 **GSEPATSGSE TPGTSESATP ESGPGSPAGS PTSTEEGSPA**
GSPTSTEEGT
2351 **STEPSEGSAP GTSESATPES GPGTSESATP ESGPGTSESA**
TPESGPGSEP
2401 **ATSGSETPGS EPATSGSETP GSPAGSPTST EEGTSTEPSE**
GSAPGTSTEP
2451 **SEGSAPGSEP ATSGSETPGT SESATPESGP GTSTEPSEGS**
APDSGGGGSG
2501 **GGGSGGGGSG GGGSGGGGSL VPRGSGG**DKT HTCPPCPAPE
LLGGPSVFLF
2551 PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV KFNWYVDGVE
VHNAKTKPRE
2601 EQYNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKALPAPIE
KTISKAKGQP
2651 REPQVYTLPP SRDELTKNQV SLTCLVKGFY PSDIAVEWES
NGQPENNYKT
2701 TPPVLDSDGS FFLYSKLTVD KSRWQQGNVF SCSVMHEALH
NHYTQKSLSL
2751 SPGK

(b) Cloning of pSYN-FVIII-168, 175, 172 and 174 (Figure 4A-4D)

[0352] pSYN-FVIII-168, 172, 174 and 175 are derivatives of pSYN-FVIII-161. R1645A/R1648A mutations were introduced into pSYN-FVIII-161 to form pSYN-FVIII-168, which produces a SC-FVIII isoform, and an AE288 XTEN was directly fused into the C-terminus of FVIII-HC for further half-life extension. To construct pSYN-FVIII-175, the D'D3 codon sequence was remove form pSYN-FVIII-168 for evaluation of the effect of Fc and XTEN technology on FVIII half-life extension.

[0353] To construct pSYN-FVIII-172, the AE288 XTEN fragment was directly fused into the C-terminus of FVIII-HC for further half-life extension, and the D'D3 codon sequence was removed from pSYN-FVIII-172 to form pSYN-FVIII-174 for evaluation of the effect of Fc and XTEN technology on FVIII half-life extension.

(c) Cloning of pSYN-FVIII-170 (Figure 4E)

[0354] pSYN-FVIII-170 was constructed to evaluate the effect of XTEN and D'D3 fragment on FVIII half-life extension. The codon sequence VWF-D1D2D'D3 fragment and BDD-FVIII were introduced into the 5' and 3' end of expression casket, an AE288 XTEN codon sequence which followed by a 35 aa

thrombin cleavable linker was used to connect the VWF and FVIII molecule. After intra cellular processing, the secreted protein comprises a polypeptide contains the D'D3 fragment of mature VWF molecule which is linked to the N-terminus of mature BDD-FVIII by an AE288 XTEN/35 aa thrombin cleavable linker.

pSYN-FVIII-170 protein sequence (SEQ ID NO: 102)

```
  1  SLSCRPPMVK LVCPADNLRA EGLECTKTCQ NYDLECMSMG
 CVSGCLCPPG
 51  MVRHENRCVA LERCPCFHQG KEYAPGETVK IGCNTCVCRD
 RKWNCTDHVC
101  DATCSTIGMA HYLTFDGLKY LFPGECQYVL VQDYCGSNPG
 TFRILVGNKG
151  CSHPSVKCKK RVTILVEGGE IELFDGEVNV KRPMKDETHF
 EVVESGRYII
201  LLLGKALSVV WDRHLSISVV LKQTYQEKVC GLCGNFDGIQ
 NNDLTSSNLQ
251  VEEDPVDFGN SWKVSSQCAD TRKVPLDSSP ATCHNNIMKQ
 TMVDSSCRIL
301  TSDVFQDCNK LVDPEPYLDV CIYDTCSCES IGDCAAFCDT
 IAAYAHVCAQ
351  HGKVVTWRTA TLCPQSCEER NLRENGYEAE WRYNSCAPAC
 QVTCQHPEPL
401  ACPVQCVEGC HAHCPPGKIL DELLQTCVDP EDCPVCEVAG
 RRFASGKKVT
451  LNPSDPEHCQ ICHCDVVNLT CEACQEPISG TSESATPESG
 PGSEPATSGS
501  ETPGTSESAT PESGPGSEPA TSGSETPGTS ESATPESGPG
 TSTEPSEGSA
551  PGSPAGSPTS TEEGTSESAT PESGPGSEPA TSGSETPGTS
 ESATPESGPG
601  SPAGSPTSTE EGSPAGSPTS TEEGTSTEPS EGSAPGTSES
 ATPESGPGTS
651  ESATPESGPG TSESATPESG PGSEPATSGS ETPGSEPATS
 GSETPGSPAG
701  SPTSTEEGTS TEPSEGSAPG TSTEPSEGSA PGSEPATSGS
 ETPGTSESAT
751  PESGPGTSTE PSEGSAPDSG GGGSGGGGSG GGGSGGGGSG
 GGGSLVPRGS
801  GGASATRRYY LGAVELSWDY MQSDLGELPV DARFPPRVPK
 SFPFNTSVVY
851  KKTLFVEFTD HLFNIAKPRP PWMGLLGPTI QAEVYDTVVI
 TLKNMASHPV
901  SLHAVGVSYW KASEGAEYDD QTSQREKEDD KVFPGGSHTY
 VWQVLKENGP
951  MASDPLCLTY SYLSHVDLVK DLNSGLIGAL LVCREGSLAK
 EKTQTLHKFI
```

1001 LLFAVFDEGK SWHSETKNSL MQDRDAASAR AWPKMHTVNG
YVNRSLPGLI
1051 GCHRKSVYWH VIGMGTTPEV HSIFLEGHTF LVRNHRQASL
EISPITFLTA
1101 QTLLMDLGQF LLFCHISSHQ HDGMEAYVKV DSCPEEPQLR
MKNNEEAEDY
1151 DDDLTDSEMD VVRFDDDNSP SFIQIRSVAK KHPKTWVHYI
AAEEEDWDYA
1201 PLVLAPDDRS YKSQYLNNGP QRIGRKYKKV RFMAYTDETF
KTREAIQHES
1251 GILGPLLYGE VGDTLLIIFK NQASRPYNIY PHGITDVRPL
YSRRLPKGVK
1301 HLKDFPILPG EIFKYKWTVT VEDGPTKSDP RCLTRYYSSF
VNMERDLASG
1351 LIGPLLICYK ESVDQRGNQI MSDKRNVILF SVFDENRSWY
LTENIQRFLP
1401 NPAGVQLEDP EFQASNIMHS INGYVFDSLQ LSVCLHEVAY
WYILSIGAQT
1451 DFLSVFFSGY TFKHKMVYED TLTLFPFSGE TVFMSMENPG
LWILGCHNSD
1501 FRNRGMTALL KVSSCDKNTG DYYEDSYEDI SAYLLSKNNA
IEPRSFSQNP
1551 PVLKRHQREI TRTTLQSDQE EIDYDDTISV EMKKEDFDIY
DEDENQSPRS
1601 FQKKTRHYFI AAVERLWDYG MSSSPHVLRN RAQSGSVPQF
KKVVFQEFTD
1651 GSFTQPLYRG ELNEHLGLLG PYIRAEVEDN IMVTFRNQAS
RPYSFYSSLI
1701 SYEEDQRQGA EPRKNFVKPN ETKTYFWKVQ HHMAPTKDEF
DCKAWAYFSD
1751 VDLEKDVHSG LIGPLLVCHT NTLNPAHGRQ VTVQEFALFF
TIFDETKSWY
1801 FTENMERNCR APCNIQMEDP TFKENYRFHA INGYIMDTLP
GLVMAQDQRI
1851 RWYLLSMGSN ENIHSIHFSG HVFTVRKKEE YKMALYNLYP
GVFETVEMLP
1901 SKAGIWRVEC LIGEHLHAGM STLFLVYSNK CQTPLGMASG
HIRDFQITAS
1951 GQYGQWAPKL ARLHYSGSIN AWSTKEPFSW IKVDLLAPMI
IHGIKTQGAR
2001 QKFSSLYISQ FIIMYSLDGK KWQTYRGNST GTLMVFFGNV
DSSGIKHNIF
2051 NPPIIARYIR LHPTHYSIRS TLRMELMGCD LNSCSMPLGM
ESKAISDAQI
2101 TASSYFTNMF ATWSPSKARL HLQGRSNAWR PQVNNPKEWL
QVDFQKTMKV
2151 TGVTTQGVKS LLTSMYVKEF LISSSQDGHQ WTLFFQNGKV
KVFQGNQDSF
2201 TPVVNSLDPP LLTRYLRIHP QSWVHQIALR MEVLGCEAQD LY

Example 4: Hydrodynamic injection of XTEN containing FVIIIF/VWF constructs in FVIII and VWF deficient mice

[0355] The XTEN containing DNA constructs in Figures 3 and 4 have combined 2-3 half-life extension elements together. To evaluate their FVIII half-life extension potential, a selective group of DNA constructs in figure 3 and figure 4 were introduced into FVIII/VWF double knockout (DKO) mice by Hydrodynamic injection (HDI) at 100ug/mouse dose. Blood samples were then collected by retro orbital blood collection at 24hr post HDI. The post HDI plasma FVIII activity was analyzed by FVIII chromogenic assay, and results were listed in Table 17 and Figure 5. Compared to wild type BDD-FVIII, all XTEN containing DNA constructs yield significantly higher FVIII plasma activity at 24hr post HDI, indicating the corresponding molecules had significant longer circulating protein half-life than BDD-FVIII. The application of the combination of those half-life extending elements was further evaluated in Hemophilic animals.

TABLE 17: FVIII plasma activity 24hr post HDI in FVIII/VWF DKO mice

DNA Construct	BDD-FVIII	FVIII-161	FVIII-168	FVIII-172	BDD-FVIII	FVIII-170
DNA Dose (μg/mouse)	100	100	100	100	50	50
FVIII Activity (mU/mL)	219±72	2446±1012	2209±609	1671±223	197±21	399±30

Hydrodynamic injection:

[0356] Hydrodynamic Injection is an efficient and safe non-viral gene delivery method to the liver in small animals, such as mice and rats. It was originally described as a rapid injection of a naked plasmid DNA/saline solution free of endotoxin at a tenth volume of the animal's body weight in about 5-7 seconds. The naked plasmid DNA contains the gene of interest and the liver produced in a tenth volume of the animal´s body weight. The targeted protein is produced in the liver from the injected DNA and can be detected within 24 hours post-injection. Plasma samples were then collected to study the therapeutic property of the expressed protein.

[0357] For all the hydrodynamic injections that were performed herein, 2 ml of plasmid DNA in 0.9% sterile saline solution was delivered via intravenous tail vein injection within about 4-7 seconds to mice weighing 20-35 grams. The mice

were closely monitored for the first couple of hours until the normal activity resumed. After the blood samples were collected via retro orbital blood collection, plasma samples were then obtained and stored at -80 °C for further analysis.

Example 5: Plasmid construction of co-transfection system for FVIIIFc-VWF Heterodimer contain XTEN insertions (Figure 6)

[0358] To increase the protein production yield, two co-transfection systems were generated for protein production, which contains three DNA constructs. The first DNA construct encoded a FVIII-Fc fusion protein in which a AE288 XTEN fragment was directly fuse to the C-terminus of the FVIII heavy chain and followed by either a wild type FVIII light chain fragment (pSYN-FVIII-173, Figure 6B) or a FVIII light chain fragment with R1645A/R1648A mutations (pSYN-FVIII-169, Figure 6A), the FVIII light chain was then directly fused to a single Fc fragment. The second DNA construct is pSYN-VWF-031 which encoding a D'D3-Fc fusion protein (Example 1). HEK293F cells were transfected with the two plasmid along with a third plasmid (PC5) at 80:15:5 ratio. The synthesized proteins were secreted as FVIII (XTEN) Fc/D'D3Fc heterodimer and D'D3Fc dimer and the FVIII (XTEN) Fc/D'D3Fc heterodimer was separated from the D'D3Fc dimer by protein purification.

pSYN-FVIII-169 mature Protein sequence (SEQ ID NO: 103):

```
  1  ATRRYYLGAV ELSWDYMQSD LGELPVDARF PPRVPKSFPF
 NTSVVYKKTL
 51  FVEFTDHLFN IAKPRPPWMG LLGPTIQAEV YDTVVITLKN
 MASHPVSLHA
101  VGVSYWKASE GAEYDDQTSQ REKEDDKVFP GGSHTYVWQV
 LKENGPMASD
151  PLCLTYSYLS HVDLVKDLNS GLIGALLVCR EGSLAKEKTQ
 TLHKFILLFA
201  VFDEGKSWHS ETKNSLMQDR DAASARAWPK MHTVNGYVNR
 SLPGLIGCHR
251  KSVYWHVIGM GTTPEVHSIF LEGHTFLVRN HRQASLEISP
 ITFLTAQTLL
301  MDLGQFLLFC HISSHQHDGM EAYVKVDSCP EEPQLRMKNN
 EEAEDYDDDL
351  TDSEMDVVRF DDDNSPSFIQ IRSVAKKHPK TWVHYIAAEE
 EDWDYAPLVL
401  APDDRSYKSQ YLNNGPQRIG RKYKKVRFMA YTDETFKTRE
 AIQHESGILG
451  PLLYGEVGDT LLIIFKNQAS RPYNIYPHGI TDVRPLYSRR
 LPKGVKHLKD
```

501 FPILPGEIFK YKWTVTVEDG PTKSDPRCLT RYYSSFVNME
RDLASGLIGP
551 LLICYKESVD QRGNQIMSDK RNVILFSVFD ENRSWYLTEN
IQRFLPNPAG
601 VQLEDPEFQA SNIMHSINGY VFDSLQLSVC LHEVAYWYIL
SIGAQTDFLS
651 VFFSGYTFKH KMVYEDTLTL FPFSGETVFM SMENPGLWIL
GCHNSDFRNR
701 GMTALLKVSS CDKNTGDYYE DSYEDISAYL LSKNNAIEPR
SFSQNGAPGT
751 SESATPESGP GSEPATSGSE TPGTSESATP ESGPGSEPAT
SGSETPGTSE
801 SATPESGPGT STEPSEGSAP GSPAGSPTST EEGTSESATP
ESGPGSEPAT
851 SGSETPGTSE SATPESGPGS PAGSPTSTEE GSPAGSPTST
EEGTSTEPSE
901 GSAPGTSESA TPESGPGTSE SATPESGPGT SESATPESGP
GSEPATSGSE
951 TPGSEPATSG SETPGSPAGS PTSTEEGTST EPSEGSAPGT
STEPSEGSAP
1001 GSEPATSGSE TPGTSESATP ESGPGTSTEP SEGSAPASSP
PVLKRHQAEI
1051 TRTTLQSDQE EIDYDDTISV EMKKEDFDIY DEDENQSPRS
FQKKTRHYFI
1101 AAVERLWDYG MSSSPHVLRN RAQSGSVPQF KKVVFQEFTD
GSFTQPLYRG
1151 ELNEHLGLLG PYIRAEVEDN IMVTFRNQAS RPYSFYSSLI
SYEEDQRQGA
1201 EPRKNFVKPN ETKTYFWKVQ HHMAPTKDEF DCKAWAYFSD
VDLEKDVHSG
1251 LIGPLLVCHT NTLNPAHGRQ VTVQEFALFF TIFDETKSWY
FTENMERNCR
1301 APCNIQMEDP TFKENYRFHA INGYIMDTLP GLVMAQDQRI
RWYLLSMGSN
1351 ENIHSIHFSG HVFTVRKKEE YKMALYNLYP GVFETVEMLP
SKAGIWRVEC
1401 LIGEHLHAGM STLFLVYSNK CQTPLGMASG HIRDFQITAS
GQYGQWAPKL
1451 ARLHYSGSIN AWSTKEPFSW IKVDLLAPMI IHGIKTQGAR
QKFSSLYISQ
1501 FIIMYSLDGK KWQTYRGNST GTLMVFFGNV DSSGIKHNIF
NPPIIARYIR
1551 LHPTHYSIRS TLRMELMGCD LNSCSMPLGM ESKAISDAQI
TASSYFTNMF
1601 ATWSPSKARL HLQGRSNAWR PQVNNPKEWL QVDFQKTMKV
TGVTTQGVKS
1651 LLTSMYVKEF LISSSQDGHQ WTLFFQNGKV KVFQGNQDSF
TPVVNSLDPP
1701 LLTRYLRIHP QSWVHQIALR MEVLGCEAQD LYDKTHTCPP
CPAPELLGGP

1751 SVFLFPPKPK DTLMISRTPE VTCVVVDVSH EDPEVKFNWY
VDGVEVHNAK
1801 TKPREEQYNS TYRVVSVLTV LHQDWLNGKE YKCKVSNKAL
PAPIEKTISK
1851 AKGQPREPQV YTLPPSRDEL TKNQVSLTCL VKGFYPSDIA
VEWESNGQPE
1901 NNYKTTPPVL DSDGSFFLYS KLTVDKSRWQ QGNVFSCSVM
HEALHNHYTQ
1951 KSLSLSPGK

pSYN-FVIII-173 mature Protein sequencing (SEQ ID NO: 104):

