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(54) Title: PROCESSABLE SINGLE CHAIN MOLECULES AND POLYPEPTIDES MADE USING SAME

(57) Abstract: The present invention features *inter alia* nucleic acid molecules which encode polypeptides comprising a single chain Fc region and the polypeptides they encode. The Fc moieties of these constructs are linked by a cleavable scFc linker which is adjacent to at least one enzymatic cleavage site, e.g., an intracellular processing site. The resulting processed molecules comprise two polypeptide chains and substantially lack the extraneous amino acid sequence found in single chain Fc linker molecule. Methods of making and using these dimeric molecules are also described.**WO 2012/006635 A1**

**PROCESSABLE
SINGLE CHAIN MOLECULES AND POLYPEPTIDES
MADE USING SAME**

5 RELATED APPLICATIONS

[0001] This patent application claims the benefit of U.S. Provisional Patent Application Serial No. 61/363,183 filed July 9, 2010; U.S. Provisional Patent Application No. 61/363,186 filed July 9, 2010; U.S. Provisional Patent Application No. 61/442,029 filed February 11, 2011; U.S. Provisional Patent Application No. 61/442,150 filed February 11, 2011; U.S. Provisional Patent Application No. 61/442,055 filed February 11, 2011; U.S. Provisional Patent Application No. 61/467,880 filed March 25, 2011; and U.S. Provisional Patent Application No. 61/491,762 filed May 31, 2011. The entire contents of the above-referenced provisional patent applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] The Fc region of an immunoglobulin mediates effector functions that have been divided into two categories. In the first are functions that occur independently of antigen binding; these functions confer persistence in circulation and the ability to be transferred across cellular barriers by transcytosis (*see Ward and Ghetie, Therapeutic*
10 *Immunology* 2:77-94, 1995, Capon et al. *Nature* 1989). The circulatory half-life of the IgG subclass of immunoglobulins is regulated by the affinity of the Fc region for the neonatal Fc receptor for FcRn (Ghetie et al. *Nature Biotechnol.* 15:637-640, 1997; Kim et. al, *Eur. J. Immunol.* 24:542-548, 1994; Dall'Acqua et al. (*J. Immunol.* 169:5171-5180, 2002). The second general category of effector functions include
15 those that operate after an immunoglobulin binds an antigen. In the case of IgG, these functions involve the participation of the complement cascade or Fc gamma receptor (FcYR)-bearing cells. Binding of the Fc region to an FcYR causes certain immune effects, for example, endocytosis of immune complexes, engulfment and destruction of immunoglobulin-coated particles or microorganisms (also called antibody-
20 dependent phagocytosis, or ADCP), clearance of immune complexes, lysis of immunoglobulin-coated target cells by killer cells (called antibody-dependent cell-

5 mediated cytotoxicity, or ADCC), release of inflammatory mediators, regulation of immune system cell activation, and regulation of immunoglobulin production.

[0003] While it is possible to generate Fc-containing, heterodimeric polypeptides, current methods require either coexpression of the two heavy chain portions of a heterodimeric Fc region or chemical conjugation of the dimeric Fc region to one or
10 more binding sites (e.g., a Fab domain). Coexpression of these constructs leads to production of complex mixtures representing all possible pairings of starting material in addition to aggregates and inactive protein. Consequently, yields of the desired functional polypeptide are relatively low. Recently, single chain Fc molecules have been developed which overcome many of these problems. These molecules comprise
15 a single chain Fc region in which the component Fc moieties are genetically-fused in a single polypeptide chain such that they form a functional, dimeric Fc region. These single chain polypeptides comprise a linker which is not present in naturally occurring Fc constructs and, therefore, may create unwanted immune responses or may prevent other protein:protein interactions. These single chain Fc constructs may also have
20 lower stability potentially due to constraints imposed by the covalent linker.

[0004] Accordingly, there is a need for Fc-containing heterodimeric polypeptides which can be produced efficiently and robustly and which minimize and preferably do not comprise extraneous amino acid sequences.

25 SUMMARY OF THE INVENTION

[0005] The present invention features *inter alia* heterodimeric polypeptides which, in unprocessed form comprise one or more genetically-fused Fc regions. In particular, unprocessed molecules of the invention comprise a single chain Fc region ("scFc") in
30 which the component Fc moieties are genetically-fused in a single polypeptide chain such that they form a functional, single chain, dimeric Fc region. The single chain Fc molecules of the invention comprise at least two Fc moieties, F1 and F2 as numbered from the amino to the carboxy terminus in the single polypeptide chain. The component Fc moieties of an scFc are genetically fused via a polypeptide linker, a
35 cleavable scFc (cscFc) linker. The cscFc linker is interposed between the Fc moieties that comprise the Fc region. At one terminus, the cscFc linker which forms the single chain Fc region is directly linked via a peptide bond to an enzymatic cleavage site (PI) which is linked to (e.g., adjacent to or directly linked to) an Fc moiety of the