1 ATRRYYLGAV ELSWDYMQSD LGELPVDARF PPRVPKSFPF
NTSVVYKKTL
51 FVEFTDHLFN IAKPRPPWMG LLGPTIQAEV YDTVVITLKN
MASHPVSLHA
101 VGVSYWKASE GAEYDDQTSQ REKEDDKVFP GGSHTYVWQV
LKENGPMASD
151 PLCLTYSYLS HVDLVKDLNS GLIGALLVCR EGSLAKEKTQ
TLHKFILLFA
201 VFDEGKSWHS ETKNSLMQDR DAASARAWPK MHTVNGYVNR
SLPGLIGCHR
251 KSVYWHVIGM GTTPEVHSIF LEGHTFLVRN HRQASLEISP
ITFLTAQTLL
301 MDLGQFLLFC HISSHQHDGM EAYVKVDSCP EEPQLRMKNN
EEAEDYDDDL
351 TDSEMDVVRF DDDNSPSFIQ IRSVAKKHPK TWVHYIAAEE
EDWDYAPLVL
401 APDDRSYKSQ YLNNGPQRIG RKYKKVRFMA YTDETFKTRE
AIQHESGILG
451 PLLYGEVGDT LLIIFKNQAS RPYNIYPHGI TDVRPLYSRR
LPKGVKHLKD
501 FPILPGEIFK YKWTVTVEDG PTKSDPRCLT RYYSSFVNME
RDLASGLIGP
551 LLICYKESVD QRGNQIMSDK RNVILFSVFD ENRSWYLTEN
IQRFLPNPAG
601 VQLEDPEFQA SNIMHSINGY VFDSLQLSVC LHEVAYWYIL
SIGAQTDFLS
651 VFFSGYTFKH KMVYEDTLTL FPFSGETVFM SMENPGLWIL
GCHNSDFRNR
701 GMTALLKVSS CDKNTGDYYE DSYEDISAYL LSKNNAIEPR
SFSQNGAPGT
751 SESATPESGP GSEPATSGSE TPGTSESATP ESGPGSEPAT
SGSETPGTSE
801 SATPESGPGT STEPSEGSAP GSPAGSPTST EEGTSESATP
ESGPGSEPAT
851 SGSETPGTSE SATPESGPGS PAGSPTSTEE GSPAGSPTST
EEGTSTEPSE
901 GSAPGTSESA TPESGPGTSE SATPESGPGT SESATPESGP
GSEPATSGSE
951 TPGSEPATSG SETPGSPAGS PTSTEEGTST EPSEGSAPGT
STEPSEGSAP

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1001  GSEPATSGSE TPGTSESATP ESGPGTSTEP SEGSAPASSP
   PVLKRHQREI
1051  TRTTLQSDQE EIDYDDTISV EMKKEDFDIY DEDENQSPRS
   FQKKTRHYFI
1101  AAVERLWDYG MSSSPHVLRN RAQSGSVPQF KKVVFQEFTD
   GSFTQPLYRG
1151  ELNEHLGLLG PYIRAEVEDN IMVTFRNQAS RPYSFYSSLI
   SYEEDQRQGA
1201  EPRKNFVKPN ETKTYFWKVQ HHMAPTKDEF DCKAWAYFSD
   VDLEKDVHSG
1251  LIGPLLVCHT NTLNPAHGRQ VTVQEFALFF TIFDETKSWY
   FTENMERNCR
1301  APCNIQMEDP TFKENYRFHA INGYIMDTLP GLVMAQDQRI
   RWYLLSMGSN
1351  ENIHSIHFSG HVFTVRKKEE YKMALYNLYP GVFETVEMLP
   SKAGIWRVEC
1401  LIGEHLHAGM STLFLVYSNK CQTPLGMASG HIRDFQITAS
   GQYGQWAPKL
1451  ARLHYSGSIN AWSTKEPFSW IKVDLLAPMI IHGIKTQGAR
   QKFSSLYISQ
1501  FIIMYSLDGK KWQTYRGNST GTLMVFFGNV DSSGIKHNIF
   NPPIIARYIR
1551  LHPTHYSIRS TLRMELMGCD LNSCSMPLGM ESKAISDAQI
   TASSYFTNMF
1601  ATWSPSKARL HLQGRSNAWR PQVNNPKEWL QVDFQKTMKV
   TGVTTQGVKS
1651  LLTSMYVKEF LISSSQDGHQ WTLFFQNGKV KVFQGNQDSF
   TPVVNSLDPP
1701  LLTRYLRIHP QSWVHQIALR MEVLGCEAQD LYDKTHTCPP
   CPAPELLGGP
1751  SVFLFPPKPK DTLMISRTPE VTCVVVDVSH EDPEVKFNWY
   VDGVEVHNAK
1801  TKPREEQYNS TYRVVSVLTV LHQDWLNGKE YKCKVSNKAL
   PAPIEKTISK
1851  AKGQPREPQV YTLPPSRDEL TKNQVSLTCL VKGFYPSDIA
   VEWESNGQPE
1901  NNYKTTPPVL DSDGSFFLYS KLTVDKSRWQ QGNVFSCSVM
   HEALHNHYTQ
1951  KSLSLSPGK
```

Example 6. Protein purification for FVIII-169/VWF-031 and FVIII-173/VWF-031

[0359] A tangential flow filtration (TFF) step was used to buffer exchange the clarified conditioned media. The FVIII-169/VWF-031 or FVIII-173/VWF-031 heterodimer was then purified using a two-step chromatography process. A weak anion exchange resin was used, followed by affinity chromatography. The final purified product had acceptable purity by SEC-HPLC. The specific activity was

compatible to B-domain deleted FVIII, as measured by FVIII chromogenic assay and A280 concentration. Purity and the presence of each moiety of this molecule were confirmed by SDS-PAGE and western blotting.

Example 7. Evaluation the VWF binding ability of FVIII-169/VWF-031 by Octet assay

[0360] The VWF binding ability of FVIII-169/VWF-031 was obtained by Bio-Layer Interferometry (BLI) based measurements (Octet assay) at 25 °C with a ForteBio Octet 384 instrument, using Tris binding buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 5 mM $CaCl_2$). The Octet assay for determining FVIII binding was based on the hydrophobic immobilization of Human von Willebrand Factor (Haematologic Technologies Catalog No. HCVWF-0191) onto the APS Biosensor, then followed by the binding of 1.0% Bovine Serum Albumin (Jackson ImmunoResearch Catalog No. 001-000-161). Briefly, hvWF (20 μg/mL) was diluted in Tris buffer and loaded across APS Biosensors for 600 sec, yielding approximately 3.0 – 3.5 nm binding on the reaction probes. Control APS probes were loaded with 1.0% BSA in the absence of hvWF for reference subtraction. After loading, all probes were incubated in Tris buffer for 300 sec to establish a new baseline. Subsequently, biosensor probes were incubated in solutions of FVIII-XTEN 169 or FVIIIFc Drug Substance (0, 0.6, 2, 6, 20, 60, 200, 600 IU/mL) for 5 min at room temperature, followed by a 5 min dissociation step. Using the Octet data analysis software, the binding response (nm) was derived from the subtracted data (Reaction probe minus Reference probe). No binding to immobilized VWF was detected for FVIII-169/VWF-031 (Figure 7), indicating a complete shielding of FVIII from full length VWF molecule by the D'D3 fragment.

Example 8. FVIII-169/VWF-031 PK in HemA and FVIII/VWF DKO mice

[0361] The PK profile of FVIII-169/VWF-031 was tested in HemA and FVIII/VWF DKO mice to evaluate the ability of the D'D3 fragment to shield the FVIII moiety from the endogenous VWF. HemA or FVIII/VWF DKO mice were treated with a single intravenous dose of FVIII-169/VWF-031 at 200 IU/kg, plasma samples were then collected at 5min, 8hr, 24hr, 48hr and 72 hours post dosing. The FVIII activity of plasma sample was tested by FVIII chromogenic

assay, and half-life of FVIII-169/VWF-031 was calculated using WinNonlin program.

[0362] Complete inhibition of the constructs' binding to immobilized VWF was demonstrated by biolayer interferometry (Figure 7) for FVIII-169/VWF-031. This indicates the D'D3 fragment in the molecule had successfully blocked the FVIII binding to native VWF molecules, therefor similar half-life of FVIII-169/VWF-031 was predicted in the two different mouse strains. As shown in Figure 8A and Table 18, as expected, FVIII-169/VWF-031 had similar PK profile in both HemA and FVIII/VWF DKO mice, which has demonstrated that the half-life of FVIIIFc/VWF heterodimer is independent from the half-life of endogenous VWF. The separation of the FVIIIFc/VWF heterodimer half-life from the endogenous VWF half-life , eliminated the FVIII extension ceiling and opened the possibility of further extending FVIII half-life beyond the 2 fold half-life limit imposed by endogenous VWF.

TABLE 18: FVIII-169/VWF-031 PK in HemA and FVIII/VWF DKO mice

Mouse Strain	Recovery (%)	$t_{1/2}$ (hr)	MRT (hr)	Cl (mL/hr/kg)	Vss (mL/kg)	AUC (hr*kg*mIU/mL/mIU)
FVIII/VWF DKO	69	17.94	20.1	4.06	81.69	0.2461
HemA	83	16.65	18.44	3.57	85.72	0.28

[0363] The FVIII protecting ability of the XTEN insertion and D'D3 fragment was evaluated by comparing the half-life of FVIII-169/VWF-031 with FVIII-169/Fc and FVIIIFc in FVIII/VWF DKO mice. After a single IV administration, blood samples were collected at 5min, 8hr, 24hr, 48hr and 72hr for FVIII-169/VWF-031, 5min, 8hr, 24hr, 32hr, 48hr for FVIII-169/Fc and at 5min, 1, 2, 4, 6 and 8hrs for FVIIIFc. The FVIII activity of plasma sample was tested by FVIII chromogenic assay, and half-life of FVIII-155/VWF-031 was calculated using WinNonlin program.

[0364] The study results were summarized in Figure 8B and Table 19, rFVIIIFc has a 1.6hr half-life in DKO mice due to the loss of VWF protection. When an XTEN insertion was introduced into the FVIIIFc molecule, the resulting FVIII-

169/Fc molecule has a 7hr half-life, a 4 fold half-life extension by the XTEN insertion. Finally, when D'D3 fragment was incorporated into the molecule to form FVIII-169/VWF-031, a 17hr half-life was observed, another 2.5 fold further increase by the D'D3 fragment. In addition of the half-life improvement, improved Mean residency time (MRT), Clearance (Cl) and AUC were also observed as shown in Table 19.

[0365] FVIII-169/VWF-031 has achieved 17-18hr $t_{1/2}$ in both HemA and FVIII/VWF DKO mice, which is the upper limit of the $t_{1/2}$ extension ceiling that imposed by VWF clearance. More $t_{1/2}$ extension elements can be further incorporated into this molecule, such as a second XTEN insertion within FVIII. The synergistic effect of D'D3 fragment and XTEN insertions provided the possibility of the complete protection for FVIII from its clearance pathway, a final breakthrough of the 2 fold FVIII $t_{1/2}$ extension limit might be achieved by the FVIIIFc/XTEN/VWF variants.

TABLE 19. FVIII-169/VWF-031 PK in FVIII/VWF DKO mice

Mouse Strain	Treatment	Recovery (%)	$t_{1/2}$ (hr)	MRT (hr)	Cl (mL/hr/kg)	Vss (mL/kg)	AUC/D (hr*kg*mIU/mL/mIU)
FVIII/VWF DKO	rFVIIIFc	35	1.6	2.1	57.7	120.2	0.0173
	rFVIII-169/Fc	77	7.0	6.2	6.4	39.2	0.1573
	rFVIII-169/VWF-031	69	17.9	20.1	4.1	81.7	0.2461

Example 9: FVIII-XTEN variants cell media concentrate PK in D'D3 expressing FVIII/VWF DKO mice

[0366] The ability of D'D3 fragment to extend the $t_{1/2}$ of FVII-XTEN was evaluated in the D'D3 expressing FVIII/VWF DKO mouse model (described in example 2). In this study, instead of using VWF-025 to introduce the D'D3 dimer into the circulation, VWF-029 construct was used to introduce the D'D3 monomer into the circulation. To prepare FVIII-XTEN variants protein, a small scale (50-100 mL) transient transfection culture media was prepared, at day 4 post transfection, cell culture was harvested and concentrated to reach 10-20 IU/mL of

FVIII activity range which is suitable for PK study. The concentrated cell media were then used for standard PK study in FVIII/VWF DKO mice with or without D'D3 in the circulation.

[0367] Total of 6 FVIII-XTEN variants that contains 1-3 XTEN insertions were tested in the system, their $t_{1/2}$ were summarized in Table 20 and data from representative variants were plotted in Figure 9A.

[0368] Longer half-life was observed for all the FVIII-XTEN variants with the presents of D'D3 fragment in the circulation (Table 20), which demonstrated the D'D3 protection for FVIII-XTEN from its clearance pathways. Furthermore, when compared to its 14hr half-life in HemA mice, LSD0055.021 has a 20.4hr $t_{1/2}$ in D'D3 expressing DKO mice (Figure 9B, Table 20), indicates the final breakthrough of the 2 fold half-life extension ceiling for FVIII molecules. By further modify the FVIII(XTEN)/VWF molecule, we could potentially achieve even longer FVIII $t_{1/2}$, and provide HemA patients a FVIII protein that only requires once weekly or less frequent dosing regimen.

TABLE 20. FVIII-XTEN $t_{1/2}$ in D'D3 expressing FVIII/VWF DKO mice

FVIII-XTEN ID	# of XTEN insertions	Insertion sites	XTEN size	$t_{1/2}$ (hr) DKO mice	$t_{1/2}$ (hr) pLIVE-D'D3/ DKO mice	$t_{1/2}$ (hr) HemA mice
pSD-0013	1	CT	144	3.3	7.9	
LSD0003.009	2	B*/CT	144/288	9.7	16.4	
LSD0038.015	2	1656/26	144/144	7.8	17.2	
LSD0049.002	3	18/B*/CT	144/144/288	12.6	17.5	
LSD0051.002	3	403/B*/CT	144/144/288	11.1	19.9	
LSD0055.021	3	1900/B*/CT	144/144/288	16	20.4	14

*B indicates an XTEN sequence (*e.g.,* 144) is inserted immediately downstream of amino acid residue 745 corresponding to mature FVIII sequence.

Example 10: Stability of VWF- and XTEN-containing FVIII variants in FVIII/VWF double knockout (DKO) plasma

[0369] Plasma stability of rFVIIIFc protein variants was tested in FVIII/VWF double knockout (DKO) mouse plasma. For the stability assay, HEK293 cells were co-transfected with plasmids directing the expression of rFVIIIFc or FVIII-169 (rFVIIIFc with 288 AE XTEN inserted at the B-domain junction) and plasmids directing the expression of either IgG-Fc or VWF-031 (VWF D'D3 region fused to IgG-Fc). At day four post-transfection, cell culture media was

harvested and concentrated to 30 IU/mL based on FVIII chromogenic activity. Concentrated cell culture medium was then added into DKO mouse plasma to yield a FVIII activity of 5 IU/mL and incubated at 37°C. Aliquots were collected at different time points for activity measurement by chromogenic assay. Activity at each time point was measured in duplicate, and the average activity was plotted as a function of time. The activity of FVIIIFc, a dual chain (dc) FVIII molecule in which heavy and light chains are held together by non-covalent interaction, decreases with time in DKO mouse plasma (Figure 10). The activity of FVIII-169:Fc, which contains a 288 AE XTEN insertion at the B-domain junction, decays at a reduced rate relative to rFVIIIFc, indicating that enhanced stability is conferred by the XTEN insertion. Given that VWF has been proposed to enhance the stability of FVIII in vivo, we evaluated the plasma stability of FVIII-169:VWF-031. This heterodimeric molecule, in which the FVIII element and the VWF D'D3 element are fused to respective hemi-domains of Fc, exhibited additional plasma stability relative to FVIII-169:Fc, indicating that the VWF D'D3 domain and XTEN have a synergistic effect on the plasma stability of rFVIIIFc.

Example 11: The effect on FVIII half-life of Fc fusion, XTEN insertion and the D'D3 fragment of VWF.

[0370] To assess the effect of Fc fusion, XTEN insertion and D'D3 fragment of VWF on the half-life of FVIII, the pharmacokinetic properties of B domain deleted recombinant FVIII (rBDD-FVIII), rFVIIIFc, FVIII-169:Fc and FVIII-169:VWF-031 were evaluated in FVIII/VWF double knockout (DKO) mice.

[0371] DKO mice were treated with a single intra venous administration of 200 IU/kg of FVIII proteins, and plasma samples were collected at designated time points as indicated in Figure 11. FVIII activity of the plasma samples were analyzed by FVIII chromogenic assay and half-life was calculated using the WinNonlin-Phoenix program. The pharmacokinetic parameters of the tested molecules are listed in Table 21. The time regression curve of plasma FVIII activity for each FVIII variants were plotted in Figure 11.

[0372] Unmodified BDD-FVIII had a half-life of 0.23 hr in DKO mice, the FVIIIFc fusion protein has an extended half-life of 1.66 hr in DKO mice due to the recycling of FVIIIFc protein through the Fc:FcRn interaction. When a 288 residue of AEXTEN polypeptide was incorporated into the B domain region of

FVIII within the FVIIIFc molecule, the half-life of the resulting FVIII169/Fc protein was further extended to 7.41 hr in DKO mice. Finally, with the addition of the D'D3 domain of VWF, the half-life of FVIII169/VWF031 heterodimer has reached 17.9 hr in DKO mice (Figure 11, Table 21). In addition of the half-life, all of the other PK parameters also improved proportionally with the addition of each element (Table 21). FVIII can tolerate multiple half-life extension elements, and this synergistic effect of the three elements on FVIII half-life extension, enabled the further improvement of the half-life of FVIII-XTEN VWF heterodimers.

Table 21: PK parameters of FVIII variants

FVIII	FVIII Isoform	XTEN Insertions		$T_{1/2}$ (hr)	MRT (hr)	Cl (mL/hr/kg)	Vss (mL/kg)	AUC_D kg*hr/mL
		Site	XTEN Length					
BDD-FVIII	dc			0.23	0.24	407.72	97.42	0.0025
FVIIIFc	**dc**			1.66	2.06	62.66	128.82	0.0161
FVIII169/Fc	**sc**	B*	AE288	7.41	6.67	6.24	41.61	0.1603
FVIII169/VWF031	**sc**	B*	AE288	17.94	20.1	4.06	81.69	0.2463

*B indicates an XTEN sequence (*e.g.,* 144) is inserted immediately downstream of amino acid residue 745 corresponding to mature FVIII sequence.

Example 12: Pharmacokinetic properties of different FVIII-XTEN_VWF heterodimers

[0373] To evaluate the combined effect of the VWF-D'D3 fragment and XTEN insertions on the FVIII half-life, the pharmacokinetic properties of FVIII-XTEN-Fc:VWF-Fc heterodimers were tested in HemA mice and compared to those of the single chain isoform of BDD-FVIII (scBDD-FVIII) and FVIII-169:VWF-031 (example 10). Seven new FVIII-XTEN-Fc constructs were generated (protein sequences were listed in Table 24). Schematic diagrams of those constructs are shown in Figure 14A-H. FVIII-195 and FVIII-199, respectively, are the FVIII dual chain and single chain isoforms that each contains two XTEN insertions at positions 1900 and 1656. FVIII-196 and FVIII-201, respectively, are the FVIII dual chain and single chain isoforms that each contains three XTEN insertions at positions 26, 1656 and 1900. FVIII-203, -204 and -205 are sc-FVIIIFc molecules with two XTEN insertions at the B domain junction and at positions 1900, 403 or 18, respectively. Each FVIII-XTEN-Fc construct was co-expressed with VWF-

031 in HEK293 cells to produce FVIII-XTEN-Fc/VWF heterodimeric proteins. At day four post-transfection, cell culture medium was harvested and either concentrated to 20 IU/mL based on FVIII chromogenic activity (FVIII-195:VWF-031, FVIII-196:VWF-031, FVIII-199:VWF-031, FVIII-203:VWF-031 and FVIII-204:VWF-031) or purified (scBDD-FVIII, FVIII-169:VWF-031, FVIII-201:VWF-031 and FVIII-205:VWF-031). Having demonstrated the complete intra-molecular shielding of FVIII molecule from the endogenous VWF by the D'D3 fragment in the FVIII-XTEN-Fc:VWF-Fc heterodimer (FVIII-169:VWF-031, Example 5), HemA mice was chosen for the PK evaluations. Purified protein or concentrated cell culture medium was administered to 8-12 week-old HemA mice by intravenous administration at a dose of 200 IU/10 mL/kg. Plasma samples were collected at 5 min, 8 hr, 16 hr, 24 hr, 32 hr, 48 hr, 72 hr and 96 hr post-dosing. FVIII activity of the plasma samples were analyzed by FVIII chromogenic assay and half-life was calculated using the WinNonlin-Phoenix program. The pharmacokinetic parameters of the tested molecules are listed in Table 22. The plasma FVIII activities at selected time points for FVIII-XTEN-Fc/VWF-Fc variants were plotted in Figures 12A-C.

[0374] When XTEN was inserted into positions 1900 and 1656 (FVIII-195, FVIII-199), moderate improvement in half-life was observed for the scFVIII isoform (FVIII-199:VWF-031) compared to FVIII-169:VWF-031. However, the dcFVIII isoform exhibited a shorter half-life than did FVIII-169:VWF-031, indicating that the single chain isoform might be significantly more stable than the corresponding dual chain isoform (Table 22 and Figure 12A). When a third XTEN insertion was incorporated into FVIII-199 at position 26, the half-life of the resulting molecule FVIII-201:VWF-031 had reached 24.6 hr, which represents greater than a threefold half-life improvement relative to scBDD-FVIII (Table 22 and Figure 12C). We have also tested the half-life extension effect of the second XTEN insertion at position 403 (A2 domain), 1900 (A3 domain) and 18 (A1 domain) each in combination with the B domain XTEN insertion. While the addition of the A2 or A3 XTEN insertion did not confer an additional half-life benefit (Table 22, Figure 12b), the addition of the A1 insertion further extended the half-life of the FVIII-XTEN-Fc:VWF-Fc heterodimer to 29.4 hr (Table 22, Figure 12C), which is greater than threefold longer than that of scBDD-FVIII.

[0375] When XTENs were incorporated into the FVIIIFc/VWF heterodimer construct, degree of half-life improvement of the resulting molecules was variable, and no obvious correlation was observed between half-lives and either the site or number of XTEN insertion, suggesting that the half-life of the FVIII-XTEN-Fc/VWF heterodimer is determined by the integrity of the whole molecule rather than by the number or placement of XTEN insertions.

[0376] The 24.6 hr and 29.4 hr half-lives observed for FVIII-XTEN-Fc:VWF-Fc heterodimers clearly exceeded the 1.6- to 2-fold limitation on FVIII half-life extension. If this finding translates for HemA patients, it will allow once-weekly or less frequent dosing for FVIII prophylaxis.

Table 22: PK parameters of FVIII-XTEN-Fc/VWF-Fc heterodimers

FVIII	FVIII Isoform	XTEN Insertions		$T_{1/2}$ (hr)	MRT (hr)	Cl (mL/hr/kg)	Vss (mL/kg)	AUC_D kg*hr/mL
		Site	XTEN Length					
scBDD-FVIII	sc			7.16	10.16	4.38	44.44	0.23
FVIII169/VWF031	sc	B*	AE288	16.65	18.44	3.57	65.79	0.28
FVIII195/VWF031	dc	1656/1900	AG144/ AE144	12.56	13.88	9.04	125.48	0.11
FVIII199/VWF031	sc	1656/1900	AG144/ AE144	18.57	20.09	6.24	125.28	0.16
FVIII201/VWF031	sc	26/1656/ 1900	AG144/AG144/AE144	24.63	33.67	1.9	63.97	0.53
FVIII203/VWF031	sc	403/B*	AE144/AE288	15.52	18	3.65	65.61	0.27
FVIII204/VWF031	sc	1900/B*	AE144/AE288	16.3	20.63	2.87	59.14	0.35
FVIII205/VWF031	sc	18/B*	AE144/AE288	29.4	37.06	1.82	67.39	0.55

*B indicates an XTEN sequence (*e.g.,* 144) is inserted immediately downstream of amino acid residue 745 corresponding to mature FVIII sequence.

[0377] In addition to incorporating XTEN into the FVIII molecule, we also evaluated the potential half-life extension benefit of incorporating XTEN as a linker between the D'D3 and Fc fragment. FVIII-155 (scFVIIIFc) was co-expressed with VWF-034 (VWF-Fc with AE 288 XTEN plus a 35 residue thrombin cleavable linker) in HEK293 cells. At day 4 post-transfection, cell culture medium was harvested and concentrated to 20 IU/mL based on FVIII activity assay. FVIII/VWF DKO mice were dosed with concentrated cell culture

media at 200 IU/10 mL/kg with a single intravenous injection. Plasma samples were collected at 5 min, 8 hr, 24 hr, 48 hr, 72 hr and 96 hr post-dosing. The FVIII activity of plasma samples was analyzed by FVIII chromogenic assay, and the regression curve of plasma FVIII activity as a function of time was plotted (Figure 13). FVIII-155/VWF-034 exhibited the same improvement in half-life as FVIII-169/VWF-031, which has AE 288 XTEN inserted into the B domain junction of FVIII, as illustrated by the over lapping regression curves for the two molecules (Figure 13). The demonstration that XTEN insertion into the VWF-Fc polypeptide confers half-life improvement of a magnitude similar to that conferred by XTEN insertion at the B domain junction of the FVIII polypeptide suggests that further half-life improvement may be possible in a heterodimeric molecule in which intra-molecular XTEN insertion in the FVIII polypeptide is combined with inter-domain XTEN insertion between the VWF and Fc elements of the VWF-Fc polypeptide.

Example 13A: Pharmacokinetic properties of additional FVIII-XTEN_VWF heterodimers

[0378] In addition to the FVIII-XTEN VWF heterodimers that were listed in Table 22, FVIII-XTEN VWF heterodimers containing different composition of XTEN insertions, single chain and dual chain version of FVIII (Table 23A) are either tested or will be tested in HemA for their pharmacokinetic properties. Various FVIII constructs (Table 23B) and VWF constructs (Table 23C) are also disclosed below. HemA mice will be treated with a single dose of intravenous administration of the heterodimer proteins at 200 IU/10 mL/kg. Plasma samples will then be collected at 5 min, 24, 48, 72, 96 and 120 hrs post-dosing. FVIII activity of the plasma samples will be analyzed by FVIII chromogenic assay and half-life will be calculated using the WinNonlin-Phoenix program. The protein sequences of the listed heterodimers were listed in Table 25.

Table 23A. Plausible FVIII-XTEN-Fc:VWF-Fc heterodimer combinations for PK and activity improvement.

	pSYN VWF-015	pSYN VWF-031	pSYN VWF-034 **	pSYN VWF-036
pSYN FVIII 010	-	$t_{1/2}$ 8.7 hr DKO mice	To be tested	-
pSYN FVIII 155	$t_{1/2}$ 6.3 hr DKO mice	$t_{1/2}$ 10.8 hr HemA mice	$t_{1/2}$ 18.6 hr HemA mice	$t_{1/2}$ 13.3 hr HemA mice

pSYN FVIII 169 **	-	$t_{1/2}$ 16.7 hr HemA mice	$t_{1/2}$ 31.1 hr HemA mice	-
pSYN FVIII 173 **	-	$t_{1/2}$ 15.2 hr DKO mice	$t_{1/2}$ 28.9 hr HemA mice	To be tested
pSYN FVIII 205	-	$t_{1/2}$ 29.4 hr HemA mice	$t_{1/2}$ 32.4 hr HemA mice	$t_{1/2}$ 29.7 hr HemA mice
pSYN FVIII 266	-	$t_{1/2}$ 24.5 hr HemA mice	$t_{1/2}$ 27.4 hr HemA mice	-
pSYN FVII 267	-	$t_{1/2}$ 23.0 hr HemA mice	$t_{1/2}$ 25.7 hr HemA mice	
pSYN FVIII 268	-	To be tested	To be tested	To be tested
Dual chain isoform of pSYN FVIII 268		To be tested	To be tested	To be tested
**Length of XTEN can be changed to 72, 144, 288, 324, 333, 576, or 864.				

Table 23B. FVIII Constructs:

pSYN FVIII 010	dual chain FVIIIFc
pSYN FVIII 169	Single chain FVIIIFc with 288 AE XTEN in B-domain
pSYN FVIII 173	dual chain FVIIIFc with 288 AE XTEN in B-domain
pSYN FVIII 195	dual chain FVIIIFc with two 144 XTENs at amino acid 1656 and 1900
pSYN FVIII 196	dual chain FVIIIFc with three 144 XTENs at amino acid 26, 1656 and 1900
pSYN FVIII 199	Single chain FVIIIFc with two 144 XTENs at amino acid 1656 and 1900
pSYN FVIII 201	Single chain FVIIIFc with three 144 XTENs at amino acid 26, 1656 and 1900
pSYN FVIII 203	Single chain FVIIIFc with 144 AE XTEN at amino acid 1900 and 288 AE XTEN in B-domain
pSYN FVIII 204	Single chain FVIIIFc with 144 AE XTEN at amino acid 403 and 288 AE XTEN in B-domain
pSYN FVIII 205	Single chain FVIIIFc with 144 AE XTEN at amino acid 18 and 288 AE XTEN in B-domain
pSYN FVIII 207	Single chain FVIII (no Fc, no XTEN)
pSYN FVIII 266	Single chain FVIIIFc with 42 AE XTEN at amino acid 18 and 288 AE XTEN in B-domain
pSYN FVIII 267	Single chain FVIIIFc with 72 AE XTEN at amino acid 18 and 288 AE XTEN in B-domain
pSYN FVIII 268	Single chain FVIIIFc with 144 AE XTEN at amino acid 18
pSYN FVIII	Single chain FVIIIFc with 72 AE XTEN at amino acid 18

269	
pSYN FVIII 271	Single chain FVIIIFc with 42 AE XTEN at amino acid 18
pSYN FVIII 272	Single chain FVIII with 144 AE XTEN at amino acid 18 and 288 AE XTEN in B-domain (no Fc)

Table 23C. VWF Constructs:

pSYN VWF031	VWF-D1D2D'D3- 48aa long thrombin cleavable GS linker-Fc with C1099A/C1142A
pSYN VWF034	VWF-D1D2D'D3- 288AE XTEN +35aa long thrombin cleavable GS linker-Fc with C1099A/C1142A
pSYN VWF035	VWF-D1D2D'D3- 72aa long thrombin cleavable GS linker-Fc with C1099A/C1142A
pSYN VWF036	VWF-D1D2D'D3- 98aa long thrombin cleavable GS linker-Fc with C1099A/C1142A
pSYN VWF041	VWF-D1D2D'D3 with 288 AE XTEN in D3 and 48aa long thrombin cleavable GS linker after D3-Fc with C1099A/C1142A

Example 13B: Pharmacokinetic properties of additional FVIII-XTEN_VWF heterodimers.

[0379] FVIII-XTEN_VWF heterodimers were tested in HemA mice for their pharmacokinetic properties. The heterodimers tested are FVIII169/VWF034, FVIII205/VWF034, FVIII205/VWF036 and FVIII266/VWF031. HemA mice were administered with a single intravenous dose of various heterodimer proteins at 200 IU/10 mL/kg. Plasma samples were collected at 5 min, 24, 48, 72, 96 and 120 hrs post-dosing. FVIII activity of the plasma samples were analyzed by FVIII chromogenic assay, and half-lives were calculated using the WinNonlin-Phoenix program. The PK results are shown below in Table 24.

Table 24. Additional FVIII-XTEN_VWF - PK in HemA Mice

Treatment	**5min recovery (%)**	**HL (hr)**	**MRT (hr)**	**Cl (mL/hr/kg)**	**Vss (mL/kg)**	**AUC_D (hr*kg*mIU/mL/mIU)**	**Fold of $t_{1/2}$ increase vs scBDD-FVIII**
ScBDD-FVIII		7.16	10.16	4.83	44.44	0.23	-
FVIII169/VWF034	76	**31.1**	34.57	1.73	59.77	0.58	4.3
FVIII205/VWF034	68	**32.41**	39.79	1.55	61.73	0.64	4.6
FVIII205/VWF036	74	29.71	36.35	1.61	58.43	0.62	4.1
FVIII266/V	66	24.45	22.75	2.67	60.83	0.37	3.4

WF031							

pSYNFVIII 010 nucleotide sequence-(Dual chain FVIIIFc) (SEQ ID NO: 125)

```
   1  ATGCAAATAG AGCTCTCCAC CTGCTTCTTT CTGTGCCTTT
  TGCGATTCTG
  51  CTTTAGTGCC ACCAGAAGAT ACTACCTGGG TGCAGTGGAA
  CTGTCATGGG
 101  ACTATATGCA AAGTGATCTC GGTGAGCTGC CTGTGGACGC
  AAGATTTCCT
 151  CCTAGAGTGC CAAAATCTTT TCCATTCAAC ACCTCAGTCG
  TGTACAAAAA
 201  GACTCTGTTT GTAGAATTCA CGGATCACCT TTTCAACATC
  GCTAAGCCAA
 251  GGCCACCCTG GATGGGTCTG CTAGGTCCTA CCATCCAGGC
  TGAGGTTTAT
 301  GATACAGTGG TCATTACACT TAAGAACATG GCTTCCCATC
  CTGTCAGTCT
 351  TCATGCTGTT GGTGTATCCT ACTGGAAAGC TTCTGAGGGA
  GCTGAATATG
 401  ATGATCAGAC CAGTCAAAGG GAGAAAGAAG ATGATAAAGT
  CTTCCCTGGT
 451  GGAAGCCATA CATATGTCTG GCAGGTCCTG AAAGAGAATG
  GTCCAATGGC
 501  CTCTGACCCA CTGTGCCTTA CCTACTCATA TCTTTCTCAT
  GTGGACCTGG
 551  TAAAAGACTT GAATTCAGGC CTCATTGGAG CCCTACTAGT
  ATGTAGAGAA
 601  GGGAGTCTGG CCAAGGAAAA GACACAGACC TTGCACAAAT
  TTATACTACT
 651  TTTTGCTGTA TTTGATGAAG GGAAAAGTTG GCACTCAGAA
  ACAAAGAACT
 701  CCTTGATGCA GGATAGGGAT GCTGCATCTG CTCGGGCCTG
  GCCTAAAATG
 751  CACACAGTCA ATGGTTATGT AAACAGGTCT CTGCCAGGTC
  TGATTGGATG
 801  CCACAGGAAA TCAGTCTATT GGCATGTGAT TGGAATGGGC
  ACCACTCCTG
 851  AAGTGCACTC AATATTCCTC GAAGGTCACA CATTTCTTGT
  GAGGAACCAT
 901  CGCCAGGCGT CCTTGGAAAT CTCGCCAATA ACTTTCCTTA
  CTGCTCAAAC
 951  ACTCTTGATG GACCTTGGAC AGTTTCTACT GTTTTGTCAT
  ATCTCTTCCC
1001  ACCAACATGA TGGCATGGAA GCTTATGTCA AAGTAGACAG
  CTGTCCAGAG
1051  GAACCCCAAC TACGAATGAA AAATAATGAA GAAGCGGAAG
  ACTATGATGA
1101  TGATCTTACT GATTCTGAAA TGGATGTGGT CAGGTTTGAT
  GATGACAACT
```

1151 CTCCTTCCTT TATCCAAATT CGCTCAGTTG CCAAGAAGCA
TCCTAAAACT
1201 TGGGTACATT ACATTGCTGC TGAAGAGGAG GACTGGGACT
ATGCTCCCTT
1251 AGTCCTCGCC CCCGATGACA GAAGTTATAA AAGTCAATAT
TTGAACAATG
1301 GCCCTCAGCG GATTGGTAGG AAGTACAAAA AAGTCCGATT
TATGGCATAC
1351 ACAGATGAAA CCTTTAAGAC TCGTGAAGCT ATTCAGCATG
AATCAGGAAT
1401 CTTGGGACCT TTACTTTATG GGGAAGTTGG AGACACACTG
TTGATTATAT
1451 TTAAGAATCA AGCAAGCAGA CCATATAACA TCTACCCTCA
CGGAATCACT
1501 GATGTCCGTC CTTTGTATTC AAGGAGATTA CCAAAAGGTG
TAAAACATTT
1551 GAAGGATTTT CCAATTCTGC CAGGAGAAAT ATTCAAATAT
AAATGGACAG
1601 TGACTGTAGA AGATGGGCCA ACTAAATCAG ATCCTCGGTG
CCTGACCCGC
1651 TATTACTCTA GTTTCGTTAA TATGGAGAGA GATCTAGCTT
CAGGACTCAT
1701 TGGCCCTCTC CTCATCTGCT ACAAAGAATC TGTAGATCAA
AGAGGAAACC
1751 AGATAATGTC AGACAAGAGG AATGTCATCC TGTTTTCTGT
ATTTGATGAG
1801 AACCGAAGCT GGTACCTCAC AGAGAATATA CAACGCTTTC
TCCCCAATCC
1851 AGCTGGAGTG CAGCTTGAGG ATCCAGAGTT CCAAGCCTCC
AACATCATGC
1901 ACAGCATCAA TGGCTATGTT TTTGATAGTT TGCAGTTGTC
AGTTTGTTTG
1951 CATGAGGTGG CATACTGGTA CATTCTAAGC ATTGGAGCAC
AGACTGACTT
2001 CCTTTCTGTC TTCTTCTCTG GATATACCTT CAAACACAAA
ATGGTCTATG
2051 AAGACACACT CACCCTATTC CCATTCTCAG GAGAAACTGT
CTTCATGTCG
2101 ATGGAAAACC CAGGTCTATG GATTCTGGGG TGCCACAACT
CAGACTTTCG
2151 GAACAGAGGC ATGACCGCCT TACTGAAGGT TTCTAGTTGT
GACAAGAACA
2201 CTGGTGATTA TTACGAGGAC AGTTATGAAG ATATTTCAGC
ATACTTGCTG
2251 AGTAAAAACA ATGCCATTGA ACCAAGAAGC TTCTCTCAAA
ACCCACCAGT
2301 CTTGAAACGC CATCAACGGG AAATAACTCG TACTACTCTT
CAGTCAGATC
2351 AAGAGGAAAT TGACTATGAT GATACCATAT CAGTTGAAAT
GAAGAAGGAA

2401 GATTTTGACA TTTATGATGA GGATGAAAAT CAGAGCCCCC
GCAGCTTTCA
2451 AAAGAAAACA CGACACTATT TTATTGCTGC AGTGGAGAGG
CTCTGGGATT
2501 ATGGGATGAG TAGCTCCCCA CATGTTCTAA GAAACAGGGC
TCAGAGTGGC
2551 AGTGTCCCTC AGTTCAAGAA AGTTGTTTTC CAGGAATTTA
CTGATGGCTC
2601 CTTTACTCAG CCCTTATACC GTGGAGAACT AAATGAACAT
TTGGGACTCC
2651 TGGGGCCATA TATAAGAGCA GAAGTTGAAG ATAATATCAT
GGTAACTTTC
2701 AGAAATCAGG CCTCTCGTCC CTATTCCTTC TATTCTAGCC
TTATTTCTTA
2751 TGAGGAAGAT CAGAGGCAAG GAGCAGAACC TAGAAAAAAC
TTTGTCAAGC
2801 CTAATGAAAC CAAAACTTAC TTTTGGAAAG TGCAACATCA
TATGGCACCC
2851 ACTAAAGATG AGTTTGACTG CAAAGCCTGG GCTTATTTCT
CTGATGTTGA
2901 CCTGGAAAAA GATGTGCACT CAGGCCTGAT TGGACCCCTT
CTGGTCTGCC
2951 ACACTAACAC ACTGAACCCT GCTCATGGGA GACAAGTGAC
AGTACAGGAA
3001 TTTGCTCTGT TTTTCACCAT CTTTGATGAG ACCAAAAGCT
GGTACTTCAC
3051 TGAAAATATG GAAAGAAACT GCAGGGCTCC CTGCAATATC
CAGATGGAAG
3101 ATCCCACTTT TAAAGAGAAT TATCGCTTCC ATGCAATCAA
TGGCTACATA
3151 ATGGATACAC TACCTGGCTT AGTAATGGCT CAGGATCAAA
GGATTCGATG
3201 GTATCTGCTC AGCATGGGCA GCAATGAAAA CATCCATTCT
ATTCATTTCA
3251 GTGGACATGT GTTCACTGTA CGAAAAAAAG AGGAGTATAA
AATGGCACTG
3301 TACAATCTCT ATCCAGGTGT TTTTGAGACA GTGGAAATGT
TACCATCCAA
3351 AGCTGGAATT TGGCGGGTGG AATGCCTTAT TGGCGAGCAT
CTACATGCTG
3401 GGATGAGCAC ACTTTTTCTG GTGTACAGCA ATAAGTGTCA
GACTCCCCTG
3451 GGAATGGCTT CTGGACACAT TAGAGATTTT CAGATTACAG
CTTCAGGACA
3501 ATATGGACAG TGGGCCCCAA AGCTGGCCAG ACTTCATTAT
TCCGGATCAA
3551 TCAATGCCTG GAGCACCAAG GAGCCCTTTT CTTGGATCAA
GGTGGATCTG
3601 TTGGCACCAA TGATTATTCA CGGCATCAAG ACCCAGGGTG
CCCGTCAGAA

3651 GTTCTCCAGC CTCTACATCT CTCAGTTTAT CATCATGTAT
AGTCTTGATG
3701 GGAAGAAGTG GCAGACTTAT CGAGGAAATT CCACTGGAAC
CTTAATGGTC
3751 TTCTTTGGCA ATGTGGATTC ATCTGGGATA AAACACAATA
TTTTTAACCC
3801 TCCAATTATT GCTCGATACA TCCGTTTGCA CCCAACTCAT
TATAGCATTC
3851 GCAGCACTCT TCGCATGGAG TTGATGGGCT GTGATTTAAA
TAGTTGCAGC
3901 ATGCCATTGG GAATGGAGAG TAAAGCAATA TCAGATGCAC
AGATTACTGC
3951 TTCATCCTAC TTTACCAATA TGTTTGCCAC CTGGTCTCCT
TCAAAAGCTC
4001 GACTTCACCT CCAAGGGAGG AGTAATGCCT GGAGACCTCA
GGTGAATAAT
4051 CCAAAAGAGT GGCTGCAAGT GGACTTCCAG AAGACAATGA
AAGTCACAGG
4101 AGTAACTACT CAGGGAGTAA AATCTCTGCT TACCAGCATG
TATGTGAAGG
4151 AGTTCCTCAT CTCCAGCAGT CAAGATGGCC ATCAGTGGAC
TCTCTTTTTT
4201 CAGAATGGCA AAGTAAAGGT TTTTCAGGGA AATCAAGACT
CCTTCACACC
4251 TGTGGTGAAC TCTCTAGACC CACCGTTACT GACTCGCTAC
CTTCGAATTC
4301 ACCCCCAGAG TTGGGTGCAC CAGATTGCCC TGAGGATGGA
GGTTCTGGGC
4351 TGCGAGGCAC AGGACCTCTA CGACAAAACT CACACATGCC
CACCGTGCCC
4401 AGCTCCAGAA CTCCTGGGCG GACCGTCAGT CTTCCTCTTC
CCCCCAAAAC
4451 CCAAGGACAC CCTCATGATC TCCCGGACCC CTGAGGTCAC
ATGCGTGGTG
4501 GTGGACGTGA GCCACGAAGA CCCTGAGGTC AAGTTCAACT
GGTACGTGGA
4551 CGGCGTGGAG GTGCATAATG CCAAGACAAA GCCGCGGGAG
GAGCAGTACA
4601 ACAGCACGTA CCGTGTGGTC AGCGTCCTCA CCGTCCTGCA
CCAGGACTGG
4651 CTGAATGGCA AGGAGTACAA GTGCAAGGTC TCCAACAAAG
CCCTCCCAGC
4701 CCCCATCGAG AAAACCATCT CCAAAGCCAA AGGGCAGCCC
CGAGAACCAC
4751 AGGTGTACAC CCTGCCCCCA TCCCGGGATG AGCTGACCAA
GAACCAGGTC
4801 AGCCTGACCT GCCTGGTCAA AGGCTTCTAT CCCAGCGACA
TCGCCGTGGA
4851 GTGGGAGAGC AATGGGCAGC CGGAGAACAA CTACAAGACC
ACGCCTCCCG

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4901  TGTTGGACTC CGACGGCTCC TTCTTCCTCT ACAGCAAGCT
   CACCGTGGAC
4951  AAGAGCAGGT GGCAGCAGGG GAACGTCTTC TCATGCTCCG
   TGATGCATGA
5001  GGCTCTGCAC AACCACTACA CGCAGAAGAG CCTCTCCCTG
   TCTCCGGGTA
5051  AATGA
```