- 5 polypeptide. In one embodiment, the cleavage site is cleaved by an intracellular processing enzyme. In one embodiment, the other terminus of the cscFc linker is directly linked to a second enzymatic cleavage site (P2). In another embodiment, the other terminus of the cscFc linker is linked to a biologically active moiety or a targeting moiety.
- 10 **[0006]** In one embodiment, cleavage during processing of the scFc containing polypeptide by a cell allows for cleavage and/or substantial excision of the linker. Alternatively, the polypeptide comprises a cleavable scFc (cscFc) linker which can be cleaved after the polypeptide has been secreted by a cell or after it has been administered to a subject. Thus, although the polypeptides of the invention comprise
- 15 a scFc region(s) in one contiguous polypeptide sequence in their unprocessed form, the cscFc linker is enzymatically cleaved (e.g., during processing in a cell, in vitro prior to administration or in vivo after administration), resulting in a processed polypeptide which comprises at least two polypeptide chains and comprises an Fc region which is not fused in a single amino acid chain.
- 20 **[0007]** The heterodimeric polypeptides of the invention have improved manufacturability as compared to conventional heteromeric Fc containing polypeptides because the complex mixture of molecules that result from coexpression of two or more chains is avoided. In addition, the substantial removal of the extraneous linker sequence reduces the risk of immunogenicity. This is of particular
- 25 importance in the case of polypeptides which are administered repeatedly to a subject, e.g., as in the case of components of the clotting cascade. Substantial removal of the extraneous linker sequence may also relieve any steric stress or hindrance that was present in the covalently linked form. In addition, cleavage of the cscFc linker allows for biologically active moieties fused to the amino terminus of each Fc moiety to have
- 30 a free N-terminus. This is particularly valuable in the case of biologically active moieties attached to the second Fc moiety such as serine protease domains which require a free N-terminus to be catalytically active or moieties which might be sterically hindered by the presence of an uncleaved polypeptide linker.
- [0008]** In one aspect, the instant invention pertains to a polypeptide, comprising (i) at
- 35 least one biologically active moiety, (ii) an Fc region comprising at least two Fc moieties and (iii) a cleavable scFc (cscFc) linker interposed between the two Fc moieties, wherein the cscFc linker is linked (e.g., is directly linked or is adjacent to) at least one enzymatic cleavage site which results in cleavage of the cscFc linker.

5 [0009] In one embodiment, at least one biologically active moiety comprises a clotting factor.

[0010] In one embodiment, the clotting factor is selected from the group consisting of FVII, FVIIa, FVIII, FIX, FIXa, FX, and FXa.

[0011] In one embodiment, the polypeptide comprises the moieties A-F1-P1-L-P2-B-
 10 F2 wherein A is a biologically active moiety, F1 is a first Fc moiety or domain, P1 is an enzymatic cleavage site, L is a cscFc linker, P2 is an enzymatic cleavage site B is a biologically active moiety, F2 is a second Fc moiety or domain and "-" represents a peptide bond. Formula (I) comprises at least an A or B and optionally both. A and B, if both present, can be the same or different. Formula (I) comprises at least a P1 or P2
 15 and optionally both. P1 and P2, if both present, can be the same or different. Formula (I) comprises at least F1 or F2. F1 and F2 can be the same or different. In one embodiment, F1 and F2 each comprise a CH2 and a CH3 domain. In one embodiment A and/or B are directly linked to F1 or F2. In another embodiment, A and/or B is linked to F1 or F2 via a spacer moiety.

20 [0012] In one embodiment, A is present and is selected from the group consisting of: an antigen binding portion of an antibody; a non immunoglobulin binding molecule, a binding portion of a ligand, and a binding portion of a receptor, and a clotting factor.

[0013] In one embodiment, A comprises the light chain of a clotting factor and B comprises the heavy chain of a clotting factor which when associated form an active
 25 molecule.

[0014] In one embodiment, B is present and is selected from the group consisting of: an antigen binding portion of an antibody; a non immunoglobulin binding molecule, a binding portion of a ligand, a binding portion of a receptor, and a clotting factor.

[0015] In one embodiment, F1 and F2 each comprise a CH2 and a CH3 domain.

30 [0016] In one embodiment, the polypeptide comprises the moieties A-F1-B-P1-L-P2-F2 or A-F1- -P1-L-P2 B-F2 in linear sequence from amino to carboxy terminus.

[0017] In one embodiment, the polypeptide comprises a structure represented by the formula selected from the group consisting of: A-F1-P1- L-P2-F2; F1-P1- L-P2-B-F2; A-F1-P1- L- F2; F1-P1- L- B-F2; A-F1- L-P2-F2; F1- L-P2-B-F2, and A-F1-P1-L-
 35 P2-B-F2 in linear sequence from amino to carboxy terminus.

[0018] In one embodiment, the polypeptide comprises one biologically active moiety. In one embodiment, two biologically active moieties, e.g, A and B of formula I, are both present and are different biologically active moieties.