pSYNFVIII 010 protein sequence-(Dual chain FVIIIFc) (SEQ ID NO: 126)

```
   1  MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQSDL
   GELPVDARFP
  51  PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL
   LGPTIQAEVY
 101  DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR
   EKEDDKVFPG
 151  GSHTYVWQVL KENGPMASDP LCLTYSYLSH VDLVKDLNSG
   LIGALLVCRE
 201  GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
   AASARAWPKM
 251  HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL
   EGHTFLVRNH
 301  RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME
   AYVKVDSCPE
 351  EPQLRMKNNE EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI
   RSVAKKHPKT
 401  WVHYIAAEEE DWDYAPLVLA PDDRSYKSQY LNNGPQRIGR
   KYKKVRFMAY
 451  TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR
   PYNIYPHGIT
 501  DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
   TKSDPRCLTR
 551  YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR
   NVILFSVFDE
 601  NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV
   FDSLQLSVCL
 651  HEVAYWYILS IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF
   PFSGETVFMS
 701  MENPGLWILG CHNSDFRNRG MTALLKVSSC DKNTGDYYED
   SYEDISAYLL
 751  SKNNAIEPRS FSQNPPVLKR HQREITRTTL QSDQEEIDYD
   DTISVEMKKE
 801  DFDIYDEDEN QSPRSFQKKT RHYFIAAVER LWDYGMSSSP
   HVLRNRAQSG
 851  SVPQFKKVVF QEFTDGSFTQ PLYRGELNEH LGLLGPYIRA
   EVEDNIMVTF
 901  RNQASRPYSF YSSLISYEED QRQGAEPRKN FVKPNETKTY
   FWKVQHHMAP
 951  TKDEFDCKAW AYFSDVDLEK DVHSGLIGPL LVCHTNTLNP
   AHGRQVTVQE
1001  FALFFTIFDE TKSWYFTENM ERNCRAPCNI QMEDPTFKEN
   YRFHAINGYI
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```
1051  MDTLPGLVMA QDQRIRWYLL SMGSNENIHS IHFSGHVFTV
   RKKEEYKMAL
1101  YNLYPGVFET VEMLPSKAGI WRVECLIGEH LHAGMSTLFL
   VYSNKCQTPL
1151  GMASGHIRDF QITASGQYGQ WAPKLARLHY SGSINAWSTK
   EPFSWIKVDL
1201  LAPMIIHGIK TQGARQKFSS LYISQFIIMY SLDGKKWQTY
   RGNSTGTLMV
1251  FFGNVDSSGI KHNIFNPPII ARYIRLHPTH YSIRSTLRME
   LMGCDLNSCS
1301  MPLGMESKAI SDAQITASSY FTNMFATWSP SKARLHLQGR
   SNAWRPQVNN
1351  PKEWLQVDFQ KTMKVTGVTT QGVKSLLTSM YVKEFLISSS
   QDGHQWTLFF
1401  QNGKVKVFQG NQDSFTPVVN SLDPPLLTRY LRIHPQSWVH
   QIALRMEVLG
1451  CEAQDLYDKT HTCPPCPAPE LLGGPSVFLF PPKPKDTLMI
   SRTPEVTCVV
1501  VDVSHEDPEV KFNWYVDGVE VHNAKTKPRE EQYNSTYRVV
   SVLTVLHQDW
1551  LNGKEYKCKV SNKALPAPIE KTISKAKGQP REPQVYTLPP
   SRDELTKNQV
1601  SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSDGS
   FFLYSKLTVD
1651  KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPGK*
```

Example 14: A new class of coagulation factor VIII molecules with greater than three-fold half-life extension in hemophilia A mice

[0380] The new class of FVIII molecules was designed to contain two polypeptides; one that consists of a single chain B-domain deleted (BDD) FVIII with XTEN inserted at one or more locations within the FVIII sequence, and one that is composed of the D'D3 region of VWF. Each polypeptide was also recombinantly fused to the Fc region of IgG1 to enable the D'D3 region to be correctly aligned to bind the FVIII moiety. The resulting FVIII variants were expressed in HEK 293 cells by transient transfection, and purified from the conditioned media. FVIII activity was evaluated by FVIII chromogenic assay and the pharmacokinetic properties were assessed in both FVIII knockout (HemA) and FVIII/VWF double knock-out (DKO) mice.

[0381] Incorporating XTEN and D'D3 region of VWF into rFVIII led to the uncoupling of the clearance of the fusion proteins from endogenous VWF while extending their circulating half-life. FVIII in this fusion configuration is completely shielded from interacting with VWF, as measured by biolayer

interferometry (Octet) analysis. Consistent with this, their pharmacokinetic profiles in HemA and DKO mice were found to be identical, indicating that their clearance rate in mice was effectively disconnected from VWF. Optimization of XTEN length and the locations for inserting XTEN identified a subset of the proteins that have exceeded the VWF limitation (16 hours), reaching a circulating half-life of up to 30 hours in HemA mice representing a 4-fold improvement over BDD-FVIII. Importantly, these proteins maintained their functionality, as judged by FVIII chromogenic assay.

[0382] The VWF dependency has set a fundamental limitation for half-life of therapeutic FVIII. Uncoupling FVIII from VWF clearance pathways while extending half-life by the fusion of D'D3 region of VWF and XTEN has generated a novel FVIII molecule with a 4-fold half-life extension. This is the first report of an engineered FVIII that has exceeded the half-life limitation observed through industry-wide efforts in development of long-lasting FVIII, representing a potentially significant advancement in prophylactic treatment of hemophilia A.

Table 25: Protein sequences of FVIII-XTEN-Fc and VWF-Fc constructs

FVIII 195 protein sequence (dual chain FVIIIFc with two 144 AE XTENs at amino acid 1656 and 1900) (SEQ ID NO: 105)

```
   1        MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQSDL
  GELPVDARFP
  51  PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL
  LGPTIQAEVY
 101  DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR
  EKEDDKVFPG
 151  GSHTYVWQVL KENGPMASDP LCLTYSYLSH VDLVKDLNSG
  LIGALLVCRE
 201  GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
  AASARAWPKM
 251  HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL
  EGHTFLVRNH
 301  RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME
  AYVKVDSCPE
 351  EPQLRMKNNE EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI
  RSVAKKHPKT
 401  WVHYIAAEEE DWDYAPLVLA PDDRSYKSQY LNNGPQRIGR
  KYKKVRFMAY
 451  TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR
  PYNIYPHGIT
 501  DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
  TKSDPRCLTR
 551  YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR
  NVILFSVFDE
```

601 NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV
FDSLQLSVCL
651 HEVAYWYILS IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF
PFSGETVFMS
701 MENPGLWILG CHNSDFRNRG MTALLKVSSC DKNTGDYYED
SYEDISAYLL
751 SKNNAIEPRS FSQNPPVLKR HQREITRTTL QGAPGTPGSG
TASSSPGASP
801 GTSSTGSPGA SPGTSSTGSP GASPGTSSTG SPGSSPSAST
GTGPGTPGSG
851 TASSSPGASP GTSSTGSPGA SPGTSSTGSP GASPGTSSTG
SPGSSTPSGA
901 TGSPGSSTPS GATGSPGASP GTSSTGSPAS SSDQEEIDYD
DTISVEMKKE
951 DFDIYDEDEN QSPRSFQKKT RHYFIAAVER LWDYGMSSSP
HVLRNRAQSG
1001 SVPQFKKVVF QEFTDGSFTQ PLYRGELNEH LGLLGPYIRA
EVEDNIMVTF
1051 RNQASRPYSF YSSLISYEED QRQGAEPRKN FVKPNETKTY
FWKVQHHMAP
1101 TKDEFDCKAW AYFSDVDLEK DVHSGLIGPL LVCHTNTLNP
AHGRQVTVQE
1151 FALFFTIFDE TKSWYFTENM ERNCRGAPTS ESATPESGPG
SEPATSGSET
1201 PGTSESATPE SGPGSEPATS GSETPGTSES ATPESGPGTS
TEPSEGSAPG
1251 TSESATPESG PGSPAGSPTS TEEGSPAGSP TSTEEGSPAG
SPTSTEEGTS
1301 ESATPESGPG TSTEPSEGSA PGASSAPCNI QMEDPTFKEN
YRFHAINGYI
1351 MDTLPGLVMA QDQRIRWYLL SMGSNENIHS IHFSGHVFTV
RKKEEYKMAL
1401 YNLYPGVFET VEMLPSKAGI WRVECLIGEH LHAGMSTLFL
VYSNKCQTPL
1451 GMASGHIRDF QITASGQYGQ WAPKLARLHY SGSINAWSTK
EPFSWIKVDL
1501 LAPMIIHGIK TQGARQKFSS LYISQFIIMY SLDGKKWQTY
RGNSTGTLMV
1551 FFGNVDSSGI KHNIFNPPII ARYIRLHPTH YSIRSTLRME
LMGCDLNSCS
1601 MPLGMESKAI SDAQITASSY FTNMFATWSP SKARLHLQGR
SNAWRPQVNN
1651 PKEWLQVDFQ KTMKVTGVTT QGVKSLLTSM YVKEFLISSS
QDGHQWTLFF
1701 QNGKVKVFQG NQDSFTPVVN SLDPPLLTRY LRIHPQSWVH
QIALRMEVLG
1751 CEAQDLYDKT HTCPPCPAPE LLGGPSVFLF PPKPKDTLMI
SRTPEVTCVV
1801 VDVSHEDPEV KFNWYVDGVE VHNAKTKPRE EQYNSTYRVV
SVLTVLHQDW

1851 LNGKEYKCKV SNKALPAPIE KTISKAKGQP REPQVYTLPP
SRDELTKNQV
1901 SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSDGS
FFLYSKLTVD
1951 KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPGK*

FVIII 196 protein sequence (dual chain FVIIIFc with three 144 AE XTENs at amino acid 26, 1656 and 1900) (SEQ ID NO: 106)

1 MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQSDL
GELPVGAPGS
51 SPSASTGTGP GSSPSASTGT GPGASPGTSS TGSPGASPGT
SSTGSPGSST
101 PSGATGSPGS SPSASTGTGP GASPGTSSTG SPGSSPSAST
GTGPGTPGSG
151 TASSSPGSST PSGATGSPGS STPSGATGSP GASPGTSSTG
SPASSDARFP
201 PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL
LGPTIQAEVY
251 DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR
EKEDDKVFPG
301 GSHTYVWQVL KENGPMASDP LCLTYSYLSH VDLVKDLNSG
LIGALLVCRE
351 GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
AASARAWPKM
401 HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL
EGHTFLVRNH
451 RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME
AYVKVDSCPE
501 EPQLRMKNNE EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI
RSVAKKHPKT
551 WVHYIAAEEE DWDYAPLVLA PDDRSYKSQY LNNGPQRIGR
KYKKVRFMAY
601 TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR
PYNIYPHGIT
651 DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
TKSDPRCLTR
701 YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR
NVILFSVFDE
751 NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV
FDSLQLSVCL
801 HEVAYWYILS IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF
PFSGETVFMS
851 MENPGLWILG CHNSDFRNRG MTALLKVSSC DKNTGDYYED
SYEDISAYLL
901 SKNNAIEPRS FSQNPPVLKR HQREITRTTL QGAPGTPGSG
TASSSPGASP
951 GTSSTGSPGA SPGTSSTGSP GASPGTSSTG SPGSSPSAST
GTGPGTPGSG
1001 TASSSPGASP GTSSTGSPGA SPGTSSTGSP GASPGTSSTG
SPGSSTPSGA

1051 TGSPGSSTPS GATGSPGASP GTSSTGSPAS SSDQEEIDYD
DTISVEMKKE
1101 DFDIYDEDEN QSPRSFQKKT RHYFIAAVER LWDYGMSSSP
HVLRNRAQSG
1151 SVPQFKKVVF QEFTDGSFTQ PLYRGELNEH LGLLGPYIRA
EVEDNIMVTF
1201 RNQASRPYSF YSSLISYEED QRQGAEPRKN FVKPNETKTY
FWKVQHHMAP
1251 TKDEFDCKAW AYFSDVDLEK DVHSGLIGPL LVCHTNTLNP
AHGRQVTVQE
1301 FALFFTIFDE TKSWYFTENM ERNCRGAPTS ESATPESGPG
SEPATSGSET
1351 PGTSESATPE SGPGSEPATS GSETPGTSES ATPESGPGTS
TEPSEGSAPG
1401 TSESATPESG PGSPAGSPTS TEEGSPAGSP TSTEEGSPAG
SPTSTEEGTS
1451 ESATPESGPG TSTEPSEGSA PGASSAPCNI QMEDPTFKEN
YRFHAINGYI
1501 MDTLPGLVMA QDQRIRWYLL SMGSNENIHS IHFSGHVFTV
RKKEEYKMAL
1551 YNLYPGVFET VEMLPSKAGI WRVECLIGEH LHAGMSTLFL
VYSNKCQTPL
1601 GMASGHIRDF QITASGQYGQ WAPKLARLHY SGSINAWSTK
EPFSWIKVDL
1651 LAPMIIHGIK TQGARQKFSS LYISQFIIMY SLDGKKWQTY
RGNSTGTLMV
1701 FFGNVDSSGI KHNIFNPPII ARYIRLHPTH YSIRSTLRME
LMGCDLNSCS
1751 MPLGMESKAI SDAQITASSY FTNMFATWSP SKARLHLQGR
SNAWRPQVNN
1801 PKEWLQVDFQ KTMKVTGVTT QGVKSLLTSM YVKEFLISSS
QDGHQWTLFF
1851 QNGKVKVFQG NQDSFTPVVN SLDPPLLTRY LRIHPQSWVH
QIALRMEVLG
1901 CEAQDLYDKT HTCPPCPAPE LLGGPSVFLF PPKPKDTLMI
SRTPEVTCVV
1951 VDVSHEDPEV KFNWYVDGVE VHNAKTKPRE EQYNSTYRVV
SVLTVLHQDW
2001 LNGKEYKCKV SNKALPAPIE KTISKAKGQP REPQVYTLPP
SRDELTKNQV
2051 SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSDGS
FFLYSKLTVD
2101 KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPGK*

FVIII 199 protein sequence (single chain FVIIIFc with three 144 AE XTENs at amino acid 1656 and 1900) (SEQ ID NO: 107)

1 MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQSDL
GELPVDARFP
51 PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL
LGPTIQAEVY

101 DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR
EKEDDKVFPG
151 GSHTYVWQVL KENGPMASDP LCLTYSYLSH VDLVKDLNSG
LIGALLVCRE
201 GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
AASARAWPKM
251 HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL
EGHTFLVRNH
301 RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME
AYVKVDSCPE
351 EPQLRMKNNE EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI
RSVAKKHPKT
401 WVHYIAAEEE DWDYAPLVLA PDDRSYKSQY LNNGPQRIGR
KYKKVRFMAY
451 TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR
PYNIYPHGIT
501 DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
TKSDPRCLTR
551 YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR
NVILFSVFDE
601 NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV
FDSLQLSVCL
651 HEVAYWYILS IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF
PFSGETVFMS
701 MENPGLWILG CHNSDFRNRG MTALLKVSSC DKNTGDYYED
SYEDISAYLL
751 SKNNAIEPRS FSQNPPVLKR HQAEITRTTL QGAPGTPGSG
TASSSPGASP
801 GTSSTGSPGA SPGTSSTGSP GASPGTSSTG SPGSSPSAST
GTGPGTPGSG
851 TASSSPGASP GTSSTGSPGA SPGTSSTGSP GASPGTSSTG
SPGSSTPSGA
901 TGSPGSSTPS GATGSPGASP GTSSTGSPAS SSDQEEIDYD
DTISVEMKKE
951 DFDIYDEDEN QSPRSFQKKT RHYFIAAVER LWDYGMSSSP
HVLRNRAQSG
1001 SVPQFKKVVF QEFTDGSFTQ PLYRGELNEH LGLLGPYIRA
EVEDNIMVTF
1051 RNQASRPYSF YSSLISYEED QRQGAEPRKN FVKPNETKTY
FWKVQHHMAP
1101 TKDEFDCKAW AYFSDVDLEK DVHSGLIGPL LVCHTNTLNP
AHGRQVTVQE
1151 FALFFTIFDE TKSWYFTENM ERNCRGAPTS ESATPESGPG
SEPATSGSET
1201 PGTSESATPE SGPGSEPATS GSETPGTSES ATPESGPGTS
TEPSEGSAPG
1251 TSESATPESG PGSPAGSPTS TEEGSPAGSP TSTEEGSPAG
SPTSTEEGTS
1301 ESATPESGPG TSTEPSEGSA PGASSAPCNI QMEDPTFKEN
YRFHAINGYI

1351 MDTLPGLVMA QDQRIRWYLL SMGSNENIHS IHFSGHVFTV
RKKEEYKMAL
1401 YNLYPGVFET VEMLPSKAGI WRVECLIGEH LHAGMSTLFL
VYSNKCQTPL
1451 GMASGHIRDF QITASGQYGQ WAPKLARLHY SGSINAWSTK
EPFSWIKVDL
1501 LAPMIIHGIK TQGARQKFSS LYISQFIIMY SLDGKKWQTY
RGNSTGTLMV
1551 FFGNVDSSGI KHNIFNPPII ARYIRLHPTH YSIRSTLRME
LMGCDLNSCS
1601 MPLGMESKAI SDAQITASSY FTNMFATWSP SKARLHLQGR
SNAWRPQVNN
1651 PKEWLQVDFQ KTMKVTGVTT QGVKSLLTSM YVKEFLISSS
QDGHQWTLFF
1701 QNGKVKVFQG NQDSFTPVVN SLDPPLLTRY LRIHPQSWVH
QIALRMEVLG
1751 CEAQDLYDKT HTCPPCPAPE LLGGPSVFLF PPKPKDTLMI
SRTPEVTCVV
1801 VDVSHEDPEV KFNWYVDGVE VHNAKTKPRE EQYNSTYRVV
SVLTVLHQDW
1851 LNGKEYKCKV SNKALPAPIE KTISKAKGQP REPQVYTLPP
SRDELTKNQV
1901 SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSDGS
FFLYSKLTVD
1951 KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPGK*

FVIII 201 protein sequence (single chain FVIIIFc with three 144 AE XTENs at amino acid 26, 1656 &1900) (SEQ ID NO: 108)

1 MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQSDL
GELPVGAPGS
51 SPSASTGTGP GSSPSASTGT GPGASPGTSS TGSPGASPGT
SSTGSPGSST
101 PSGATGSPGS SPSASTGTGP GASPGTSSTG SPGSSPSAST
GTGPGTPGSG
151 TASSSPGSST PSGATGSPGS STPSGATGSP GASPGTSSTG
SPASSDARFP
201 PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL
LGPTIQAEVY
251 DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR
EKEDDKVFPG
301 GSHTYVWQVL KENGPMASDP LCLTYSYLSH VDLVKDLNSG
LIGALLVCRE
351 GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
AASARAWPKM
401 HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL
EGHTFLVRNH
451 RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME
AYVKVDSCPE
501 EPQLRMKNNE EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI
RSVAKKHPKT

551 WVHYIAAEEE DWDYAPLVLA PDDRSYKSQY LNNGPQRIGR
KYKKVRFMAY
601 TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR
PYNIYPHGIT
651 DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
TKSDPRCLTR
701 YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR
NVILFSVFDE
751 NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV
FDSLQLSVCL
801 HEVAYWYILS IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF
PFSGETVFMS
851 MENPGLWILG CHNSDFRNRG MTALLKVSSC DKNTGDYYED
SYEDISAYLL
901 SKNNAIEPRS FSQNPPVLKR HQAEITRTTL QGAPGTPGSG
TASSSPGASP
951 GTSSTGSPGA SPGTSSTGSP GASPGTSSTG SPGSSPSAST
GTGPGTPGSG
1001 TASSSPGASP GTSSTGSPGA SPGTSSTGSP GASPGTSSTG
SPGSSTPSGA
1051 TGSPGSSTPS GATGSPGASP GTSSTGSPAS SSDQEEIDYD
DTISVEMKKE
1101 DFDIYDEDEN QSPRSFQKKT RHYFIAAVER LWDYGMSSSP
HVLRNRAQSG
1151 SVPQFKKVVF QEFTDGSFTQ PLYRGELNEH LGLLGPYIRA
EVEDNIMVTF
1201 RNQASRPYSF YSSLISYEED QRQGAEPRKN FVKPNETKTY
FWKVQHHMAP
1251 TKDEFDCKAW AYFSDVDLEK DVHSGLIGPL LVCHTNTLNP
AHGRQVTVQE
1301 FALFFTIFDE TKSWYFTENM ERNCRGAPTS ESATPESGPG
SEPATSGSET
1351 PGTSESATPE SGPGSEPATS GSETPGTSES ATPESGPGTS
TEPSEGSAPG
1401 TSESATPESG PGSPAGSPTS TEEGSPAGSP TSTEEGSPAG
SPTSTEEGTS
1451 ESATPESGPG TSTEPSEGSA PGASSAPCNI QMEDPTFKEN
YRFHAINGYI
1501 MDTLPGLVMA QDQRIRWYLL SMGSNENIHS IHFSGHVFTV
RKKEEYKMAL
1551 YNLYPGVFET VEMLPSKAGI WRVECLIGEH LHAGMSTLFL
VYSNKCQTPL
1601 GMASGHIRDF QITASGQYGQ WAPKLARLHY SGSINAWSTK
EPFSWIKVDL
1651 LAPMIIHGIK TQGARQKFSS LYISQFIIMY SLDGKKWQTY
RGNSTGTLMV
1701 FFGNVDSSGI KHNIFNPPII ARYIRLHPTH YSIRSTLRME
LMGCDLNSCS
1751 MPLGMESKAI SDAQITASSY FTNMFATWSP SKARLHLQGR
SNAWRPQVNN

1801 PKEWLQVDFQ KTMKVTGVTT QGVKSLLTSM YVKEFLISSS
QDGHQWTLFF
1851 QNGKVKVFQG NQDSFTPVVN SLDPPLLTRY LRIHPQSWVH
QIALRMEVLG
1901 CEAQDLYDKT HTCPPCPAPE LLGGPSVFLF PPKPKDTLMI
SRTPEVTCVV
1951 VDVSHEDPEV KFNWYVDGVE VHNAKTKPRE EQYNSTYRVV
SVLTVLHQDW
2001 LNGKEYKCKV SNKALPAPIE KTISKAKGQP REPQVYTLPP
SRDELTKNQV
2051 SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSDGS
FFLYSKLTVD
2101 KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPGK*

FVIII 203 protein sequence (single chain FVIIIFc with two AE XTENs; one 288AE XTEN
in B-domain and one 144 AE XTEN at amino acid 1900) (SEQ ID NO: 109)

1 MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQSDL
GELPVDARFP
51 PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL
LGPTIQAEVY
101 DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR
EKEDDKVFPG
151 GSHTYVWQVL KENGPMASDP LCLTYSYLSH VDLVKDLNSG
LIGALLVCRE
201 GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
AASARAWPKM
251 HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL
EGHTFLVRNH
301 RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME
AYVKVDSCPE
351 EPQLRMKNNE EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI
RSVAKKHPKT
401 WVHYIAAEEE DWDYAPLVLA PDDRSYKSQY LNNGPQRIGR
KYKKVRFMAY
451 TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR
PYNIYPHGIT
501 DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
TKSDPRCLTR
551 YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR
NVILFSVFDE
601 NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV
FDSLQLSVCL
651 HEVAYWYILS IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF
PFSGETVFMS
701 MENPGLWILG CHNSDFRNRG MTALLKVSSC DKNTGDYYED
SYEDISAYLL
751 SKNNAIEPRS FSQNGAPGTS ESATPESGPG SEPATSGSET
PGTSESATPE
801 SGPGSEPATS GSETPGTSES ATPESGPGTS TEPSEGSAPG
SPAGSPTSTE

851 EGTSESATPE SGPGSEPATS GSETPGTSES ATPESGPGSP
AGSPTSTEEG
901 SPAGSPTSTE EGTSTEPSEG SAPGTSESAT PESGPGTSES
ATPESGPGTS
951 ESATPESGPG SEPATSGSET PGSEPATSGS ETPGSPAGSP
TSTEEGTSTE
1001 PSEGSAPGTS TEPSEGSAPG SEPATSGSET PGTSESATPE
SGPGTSTEPS
1051 EGSAPASSPP VLKRHQAEIT RTTLQSDQEE IDYDDTISVE
MKKEDFDIYD
1101 EDENQSPRSF QKKTRHYFIA AVERLWDYGM SSSPHVLRNR
AQSGSVPQFK
1151 KVVFQEFTDG SFTQPLYRGE LNEHLGLLGP YIRAEVEDNI
MVTFRNQASR
1201 PYSFYSSLIS YEEDQRQGAE PRKNFVKPNE TKTYFWKVQH
HMAPTKDEFD
1251 CKAWAYFSDV DLEKDVHSGL IGPLLVCHTN TLNPAHGRQV
TVQEFALFFT
1301 IFDETKSWYF TENMERNCRG APTSESATPE SGPGSEPATS
GSETPGTSES
1351 ATPESGPGSE PATSGSETPG TSESATPESG PGTSTEPSEG
SAPGTSESAT
1401 PESGPGSPAG SPTSTEEGSP AGSPTSTEEG SPAGSPTSTE
EGTSESATPE
1451 SGPGTSTEPS EGSAPGASSA PCNIQMEDPT FKENYRFHAI
NGYIMDTLPG
1501 LVMAQDQRIR WYLLSMGSNE NIHSIHFSGH VFTVRKKEEY
KMALYNLYPG
1551 VFETVEMLPS KAGIWRVECL IGEHLHAGMS TLFLVYSNKC
QTPLGMASGH
1601 IRDFQITASG QYGQWAPKLA RLHYSGSINA WSTKEPFSWI
KVDLLAPMII
1651 HGIKTQGARQ KFSSLYISQF IIMYSLDGKK WQTYRGNSTG
TLMVFFGNVD
1701 SSGIKHNIFN PPIIARYIRL HPTHYSIRST LRMELMGCDL
NSCSMPLGME
1751 SKAISDAQIT ASSYFTNMFA TWSPSKARLH LQGRSNAWRP
QVNNPKEWLQ
1801 VDFQKTMKVT GVTTQGVKSL LTSMYVKEFL ISSSQDGHQW
TLFFQNGKVK
1851 VFQGNQDSFT PVVNSLDPPL LTRYLRIHPQ SWVHQIALRM
EVLGCEAQDL
1901 YDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV
TCVVVDVSHE
1951 DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL
HQDWLNGKEY
2001 KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELT
KNQVSLTCLV
2051 KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK
LTVDKSRWQQ
2101 GNVFSCSVMH EALHNHYTQK SLSLSPGK*

FVIII 204 protein sequence (single chain FVIIIFc with two AE XTENs; one 288AE XTEN in B-domain and one 144 AE XTEN at amino acid 403) (SEQ ID NO: 110)

```
   1  MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQSDL
  GELPVDARFP
  51  PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL
  LGPTIQAEVY
 101  DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR
  EKEDDKVFPG
 151  GSHTYVWQVL KENGPMASDP LCLTYSYLSH VDLVKDLNSG
  LIGALLVCRE
 201  GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
  AASARAWPKM
 251  HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL
  EGHTFLVRNH
 301  RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME
  AYVKVDSCPE
 351  EPQLRMKNNE EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI
  RSVAKKHPKT
 401  WVHYIAAEEE DWDYAPLVLA PDGAPTSTEP SEGSAPGSPA
  GSPTSTEEGT
 451  STEPSEGSAP GTSTEPSEGS APGTSESATP ESGPGTSTEP
  SEGSAPGTSE
 501  SATPESGPGS EPATSGSETP GTSTEPSEGS APGTSTEPSE
  GSAPGTSESA
 551  TPESGPGTSE SATPESGPGA SSDRSYKSQY LNNGPQRIGR
  KYKKVRFMAY
 601  TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR
  PYNIYPHGIT
 651  DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
  TKSDPRCLTR
 701  YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR
  NVILFSVFDE
 751  NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV
  FDSLQLSVCL
 801  HEVAYWYILS IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF
  PFSGETVFMS
 851  MENPGLWILG CHNSDFRNRG MTALLKVSSC DKNTGDYYED
  SYEDISAYLL
 901  SKNNAIEPRS FSQNGAPGTS ESATPESGPG SEPATSGSET
  PGTSESATPE
 951  SGPGSEPATS GSETPGTSES ATPESGPGTS TEPSEGSAPG
  SPAGSPTSTE
1001  EGTSESATPE SGPGSEPATS GSETPGTSES ATPESGPGSP
  AGSPTSTEEG
1051  SPAGSPTSTE EGTSTEPSEG SAPGTSESAT PESGPGTSES
  ATPESGPGTS
1101  ESATPESGPG SEPATSGSET PGSEPATSGS ETPGSPAGSP
  TSTEEGTSTE
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1151 PSEGSAPGTS TEPSEGSAPG SEPATSGSET PGTSESATPE
SGPGTSTEPS
1201 EGSAPASSPP VLKRHQAEIT RTTLQSDQEE IDYDDTISVE
MKKEDFDIYD
1251 EDENQSPRSF QKKTRHYFIA AVERLWDYGM SSSPHVLRNR
AQSGSVPQFK
1301 KVVFQEFTDG SFTQPLYRGE LNEHLGLLGP YIRAEVEDNI
MVTFRNQASR
1351 PYSFYSSLIS YEEDQRQGAE PRKNFVKPNE TKTYFWKVQH
HMAPTKDEFD
1401 CKAWAYFSDV DLEKDVHSGL IGPLLVCHTN TLNPAHGRQV
TVQEFALFFT
1451 IFDETKSWYF TENMERNCRA PCNIQMEDPT FKENYRFHAI
NGYIMDTLPG
1501 LVMAQDQRIR WYLLSMGSNE NIHSIHFSGH VFTVRKKEEY
KMALYNLYPG
1551 VFETVEMLPS KAGIWRVECL IGEHLHAGMS TLFLVYSNKC
QTPLGMASGH
1601 IRDFQITASG QYGQWAPKLA RLHYSGSINA WSTKEPFSWI
KVDLLAPMII
1651 HGIKTQGARQ KFSSLYISQF IIMYSLDGKK WQTYRGNSTG
TLMVFFGNVD
1701 SSGIKHNIFN PPIIARYIRL HPTHYSIRST LRMELMGCDL
NSCSMPLGME
1751 SKAISDAQIT ASSYFTNMFA TWSPSKARLH LQGRSNAWRP
QVNNPKEWLQ
1801 VDFQKTMKVT GVTTQGVKSL LTSMYVKEFL ISSSQDGHQW
TLFFQNGKVK
1851 VFQGNQDSFT PVVNSLDPPL LTRYLRIHPQ SWVHQIALRM
EVLGCEAQDL
1901 YDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV
TCVVVDVSHE
1951 DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL
HQDWLNGKEY
2001 KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELT
KNQVSLTCLV
2051 KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK
LTVDKSRWQQ
2101 GNVFSCSVMH EALHNHYTQK SLSLSPGK*

FVIII 205 protein sequence (single chain FVIIIFc with two AE XTENs; one 288AE XTEN in B-domain and one 144 AE XTEN at amino acid 18) (SEQ ID NO: 111)

1 MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQGAP
TSESATPESG
51 PGSEPATSGS ETPGTSESAT PESGPGSEPA TSGSETPGTS
ESATPESGPG
101 TSTEPSEGSA PGSPAGSPTS TEEGTSESAT PESGPGSEPA
TSGSETPGTS

151 ESATPESGPG SPAGSPTSTE EGSPAGSPTS TEEGASSSDL
GELPVDARFP
201 PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL
LGPTIQAEVY
251 DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR
EKEDDKVFPG
301 GSHTYVWQVL KENGPMASDP LCLTYSYLSH VDLVKDLNSG
LIGALLVCRE
351 GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
AASARAWPKM
401 HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL
EGHTFLVRNH
451 RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME
AYVKVDSCPE
501 EPQLRMKNNE EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI
RSVAKKHPKT
551 WVHYIAAEEE DWDYAPLVLA PDDRSYKSQY LNNGPQRIGR
KYKKVRFMAY
601 TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR
PYNIYPHGIT
651 DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
TKSDPRCLTR
701 YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR
NVILFSVFDE
751 NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV
FDSLQLSVCL
801 HEVAYWYILS IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF
PFSGETVFMS
851 MENPGLWILG CHNSDFRNRG MTALLKVSSC DKNTGDYYED
SYEDISAYLL
901 SKNNAIEPRS FSQNGAPGTS ESATPESGPG SEPATSGSET
PGTSESATPE
951 SGPGSEPATS GSETPGTSES ATPESGPGTS TEPSEGSAPG
SPAGSPTSTE
1001 EGTSESATPE SGPGSEPATS GSETPGTSES ATPESGPGSP
AGSPTSTEEG
1051 SPAGSPTSTE EGTSTEPSEG SAPGTSESAT PESGPGTSES
ATPESGPGTS
1101 ESATPESGPG SEPATSGSET PGSEPATSGS ETPGSPAGSP
TSTEEGTSTE
1151 PSEGSAPGTS TEPSEGSAPG SEPATSGSET PGTSESATPE
SGPGTSTEPS
1201 EGSAPASSPP VLKRHQAEIT RTTLQSDQEE IDYDDTISVE
MKKEDFDIYD
1251 EDENQSPRSF QKKTRHYFIA AVERLWDYGM SSSPHVLRNR
AQSGSVPQFK
1301 KVVFQEFTDG SFTQPLYRGE LNEHLGLLGP YIRAEVEDNI
MVTFRNQASR
1351 PYSFYSSLIS YEEDQRQGAE PRKNFVKPNE TKTYFWKVQH
HMAPTKDEFD

1401 CKAWAYFSDV DLEKDVHSGL IGPLLVCHTN TLNPAHGRQV
TVQEFALFFT
1451 IFDETKSWYF TENMERNCRA PCNIQMEDPT FKENYRFHAI
NGYIMDTLPG
1501 LVMAQDQRIR WYLLSMGSNE NIHSIHFSGH VFTVRKKEEY
KMALYNLYPG
1551 VFETVEMLPS KAGIWRVECL IGEHLHAGMS TLFLVYSNKC
QTPLGMASGH
1601 IRDFQITASG QYGQWAPKLA RLHYSGSINA WSTKEPFSWI
KVDLLAPMII
1651 HGIKTQGARQ KFSSLYISQF IIMYSLDGKK WQTYRGNSTG
TLMVFFGNVD
1701 SSGIKHNIFN PPIIARYIRL HPTHYSIRST LRMELMGCDL
NSCSMPLGME
1751 SKAISDAQIT ASSYFTNMFA TWSPSKARLH LQGRSNAWRP
QVNNPKEWLQ
1801 VDFQKTMKVT GVTTQGVKSL LTSMYVKEFL ISSSQDGHQW
TLFFQNGKVK
1851 VFQGNQDSFT PVVNSLDPPL LTRYLRIHPQ SWVHQIALRM
EVLGCEAQDL
1901 YDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV
TCVVVDVSHE
1951 DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL
HQDWLNGKEY
2001 KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELT
KNQVSLTCLV
2051 KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK
LTVDKSRWQQ
2101 GNVFSCSVMH EALHNHYTQK SLSLSPGK*

pSYN FVIII 266 protein sequence (FVIII Fc with 42 AE-XTEN at amino acid 18 and 288 AE XTEN in B-domain) SEQ ID NO: 112

1 MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQGAP
GSPAGSPTST
51 EEGTSESATP ESGPGSEPAT SGSETPASSS DLGELPVDAR
FPPRVPKSFP
101 FNTSVVYKKT LFVEFTDHLF NIAKPRPPWM GLLGPTIQAE
VYDTVVITLK
151 NMASHPVSLH AVGVSYWKAS EGAEYDDQTS QREKEDDKVF
PGGSHTYVWQ
201 VLKENGPMAS DPLCLTYSYL SHVDLVKDLN SGLIGALLVC
REGSLAKEKT
251 QTLHKFILLF AVFDEGKSWH SETKNSLMQD RDAASARAWP
KMHTVNGYVN
301 RSLPGLIGCH RKSVYWHVIG MGTTPEVHSI FLEGHTFLVR
NHRQASLEIS
351 PITFLTAQTL LMDLGQFLLF CHISSHQHDG MEAYVKVDSC
PEEPQLRMKN
401 NEEAEDYDDD LTDSEMDVVR FDDDNSPSFI QIRSVAKKHP
KTWVHYIAAE

451 EEDWDYAPLV LAPDDRSYKS QYLNNGPQRI GRKYKKVRFM
AYTDETFKTR
501 EAIQHESGIL GPLLYGEVGD TLLIIFKNQA SRPYNIYPHG
ITDVRPLYSR
551 RLPKGVKHLK DFPILPGEIF KYKWTVTVED GPTKSDPRCL
TRYYSSFVNM
601 ERDLASGLIG PLLICYKESV DQRGNQIMSD KRNVILFSVF
DENRSWYLTE
651 NIQRFLPNPA GVQLEDPEFQ ASNIMHSING YVFDSLQLSV
CLHEVAYWYI
701 LSIGAQTDFL SVFFSGYTFK HKMVYEDTLT LFPFSGETVF
MSMENPGLWI
751 LGCHNSDFRN RGMTALLKVS SCDKNTGDYY EDSYEDISAY
LLSKNNAIEP
801 RSFSQNGAPG TSESATPESG PGSEPATSGS ETPGTSESAT
PESGPGSEPA
851 TSGSETPGTS ESATPESGPG TSTEPSEGSA PGSPAGSPTS
TEEGTSESAT
901 PESGPGSEPA TSGSETPGTS ESATPESGPG SPAGSPTSTE
EGSPAGSPTS
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TSESATPESG
1001 PGSEPATSGS ETPGSEPATS GSETPGSPAG SPTSTEEGTS
TEPSEGSAPG
1051 TSTEPSEGSA PGSEPATSGS ETPGTSESAT PESGPGTSTE
PSEGSAPASS
1101 PPVLKRHQAE ITRTTLQSDQ EEIDYDDTIS VEMKKEDFDI
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1151 SFQKKTRHYF IAAVERLWDY GMSSSPHVLR NRAQSGSVPQ
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1201 DGSFTQPLYR GELNEHLGLL GPYIRAEVED NIMVTFRNQA
SRPYSFYSSL
1251 ISYEEDQRQG AEPRKNFVKP NETKTYFWKV QHHMAPTKDE
FDCKAWAYFS
1301 DVDLEKDVHS GLIGPLLVCH TNTLNPAHGR QVTVQEFALF
FTIFDETKSW
1351 YFTENMERNC RAPCNIQMED PTFKENYRFH AINGYIMDTL
PGLVMAQDQR
1401 IRWYLLSMGS NENIHSIHFS GHVFTVRKKE EYKMALYNLY
PGVFETVEML
1451 PSKAGIWRVE CLIGEHLHAG MSTLFLVYSN KCQTPLGMAS
GHIRDFQITA
1501 SGQYGQWAPK LARLHYSGSI NAWSTKEPFS WIKVDLLAPM
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1601 FNPPIIARYI RLHPTHYSIR STLRMELMGC DLNSCSMPLG
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1651 ITASSYFTNM FATWSPSKAR LHLQGRSNAW RPQVNNPKEW
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1701 VTGVTTQGVK SLLTSMYVKE FLISSSQDGH QWTLFFQNGK
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1751 FTPVVNSLDP PLLTRYLRIH PQSWVHQIAL RMEVLGCEAQ
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1801 PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS
HEDPEVKFNW
1851 YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK
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1901 LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE LTKNQVSLTC
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1951 AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
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2001 MHEALHNHYT QKSLSLSPGK *

pSYN FVIII 267 protein sequence (FVIII Fc with 72 AE-XTEN at amino acid 18 and 288
AE XTEN in B-domain) SEQ ID NO: 113

1 MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQGAP
TSESATPESG
51 PGSEPATSGS ETPGTSESAT PESGPGSEPA TSGSETPGTS
ESATPESGPG
101 TSTEPSEGSA PGASSSDLGE LPVDARFPPR VPKSFPFNTS
VVYKKTLFVE
151 FTDHLFNIAK PRPPWMGLLG PTIQAEVYDT VVITLKNMAS
HPVSLHAVGV
201 SYWKASEGAE YDDQTSQREK EDDKVFPGGS HTYVWQVLKE
NGPMASDPLC
251 LTYSYLSHVD LVKDLNSGLI GALLVCREGS LAKEKTQTLH
KFILLFAVFD
301 EGKSWHSETK NSLMQDRDAA SARAWPKMHT VNGYVNRSLP
GLIGCHRKSV
351 YWHVIGMGTT PEVHSIFLEG HTFLVRNHRQ ASLEISPITF
LTAQTLLMDL
401 GQFLLFCHIS SHQHDGMEAY VKVDSCPEEP QLRMKNNEEA
EDYDDDLTDS
451 EMDVVRFDDD NSPSFIQIRS VAKKHPKTWV HYIAAEEEDW
DYAPLVLAPD
501 DRSYKSQYLN NGPQRIGRKY KKVRFMAYTD ETFKTREAIQ
HESGILGPLL
551 YGEVGDTLLI IFKNQASRPY NIYPHGITDV RPLYSRRLPK
GVKHLKDFPI
601 LPGEIFKYKW TVTVEDGPTK SDPRCLTRYY SSFVNMERDL
ASGLIGPLLI
651 CYKESVDQRG NQIMSDKRNV ILFSVFDENR SWYLTENIQR
FLPNPAGVQL
701 EDPEFQASNI MHSINGYVFD SLQLSVCLHE VAYWYILSIG
AQTDFLSVFF
751 SGYTFKHKMV YEDTLTLFPF SGETVFMSME NPGLWILGCH
NSDFRNRGMT
801 ALLKVSSCDK NTGDYYEDSY EDISAYLLSK NNAIEPRSFS
QNGAPGTSES

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  ETPGTSESAT
 901  PESGPGTSTE PSEGSAPGSP AGSPTSTEEG TSESATPESG
  PGSEPATSGS
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1001  PGTSESATPE SGPGTSESAT PESGPGTSES ATPESGPGSE
  PATSGSETPG
1051  SEPATSGSET PGSPAGSPTS TEEGTSTEPS EGSAPGTSTE
  PSEGSAPGSE
1101  PATSGSETPG TSESATPESG PGTSTEPSEG SAPASSPPVL
  KRHQAEITRT
1151  TLQSDQEEID YDDTISVEMK KEDFDIYDED ENQSPRSFQK
  KTRHYFIAAV
1201  ERLWDYGMSS SPHVLRNRAQ SGSVPQFKKV VFQEFTDGSF
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  EKDVHSGLIG
1351  PLLVCHTNTL NPAHGRQVTV QEFALFFTIF DETKSWYFTE
  NMERNCRAPC
1401  NIQMEDPTFK ENYRFHAING YIMDTLPGLV MAQDQRIRWY
  LLSMGSNENI
1451  HSIHFSGHVF TVRKKEEYKM ALYNLYPGVF ETVEMLPSKA
  GIWRVECLIG
1501  EHLHAGMSTL FLVYSNKCQT PLGMASGHIR DFQITASGQY
  GQWAPKLARL
1551  HYSGSINAWS TKEPFSWIKV DLLAPMIIHG IKTQGARQKF
  SSLYISQFII
1601  MYSLDGKKWQ TYRGNSTGTL MVFFGNVDSS GIKHNIFNPP
  IIARYIRLHP
1651  THYSIRSTLR MELMGCDLNS CSMPLGMESK AISDAQITAS
  SYFTNMFATW
1701  SPSKARLHLQ GRSNAWRPQV NNPKEWLQVD FQKTMKVTGV
  TTQGVKSLLT
1751  SMYVKEFLIS SSQDGHQWTL FFQNGKVKVF QGNQDSFTPV
  VNSLDPPLLT
1801  RYLRIHPQSW VHQIALRMEV LGCEAQDLYD KTHTCPPCPA
  PELLGGPSVF
1851  LFPPKPKDTL MISRTPEVTC VVVDVSHEDP EVKFNWYVDG
  VEVHNAKTKP
1901  REEQYNSTYR VVSVLTVLHQ DWLNGKEYKC KVSNKALPAP
  IEKTISKAKG
1951  QPREPQVYTL PPSRDELTKN QVSLTCLVKG FYPSDIAVEW
  ESNGQPENNY
2001  KTTPPVLDSD GSFFLYSKLT VDKSRWQQGN VFSCSVMHEA
  LHNHYTQKSL
2051  SLSPGK*
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pSYN FVIII 268 protein sequence (FVIII Fc with 144 AE-XTEN at amino acid 18) SEQ ID NO: 114

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   1  MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQGAP
  TSESATPESG
  51  PGSEPATSGS ETPGTSESAT PESGPGSEPA TSGSETPGTS
  ESATPESGPG
 101  TSTEPSEGSA PGSPAGSPTS TEEGTSESAT PESGPGSEPA
  TSGSETPGTS
 151  ESATPESGPG SPAGSPTSTE EGSPAGSPTS TEEGASSSDL
  GELPVDARFP
 201  PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL
  LGPTIQAEVY
 251  DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR
  EKEDDKVFPG
 301  GSHTYVWQVL KENGPMASDP LCLTYSYLSH VDLVKDLNSG
  LIGALLVCRE
 351  GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
  AASARAWPKM
 401  HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL
  EGHTFLVRNH
 451  RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME
  AYVKVDSCPE
 501  EPQLRMKNNE EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI
  RSVAKKHPKT
 551  WVHYIAAEEE DWDYAPLVLA PDDRSYKSQY LNNGPQRIGR
  KYKKVRFMAY
 601  TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR
  PYNIYPHGIT
 651  DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
  TKSDPRCLTR
 701  YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR
  NVILFSVFDE
 751  NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV
  FDSLQLSVCL
 801  HEVAYWYILS IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF
  PFSGETVFMS
 851  MENPGLWILG CHNSDFRNRG MTALLKVSSC DKNTGDYYED
  SYEDISAYLL
 901  SKNNAIEPRS FSQNPPVLKR HQAEITRTTL QSDQEEIDYD
  DTISVEMKKE
 951  DFDIYDEDEN QSPRSFQKKT RHYFIAAVER LWDYGMSSSP
  HVLRNRAQSG
1001  SVPQFKKVVF QEFTDGSFTQ PLYRGELNEH LGLLGPYIRA
  EVEDNIMVTF
1051  RNQASRPYSF YSSLISYEED QRQGAEPRKN FVKPNETKTY
  FWKVQHHMAP
1101  TKDEFDCKAW AYFSDVDLEK DVHSGLIGPL LVCHTNTLNP
  AHGRQVTVQE
1151  FALFFTIFDE TKSWYFTENM ERNCRAPCNI QMEDPTFKEN
  YRFHAINGYI
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1201 MDTLPGLVMA QDQRIRWYLL SMGSNENIHS IHFSGHVFTV
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1251 YNLYPGVFET VEMLPSKAGI WRVECLIGEH LHAGMSTLFL
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1301 GMASGHIRDF QITASGQYGQ WAPKLARLHY SGSINAWSTK
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1351 LAPMIIHGIK TQGARQKFSS LYISQFIIMY SLDGKKWQTY
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1401 FFGNVDSSGI KHNIFNPPII ARYIRLHPTH YSIRSTLRME
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1501 PKEWLQVDFQ KTMKVTGVTT QGVKSLLTSM YVKEFLISSS
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1551 QNGKVKVFQG NQDSFTPVVN SLDPPLLTRY LRIHPQSWVH
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1601 CEAQDLYDKT HTCPPCPAPE LLGGPSVFLF PPKPKDTLMI
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1651 VDVSHEDPEV KFNWYVDGVE VHNAKTKPRE EQYNSTYRVV
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1701 LNGKEYKCKV SNKALPAPIE KTISKAKGQP REPQVYTLPP
SRDELTKNQV
1751 SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSDGS
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1801 KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPGK*

pSYN FVIII 269 protein sequence (FVIII Fc with 72 AE-XTEN at amino acid 18) SEQ ID NO: 115

1 MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQGAP
TSESATPESG
51 PGSEPATSGS ETPGTSESAT PESGPGSEPA TSGSETPGTS
ESATPESGPG
101 TSTEPSEGSA PGASSSDLGE LPVDARFPPR VPKSFPFNTS
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151 FTDHLFNIAK PRPPWMGLLG PTIQAEVYDT VVITLKNMAS
HPVSLHAVGV
201 SYWKASEGAE YDDQTSQREK EDDKVFPGGS HTYVWQVLKE
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251 LTYSYLSHVD LVKDLNSGLI GALLVCREGS LAKEKTQTLH
KFILLFAVFD
301 EGKSWHSETK NSLMQDRDAA SARAWPKMHT VNGYVNRSLP
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351 YWHVIGMGTT PEVHSIFLEG HTFLVRNHRQ ASLEISPITF
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501 DRSYKSQYLN NGPQRIGRKY KKVRFMAYTD ETFKTREAIQ
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1651 ISKAKGQPRE PQVYTLPPSR DELTKNQVSL TCLVKGFYPS
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1701 QPENNYKTTP PVLDSDGSFF LYSKLTVDKS RWQQGNVFSC
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1751 YTQKSLSLSP GK*

pSYNFVIII 271 protein sequence (FVIII Fc with 42 AE-XTEN at amino acid 18) SEQ ID NO: 116

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 151  NMASHPVSLH AVGVSYWKAS EGAEYDDQTS QREKEDDKVF
  PGGSHTYVWQ
 201  VLKENGPMAS DPLCLTYSYL SHVDLVKDLN SGLIGALLVC
  REGSLAKEKT
 251  QTLHKFILLF AVFDEGKSWH SETKNSLMQD RDAASARAWP
  KMHTVNGYVN
 301  RSLPGLIGCH RKSVYWHVIG MGTTPEVHSI FLEGHTFLVR
  NHRQASLEIS
 351  PITFLTAQTL LMDLGQFLLF CHISSHQHDG MEAYVKVDSC
  PEEPQLRMKN
 401  NEEAEDYDDD LTDSEMDVVR FDDDNSPSFI QIRSVAKKHP
  KTWVHYIAAE
 451  EEDWDYAPLV LAPDDRSYKS QYLNNGPQRI GRKYKKVRFM
  AYTDETFKTR
 501  EAIQHESGIL GPLLYGEVGD TLLIIFKNQA SRPYNIYPHG
  ITDVRPLYSR
 551  RLPKGVKHLK DFPILPGEIF KYKWTVTVED GPTKSDPRCL
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 601  ERDLASGLIG PLLICYKESV DQRGNQIMSD KRNVILFSVF
  DENRSWYLTE
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1051  DETKSWYFTE NMERNCRAPC NIQMEDPTFK ENYRFHAING
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1101  MAQDQRIRWY LLSMGSNENI HSIHFSGHVF TVRKKEEYKM
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1151  ETVEMLPSKA GIWRVECLIG EHLHAGMSTL FLVYSNKCQT
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1351 AISDAQITAS SYFTNMFATW SPSKARLHLQ GRSNAWRPQV
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1401 FQKTMKVTGV TTQGVKSLLT SMYVKEFLIS SSQDGHQWTL
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1451 QGNQDSFTPV VNSLDPPLLT RYLRIHPQSW VHQIALRMEV
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1551 EVKFNWYVDG VEVHNAKTKP REEQYNSTYR VVSVLTVLHQ
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1601 KVSNKALPAP IEKTISKAKG QPREPQVYTL PPSRDELTKN
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1651 FYPSDIAVEW ESNGQPENNY KTTPPVLDSD GSFFLYSKLT
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1701 VFSCSVMHEA LHNHYTQKSL SLSPGK*

pSYN FVIII protein sequence 272 (FVIII with 144 AE XTEN at amino acid 18 and 244 AE XTEN in B-domain- no Fc) SEQ ID NO: 117

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101 TSTEPSEGSA PGSPAGSPTS TEEGTSESAT PESGPGSEPA
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201 PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL
LGPTIQAEVY
251 DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR
EKEDDKVFPG
301 GSHTYVWQVL KENGPMASDP LCLTYSYLSH VDLVKDLNSG
LIGALLVCRE
351 GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
AASARAWPKM
401 HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL
EGHTFLVRNH
451 RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME
AYVKVDSCPE
501 EPQLRMKNNE EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI
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551 WVHYIAAEEE DWDYAPLVLA PDDRSYKSQY LNNGPQRIGR
KYKKVRFMAY
601 TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR
PYNIYPHGIT

651 DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
TKSDPRCLTR
701 YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR
NVILFSVFDE
751 NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV
FDSLQLSVCL
801 HEVAYWYILS IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF
PFSGETVFMS
851 MENPGLWILG CHNSDFRNRG MTALLKVSSC DKNTGDYYED
SYEDISAYLL
901 SKNNAIEPRS FSQNGAPGTS ESATPESGPG SEPATSGSET
PGTSESATPE
951 SGPGSEPATS GSETPGTSES ATPESGPGTS TEPSEGSAPG
SPAGSPTSTE
1001 EGTSESATPE SGPGSEPATS GSETPGTSES ATPESGPGSP
AGSPTSTEEG
1051 SPAGSPTSTE EGTSTEPSEG SAPGTSESAT PESGPGTSES
ATPESGPGTS
1101 ESATPESGPG SEPATSGSET PGSEPATSGS ETPGSPAGSP
TSTEEGTSTE
1151 PSEGSAPGTS TEPSEGSAPG SEPATSGSET PGTSESATPE
SGPGTSTEPS
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TVQEFALFFT
1451 IFDETKSWYF TENMERNCRA PCNIQMEDPT FKENYRFHAI
NGYIMDTLPG
1501 LVMAQDQRIR WYLLSMGSNE NIHSIHFSGH VFTVRKKEEY
KMALYNLYPG
1551 VFETVEMLPS KAGIWRVECL IGEHLHAGMS TLFLVYSNKC
QTPLGMASGH
1601 IRDFQITASG QYGQWAPKLA RLHYSGSINA WSTKEPFSWI
KVDLLAPMII
1651 HGIKTQGARQ KFSSLYISQF IIMYSLDGKK WQTYRGNSTG
TLMVFFGNVD
1701 SSGIKHNIFN PPIIARYIRL HPTHYSIRST LRMELMGCDL
NSCSMPLGME
1751 SKAISDAQIT ASSYFTNMFA TWSPSKARLH LQGRSNAWRP
QVNNPKEWLQ
1801 VDFQKTMKVT GVTTQGVKSL LTSMYVKEFL ISSSQDGHQW
TLFFQNGKVK
1851 VFQGNQDSFT PVVNSLDPPL LTRYLRIHPQ SWVHQIALRM
EVLGCEAQDL
1901 Y*

pSYN VWF 031 protein sequence (VWF D1D2D'D3- 48aa long thrombin cleavable GS linker-Fc) SEQ ID NO: 118

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   1  MIPARFAGVL LALALILPGT LCAEGTRGRS STARCSLFGS
  DFVNTFDGSM
  51  YSFAGYCSYL LAGGCQKRSF SIIGDFQNGK RVSLSVYLGE
  FFDIHLFVNG
 101  TVTQGDQRVS MPYASKGLYL ETEAGYYKLS GEAYGFVARI
  DGSGNFQVLL
 151  SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL TSDPYDFANS
  WALSSGEQWC
 201  ERASPPSSSC NISSGEMQKG LWEQCQLLKS TSVFARCHPL
  VDPEPFVALC
 251  EKTLCECAGG LECACPALLE YARTCAQEGM VLYGWTDHSA
  CSPVCPAGME
 301  YRQCVSPCAR TCQSLHINEM CQERCVDGCS CPEGQLLDEG
  LCVESTECPC
 351  VHSGKRYPPG TSLSRDCNTC ICRNSQWICS NEECPGECLV
  TGQSHFKSFD
 401  NRYFTFSGIC QYLLARDCQD HSFSIVIETV QCADDRDAVC
  TRSVTVRLPG
 451  LHNSLVKLKH GAGVAMDGQD IQLPLLKGDL RIQHTVTASV
  RLSYGEDLQM
 501  DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN QGDDFLTPSG
  LAEPRVEDFG
 551  NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS EEACAVLTSP
  TFEACHRAVS
 601  PLPYLRNCRY DVCSCSDGRE CLCGALASYA AACAGRGVRV
  AWREPGRCEL
 651  NCPKGQVYLQ CGTPCNLTCR SLSYPDEECN EACLEGCFCP
  PGLYMDERGD
 701  CVPKAQCPCY YDGEIFQPED IFSDHHTMCY CEDGFMHCTM
  SGVPGSLLPD
 751  AVLSSPLSHR SKRSLSCRPP MVKLVCPADN LRAEGLECTK
  TCQNYDLECM
 801  SMGCVSGCLC PPGMVRHENR CVALERCPCF HQGKEYAPGE
  TVKIGCNTCV
 851  CRDRKWNCTD HVCDATCSTI GMAHYLTFDG LKYLFPGECQ
  YVLVQDYCGS
 901  NPGTFRILVG NKGCSHPSVK CKKRVTILVE GGEIELFDGE
  VNVKRPMKDE
 951  THFEVVESGR YIILLLGKAL SVVWDRHLSI SVVLKQTYQE
  KVCGLCGNFD
1001  GIQNNDLTSS NLQVEEDPVD FGNSWKVSSQ CADTRKVPLD
  SSPATCHNNI
1051  MKQTMVDSSC RILTSDVFQD CNKLVDPEPY LDVCIYDTCS
  CESIGDCAAF
1101  CDTIAAYAHV CAQHGKVVTW RTATLCPQSC EERNLRENGY
  EAEWRYNSCA
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1151 PACQVTCQHP EPLACPVQCV EGCHAHCPPG KILDELLQTC
VDPEDCPVCE
1201 VAGRRFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP
ISGGGGSGGG
1251 GSGGGGSGGG GSGGGGSGGG GSLVPRGSGG GGSGGGGSDK
THTCPPCPAP
1301 ELLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE
VKFNWYVDGV
1351 EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK
VSNKALPAPI
1401 EKTISKAKGQ PREPQVYTLP PSRDELTKNQ VSLTCLVKGF
YPSDIAVEWE
1451 SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV
FSCSVMHEAL
1501 HNHYTQKSLS LSPGK*

pSYN VWF 034 protein sequence (VWF D1D2D'D3- 288AE XTEN- 35aa long thrombin cleavable GS linker-Fc) SEQ ID NO: 119

1 MIPARFAGVL LALALILPGT LCAEGTRGRS STARCSLFGS
DFVNTFDGSM
51 YSFAGYCSYL LAGGCQKRSF SIIGDFQNGK RVSLSVYLGE
FFDIHLFVNG
101 TVTQGDQRVS MPYASKGLYL ETEAGYYKLS GEAYGFVARI
DGSGNFQVLL
151 SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL TSDPYDFANS
WALSSGEQWC
201 ERASPPSSSC NISSGEMQKG LWEQCQLLKS TSVFARCHPL
VDPEPFVALC
251 EKTLCECAGG LECACPALLE YARTCAQEGM VLYGWTDHSA
CSPVCPAGME
301 YRQCVSPCAR TCQSLHINEM CQERCVDGCS CPEGQLLDEG
LCVESTECPC
351 VHSGKRYPPG TSLSRDCNTC ICRNSQWICS NEECPGECLV
TGQSHFKSFD
401 NRYFTFSGIC QYLLARDCQD HSFSIVIETV QCADDRDAVC
TRSVTVRLPG
451 LHNSLVKLKH GAGVAMDGQD IQLPLLKGDL RIQHTVTASV
RLSYGEDLQM
501 DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN QGDDFLTPSG
LAEPRVEDFG
551 NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS EEACAVLTSP
TFEACHRAVS
601 PLPYLRNCRY DVCSCSDGRE CLCGALASYA AACAGRGVRV
AWREPGRCEL
651 NCPKGQVYLQ CGTPCNLTCR SLSYPDEECN EACLEGCFCP
PGLYMDERGD
701 CVPKAQCPCY YDGEIFQPED IFSDHHTMCY CEDGFMHCTM
SGVPGSLLPD
751 AVLSSPLSHR SKRSLSCRPP MVKLVCPADN LRAEGLECTK
TCQNYDLECM

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   YVLVQDYCGS
 901  NPGTFRILVG NKGCSHPSVK CKKRVTILVE GGEIELFDGE
   VNVKRPMKDE
 951  THFEVVESGR YIILLLGKAL SVVWDRHLSI SVVLKQTYQE
   KVCGLCGNFD
1001  GIQNNDLTSS NLQVEEDPVD FGNSWKVSSQ CADTRKVPLD
   SSPATCHNNI
1051  MKQTMVDSSC RILTSDVFQD CNKLVDPEPY LDVCIYDTCS
   CESIGDCAAF
1101  CDTIAAYAHV CAQHGKVVTW RTATLCPQSC EERNLRENGY
   EAEWRYNSCA
1151  PACQVTCQHP EPLACPVQCV EGCHAHCPPG KILDELLQTC
   VDPEDCPVCE
1201  VAGRRFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP
   ISGTSESATP
1251  ESGPGSEPAT SGSETPGTSE SATPESGPGS EPATSGSETP
   GTSESATPES
1301  GPGTSTEPSE GSAPGSPAGS PTSTEEGTSE SATPESGPGS
   EPATSGSETP
1351  GTSESATPES GPGSPAGSPT STEEGSPAGS PTSTEEGTST
   EPSEGSAPGT
1401  SESATPESGP GTSESATPES GPGTSESATP ESGPGSEPAT
   SGSETPGSEP
1451  ATSGSETPGS PAGSPTSTEE GTSTEPSEGS APGTSTEPSE
   GSAPGSEPAT
1501  SGSETPGTSE SATPESGPGT STEPSEGSAP DIGGGGGSGG
   GGSLVPRGSG
1551  GDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV
   TCVVVDVSHE
1601  DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL
   HQDWLNGKEY
1651  KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELT
   KNQVSLTCLV
1701  KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK
   LTVDKSRWQQ
1751  GNVFSCSVMH EALHNHYTQK SLSLSPGK*
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pSYN VWF 036 protein sequence (VWF D1D2D'D-98aa long thrombin cleavable GS linker-Fc) SEQ ID NO: 120

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   FFDIHLFVNG
 101  TVTQGDQRVS MPYASKGLYL ETEAGYYKLS GEAYGFVARI
   DGSGNFQVLL
 151  SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL TSDPYDFANS
   WALSSGEQWC
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201 ERASPPSSSC NISSGEMQKG LWEQCQLLKS TSVFARCHPL
VDPEPFVALC
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CSPVCPAGME
301 YRQCVSPCAR TCQSLHINEM CQERCVDGCS CPEGQLLDEG
LCVESTECPC
351 VHSGKRYPPG TSLSRDCNTC ICRNSQWICS NEECPGECLV
TGQSHFKSFD
401 NRYFTFSGIC QYLLARDCQD HSFSIVIETV QCADDRDAVC
TRSVTVRLPG
451 LHNSLVKLKH GAGVAMDGQD IQLPLLKGDL RIQHTVTASV
RLSYGEDLQM
501 DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN QGDDFLTPSG
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551 NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS EEACAVLTSP
TFEACHRAVS
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AWREPGRCEL
651 NCPKGQVYLQ CGTPCNLTCR SLSYPDEECN EACLEGCFCP
PGLYMDERGD
701 CVPKAQCPCY YDGEIFQPED IFSDHHTMCY CEDGFMHCTM
SGVPGSLLPD
751 AVLSSPLSHR SKRSLSCRPP MVKLVCPADN LRAEGLECTK
TCQNYDLECM
801 SMGCVSGCLC PPGMVRHENR CVALERCPCF HQGKEYAPGE
TVKIGCNTCV
851 CRDRKWNCTD HVCDATCSTI GMAHYLTFDG LKYLFPGECQ
YVLVQDYCGS
901 NPGTFRILVG NKGCSHPSVK CKKRVTILVE GGEIELFDGE
VNVKRPMKDE
951 THFEVVESGR YIILLLGKAL SVVWDRHLSI SVVLKQTYQE
KVCGLCGNFD
1001 GIQNNDLTSS NLQVEEDPVD FGNSWKVSSQ CADTRKVPLD
SSPATCHNNI
1051 MKQTMVDSSC RILTSDVFQD CNKLVDPEPY LDVCIYDTCS
CESIGDCAAF
1101 CDTIAAYAHV CAQHGKVVTW RTATLCPQSC EERNLRENGY
EAEWRYNSCA
1151 PACQVTCQHP EPLACPVQCV EGCHAHCPPG KILDELLQTC
VDPEDCPVCE
1201 VAGRRFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP
ISGGGGSGGG
1251 GSGGGGSGGG GSGGGGSGGG GSGGGGSGGG GSGGGGSGGG
GSGGGGSGGG
1301 GSGGGGSGGG GSGGGGSGGG GSLVPRGSGG GGSGGGGSDK
THTCPPCPAP
1351 ELLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE
VKFNWYVDGV
1401 EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK
VSNKALPAPI

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1451  EKTISKAKGQ PREPQVYTLP PSRDELTKNQ VSLTCLVKGF
  YPSDIAVEWE
1501  SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV
  FSCSVMHEAL
1551  HNHYTQKSLS LSPGK*
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pSYN Fc-015 protein sequence (IgG-Fc domain) SEQ ID NO: 121

```
   1  METDTLLLWV LLLWVPGSTG DKTHTCPPCP APELLGGPSV
  FLFPPKPKDT
  51  LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK
  PREEQYNSTY
 101  RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK
  GQPREPQVYT
 151  LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN
  YKTTPPVLDS
 201  DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS
  LSLSPGK*
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Example 15: FVIII-XTEN-Fc:VWF-Fc heterodimers have maintained normal FVIII specific activity as compared to wild type BDD-FVIII.

[0383] The FVIII specific activity of FVIII-XTEN-Fc:VWF-Fc heterodimers were determined. Heterodimers were purified using a two-step chromatography process. A weak anion exchange resin was used, followed by affinity chromatography. The final purified product had acceptable purity by SEC-HPLC. The specific activity was compared to B-domain deleted FVIII (BDD-FVIII), as measured by FVIII chromogenic assay and A280 concentration. The data are presented in Table 26. All tested molecules had demonstrated comparable FVIII specific activities to BDD-FVIII. Purity and the presence of each moiety of the molecules were confirmed by SDS-PAGE and western blotting.

Table 26: FVIII specific activity of FVIII-XTEN-Fc:VWF-Fc heterodimers

Construct	FVIII 207 scBDDFVIII	FVIII-66 dcBDD FVIII)	FVIII 155 / vWF31	FVIII 155 / vWF39	FVIII 169 / vWF31	FVIII 205 / vWF31	FVIII 169 / vWF34
Measured Specific Activity (IU/nmol)	1473	1592	1534	1796	1511	1345	1505

[0384] The half-lives of rFVIII-XTEN/D'D3 and BDD-FVIII were compared in HemA Mice (Figure 15; Table 27). As Figure 15 shows, rFVIII-XTEN/D'D3

achieved a half-life that was four fold longer than the half-life achieved by BDD-FVIII.

Table 27: rFVIII-XTEN/D'D3 and BDD-FVIII in HemA mice

Treatment	5 minutes Recovery (%)	HL (hr)	MRT (hr)	Cl (mL/hr/kg)	Vss (mL/kg)	AUC_D (hr*kg*mIU/mL/mIU)
BDD-FVIII	89	7.6	11	4.5	49.2	0.22
rFVIIIFc	78	16	20	2.9	57.8	0.35
rFVIII-XTEN/D'D3	86	30	36	1.8	63.4	0.57

Example 16: FVIII-XTEN-Fc:VWF-Fc heterodimer's potency (FVIII activity) in hemostasis as measured by one stage aPTT assay

[0385] The potency of FVIII-XTEN-Fc:VWF-Fc heterodimers in hemostasis was evaluated by their FVIII specific aPTT activity as summarized in Table 28. As demonstrated by Table 28, while the addition of the VWF D'D3 fragment and the insertion of XTEN into the intra-domains of FVIII reduce the FVIII specific aPTT activity of the heterodimers (as indicated by the FVIII155/VWF031 data and the FVIII205/VWF031 data), XTEN insertions in the FVIII B domain region or C-terminus of the VWF D'D3 fragment have no negative effect on the FVIII specific aPTT activity (as indicated by the FVIII169/VWF031 data and the FVIII169/VWF034 data). Compared to dual-chain BDD-FVIII (dcBDD-FVIII), FVIII155/VWF031, FVIII169/VWF031, FVIII169/VWF034 and VWF205/VWF031 showed reduction of specific aPTT activity by 2.5-fold, 2.8-fold, 2.6-fold and 5.5-fold, respectively.

Table 28: FVIII specific aPTT activity of FVIII-XTEN-Fc:VWF-Fc heterodimers

Construct	FVIII 207 scBDD-FVIII	FVIII-66 dcBDD-FVIII	FVIII 155 / VWF31	FVIII 169 / VWF31	FVIII 205 / VWF31	FVIII 169 / VWF34
Measured Specific aPTT Activity (IU/nmol)	818 ± 153	1188 ± 213	448 ± 111	416 ± 70	214 ± 38	436 ± 189

FVIII specific aPTT assay

[0386] FVIII variants were diluted with aPTT buffer (0.15 M NaCl, 0.05 M Tris-HCl, 1% BSA, pH 7.4) to the linear assay range (200- 1.6 mU/mL). 50 µL of diluted samples or standards were sequentially mixed with 50 µL of 37°C naïve human HemA pooled plasma, 50 µL of 37°C aPTT reagent (ACTIN® FSL activated cephaloplastin reagent - Dade Behring, reference # B4219-2) and incubated at 37 °C for 4 minutes. 50 µl of 20 mM $CaCl_2$ (Dade Behring [reference # ORFO37]) was then added to the reaction mixture to start the clotting reactions. Using the clotting time of each sample (the length of time from the addition of $CaCl_2$ until the onset of clot formation), the aPTT activity was calculated against the standard that was generated with the 8th international standard FVIII concentrate. Specific aPTT activity was calculated against the protein concentration of each molecule that measured by OD280.

Example 17: *In vivo* efficacy of FVIII-XTEN-Fc:VWF-Fc heterodimer in HemA mice Tail Clip bleeding model

[0387] To further access the hemostasis potency of the heterodimers, the acute efficacy of FVIII169/VWF034 and FVIII205/VWF031 was evaluated in comparison with BDD-FVIII in the HemA mice Tail clip bleeding model. HemA mice were treated with a single IV injection of BDD-FVIII at 200, 65 and 20 IU/kg to generate the post tail clip injury blood loss control level. Blood loss from mice treated with 200 IU/kg of FVIII169/VWF034 or FVIII205/VWF031 was compared to that of the BDD-FVIII treated control group mice to estimate their potency on hemostasis. Vehicle treated animals were used to generate blood loss baseline for the model. As shown in Figure 16, significant reduction in blood loss was observed from all FVIII treatment groups compared to that of the vehicle treated animals ($p<0.05$). Both FVIII169/VWF034 and FVIII205/VWF031 are efficacious in the HemA mice Tail Clip model. Compared to BDD-FVIII, about 3 fold lower potency was observed for FVIII169/VWF034, as demonstrated by the similar blood loss reduction achieved by 65 IU/kg BDD-FVIII and 200 IU/kg FVIII169/VWF034. As for FVIII205/VWF034, a 10 fold potency reduction has been observed, as demonstrated by the similar blood loss reduction achieved by 20 IU/kg BDD-FVIII and 200 IU/kg FVIII205/VWF031.

[0388] Even though FVIII69/VWF034 and FVIII205/VWF031 had similar specific FVIII chromogenic activity compared to rBDD-FVIII, their FVIII aPTT activity and *in vivo* potency were both reduced due to the modifications of the molecules. Those data indicate that the aPTT activity of a FVIII molecule is a more accurate measurement on predicating its *in vivo* potency on hemostasis than the FVIII chromogenic activity.

HemA mice Tail clip bleeding model

[0389] 8-10 weeks old male HemA mice were used for the study. Prior to tail clip injury, mice were anesthetized with a 50 mg/kg Ketamine/0.5 mg/kg Dexmedetomidine cocktail and placed on a 37°C heating pad to help maintain the body temperature. The tails of the mice were then be immersed in 37°C water for 10 minutes to dilate the lateral vein. After vein dilation, rFVIII or vehicle solution were injected via the tail vein and 5 min later, the distal 1 cm of the tail was cut off using a #11 scalpel with straight edge. The shed blood was collected into 13 ml of 37°C warm saline for 30 minutes and the mice were then euthanized while still under anesthesia by bilateral thoracotomy. Blood loss was quantified gravimetrically by weight change of the blood collection tubes before and after blood was collected in gram, which translated into milliliter (mL) of blood loss volume (1g weight change = 1 mL blood loss).

[0390] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art, readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance.

[0391] Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as

exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

[0392] All patents and publications cited herein are incorporated by reference herein in their entirety.

CLAIMS

1. A chimeric protein comprising (i) a first polypeptide comprising (1) a von Willebrand Factor (VWF) fragment comprising a D' domain and a D3 domain of VWF, (2) an extended length polypeptide (XTEN) and (3) a first Fc region and (ii) a second polypeptide comprising (4) a factor VIII (FVIII) protein and (5) a second Fc region, wherein the XTEN comprises an amino acid sequence having at least 80% identity to an amino acid sequence as set forth in SEQ ID NO: 141-169, or 170, wherein the VWF fragment does not comprise amino acids 1274 to 2813 corresponding to SEQ ID NO: 2, and wherein the first Fc region is associated with the second Fc region by a disulfide bond.

2. The chimeric protein of claim 1, wherein the first polypeptide and the second polypeptide are not linked by a peptide bond.

3. The chimeric protein of claim 1, wherein the first Fc region is linked to the XTEN.

4. The chimeric protein of claim 3, wherein the first Fc region is linked to the XTEN by a cleavable linker.

5. The chimeric protein of claim 1, wherein the second Fc region is further linked to the first Fc region by a processable linker.

6. The chimeric protein of claim 1, wherein the first polypeptide comprises an amino acid sequence at least 80% identical to an amino acid sequence as set forth in SEQ ID NO: 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, or 117; and wherein the VWF fragment comprises an amino acid sequence at least 80% identical to an amino acid sequence as set forth in SEQ ID NO: 118, 119, or 120.

7. The chimeric protein of claim 1, wherein the FVIII protein comprises a B domain or a portion thereof.

8. The chimeric protein of claim 7, wherein the FVIII protein is SQ B domain deleted FVIII.

9. The chimeric protein of claim 7, wherein an additional XTEN is inserted within the B domain or a portion thereof.

10. The chimeric protein of claim 1, wherein the VWF fragment comprises an amino acid sequence at least 90% identical to amino acids 764 to 1240 of SEQ ID NO: 119.

11. The chimeric protein of claim 10, wherein the VWF fragment consists essentially of amino acids 764 to 1240 of SEQ ID NO: 119.

12. The chimeric protein of claim 1, wherein the XTEN is an amino acid sequence at least 95% identical to SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, or SEQ ID NO: 44.

13. The chimeric protein of claim 1, wherein the XTEN is an amino acid sequence at least 80% identical to SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, or SEQ ID NO: 44.

14. A pharmaceutical composition comprising the chimeric protein of claim 1 and a pharmaceutically acceptable carrier.

15. The chimeric protein of claim 1, wherein the FVIII protein comprises (i) a heavy chain and a light

chain, wherein the heavy chain of the FVIII protein comprises an amino acid sequence at least 90% identical to amino acids 1-740 of SEQ ID NO: 4, and the light chain of the FVIII protein comprises an amino acid sequence at least 90% identical to amino acids 1649-2332 of SEQ ID NO: 4.

16. The chimeric protein of claim 15, wherein the FVIII protein comprises a B-domain or a portion thereof.

17. The chimeric protein of claim 1, wherein the second polypeptide comprises an amino acid sequence at least 90% identical to the amino acid sequence as set forth in SEQ ID NO: 103.

18. The chimeric protein of claim 1, wherein the second polypeptide comprises an amino acid sequence at least 95% identical to the amino acid sequence as set forth in SEQ ID NO:103.

19. The chimeric protein of claim 1, wherein the second polypeptide comprises the amino acid sequence as set forth in SEQ ID NO:103.

20. The chimeric protein of claim 1, wherein the second polypeptide comprises an amino acid sequence at least 90% identical to the amino acid sequence as set forth in SEQ ID NO:103, and the first polypeptide comprises an amino acid sequence at least 90% identical to the amino acid sequence as set forth in SEQ ID NO:119.

21. The chimeric protein of claim 1, wherein the second polypeptide comprises an amino acid sequence at least 95% identical to the amino acid sequence as set forth in SEQ ID NO:103, and the first polypeptide comprises an amino acid sequence at least 95% identical to the amino acid sequence as set forth in SEQ ID NO:119.

22. The chimeric protein of claim 4, wherein the cleavable linker comprises one or more cleavage sites that can be cleaved by a protease.

23. The chimeric protein of claim 22, wherein the protease is factor XIa, factor XIIa, kallikrein, factor VIIa, factor IXa, factor Xa, factor IIa (thrombin), Elastase-2, MMP-12, MMP-13, MMP-17, or MMP-20.

24. The chimeric protein of claim 4, wherein the cleavable linker comprises one or more thrombin cleavage sites.

25. The chimeric protein of claim 9, wherein the additional XTEN comprises an amino acid sequence of 288 amino acids in length.

26. The chimeric protein of claim 1, wherein the XTEN comprises an amino acid sequence having at least 90% identity to an amino acid sequence as set forth in SEQ ID NO: 145, 146, 147, or 148.

27. The chimeric protein of claim 11, wherein the VWF fragment consists of amino acids 764 to 1240 of SEQ ID NO: 119.

28. The chimeric protein of claim 1, wherein the XTEN comprises an amino acid sequence as set forth in SEQ ID NO: 145, 146, 147, or 148.

29. A chimeric protein comprising (i) a first polypeptide and (ii) a second polypeptide, the first polypeptide comprising (1) a VWF fragment, (2) a first XTEN, (3) a thrombin cleavable linker, and (4) a first Fc region, and the second polypeptide comprising (5) a FVIII protein, (6) a second XTEN, and (7) a second Fc region, wherein the VWF fragment consists of amino acids 764 to 1240 of SEQ ID NO: 119; wherein the first XTEN comprises the amino acid sequence as set forth in SEQ ID NO:145, 146, 147, or 148; wherein the FVIII protein comprises a full or partial deletion of the B domain; wherein the second XTEN comprises the amino acid sequence as set forth in SEQ ID NO: 145, 146, 147, or 148, wherein the second XTEN is inserted within the B domain or a portion thereof, and

wherein the XTEN is about 36 to about 3000 amino acid in length; and wherein the first Fc region and the second Fc region form a disulfide bond.

30. The chimeric protein of claim 1, wherein the XTEN is about 36 to about 3000 amino acid in length.

31. A chimeric protein comprising (i) a first polypeptide, (ii) a second polypeptide, and (iii) a third polypeptide, wherein the first polypeptide comprises (1) a VWF fragment, (2) a first XTEN, (3) a thrombin cleavable linker, and (4) a first Fc region; wherein the second polypeptide comprises (5) a heavy chain of a FVIII protein and (6) a second XTEN; and wherein the third polypeptide comprises (7) a light chain of a FVIII protein and (8) a second Fc region; wherein the heavy chain of the FVIII protein and the light chain of the FVIII protein form a metal bond; wherein the first XTEN comprises an amino acid sequence having at least 80% identity to an amino acid sequence as set forth in SEQ ID NO: 141-169, or 170; wherein the second XTEN comprises an amino acid sequence having at least 80% identity to an amino acid sequence as set forth in SEQ ID NO: 141-169, or 170; wherein the VWF fragment does not comprise amino acids 1274 to 2813 corresponding to SEQ ID NO: 2; and wherein the first Fc region and the second Fc region form a disulfide bond.

32. The chimeric protein of claim 31, wherein the first and second XTENs are about 36 to about 3000 amino acid in length.

33. The chimeric protein of claim 1, wherein the VWF fragment in the first polypeptide comprises an amino acid sequence at least 90% identical to amino acids 764 to 1240 of SEQ ID NO: 119 and the second polypeptide comprises an amino acid sequence at least 90% identical to the amino acid sequence as set forth in SEQ ID NO:103.

34. The chimeric protein of claim 1, wherein the VWF fragment in the first polypeptide comprises an amino acid sequence at least 95% identical to amino acids 764 to 1240 of SEQ ID NO: 119 and the second polypeptide comprises an amino acid sequence at least 95% identical to the amino acid sequence as set forth in SEQ ID NO:103.

35. The chimeric protein of claim 31, wherein the VWF fragment in the first polypeptide comprises an amino acid sequence at least 90% identical to amino acids 764 to 1240 of SEQ ID NO: 119.

36. The chimeric protein of claim 31, wherein the VWF fragment in the first polypeptide comprises an amino acid sequence at least 95% identical to amino acids 764 to 1240 of SEQ ID NO: 119.