- 5 [0019] In one embodiment, PI and P2 are both present and are recognized by the same or by different enzymes. In one embodiment, at least one of PI or P2 comprises comprises the amino acid sequence Arg-Arg-Arg-Arg. In one embodiment, at least one of PI or P2 comprises the amino acid sequence Arg-Lys-Arg-Arg-Lys-Arg. In one embodiment, at least one of PI or P2 comprises the amino acid sequence Arg-Arg-Arg-Ser. In one embodiment, PI and P2 are both present and PI comprise the sequence Arg-Arg-Arg-Arg and P2 comprises the sequence Arg-Lys-Arg-Arg-Lys-Arg. In one embodiment, at least one of PI or P2 comprises an amino acid sequence selected from the group consisting of: TQSFNDFTR and SVSQTSLTR, DFLAEGGGVR, TTKIKPR, LVPRG, and ALRPR.
- 10 [0020] In one embodiment, the cscFc linker has a length of about 1 to about 50 amino acids. In one embodiment, the cscFc linker has a length of about 20 to about 30 amino acids.
- [0021] In one embodiment, the cscFc linker comprises a gly/ser peptide. In one embodiment, the gly/ser peptide is of the formula $(\text{Gly}_4\text{Ser})_n$ or $\text{S}(\text{Gly}_4\text{Ser})_n$, wherein
- 20 n is a positive integer selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10.
- [0022] In one embodiment, the $\text{S}(\text{Gly}_4\text{Ser})_n$ linker is $\text{S}(\text{Gly}_4\text{Ser})_6$ or $\text{S}(\text{Gly}_4\text{Ser})_4$.
- [0023] In another aspect, the invention pertains to a polypeptide comprising two polypeptide chains, wherein the first polypeptide chain comprises a light chain of a
- 25 clotting factor linked to a first Fc moiety and a second polypeptide chain comprises a heavy chain of a clotting factor linked to a second Fc moiety, wherein the wherein the light chain and the heavy chain associate to form an enzymatically active clotting factor
- [0024] In one embodiment, the light chain of the clotting factor is linked to the first
- 30 Fc moiety and the heavy chain of the clotting factor is linked to the second Fc moiety and wherein the clotting factor is enzymatically active upon secretion by a cell.
- [0025] In one embodiment, wherein the clotting factor is selected from the group consisting of FVII, FVIIa, FFX, FFXa, FX, and FXa. In another embodiment, at least one biologically active moiety is factor VII, factor Vila or a portion thereof. In one
- 35 embodiment, at least one biologically active moiety is factor IX, factor FXa or a portion thereof. In one embodiment, at least one biologically active moiety is factor VIII, factor Villa or a portion thereof. In one embodiment, at least one biologically active moiety is factor X, factor Xa, or a portion thereof.

- 5 **[0026]** In one embodiment, a polypeptide of the invention comprises a targeting moiety.
- [0027]** In one embodiment, the targeting moiety binds to resting platelets.
- [0028]** In one embodiment, the targeting moiety selectively binds to activated platelets.
- 10 **[0029]** In one embodiment, the targeting moiety selectively binds to a target selected from the group consisting of: GPIba, GPVI, and the nonactive form of GPIIb/IIIa.
- [0030]** In one embodiment, wherein the targeting moiety selectively binds to a target selected from the group consisting of: the active form of GPIIb/IIIa, P selectin, GMP-33, LAMP-1, LAMP-2, CD40L, and LOX-1.
- 15 **[0031]** In one embodiment, the targeting moiety binds to the GPIb complex
- [0032]** In one embodiment, comprises a targeting moiety, wherein the targeting moiety is a peptide selected from the group consisting of: PS4, OS1, and OS2.
- [0033]** In one embodiment, the targeting moiety comprises an antibody variable region from an antibody selected from the group consisting of: SCE5, MB9, and
- 20 AP3.
- [0034]** In one embodiment, A is the light chain of FVII and B is the heavy chain of FVII.
- [0035]** In another aspect, the invention pertains to a composition comprising a polypeptide of the invention
- 25 **[0036]** In one embodiment, a composition of the invention comprises a cell culture supernatant.
- [0037]** In another aspect, the invention pertains to a nucleic acid molecule encoding the polypeptide of the invention. Exemplary such nucleic acid molecules are described in the instant Examples and set forth in the sequence listing.
- 30 **[0038]** In another aspect, the invention pertains to a nucleic acid molecule encoding a polypeptide, and the polypeptide encoded thereby, wherein the polypeptide comprises (i) at least one biologically active moiety, (ii) an Fc region comprising at least two Fc moieties and (iii) a cleavable scFc linker interposed between the two Fc moieties, wherein the cleavable scFc linker is adjacent to at least one enzymatic cleavage site,
- 35 e.g., an intracellular processing site which results in cleavage of the cscFc linker. In one embodiment, the cscFc linker is adjacent to two enzymatic cleavage sites, e.g., is flanked by an enzymatic cleavage site both upstream and downstream of the cscFc

- 5 linker which links the Fc moieties of the Fc region and cleavage at these two sites results in substantial removal of the cscFc linker.
- [0039] In one embodiment, the invention pertains to a heterodimeric polypeptide comprising two amino acid chains which polypeptide is encoded by the nucleic acid molecule of the invention.
- 10 [0040] In another embodiment, the invention pertains to a heterodimeric polypeptide , wherein said heterodimeric polypeptide is made by expressing the vector containing a nucleic molecule of the invention in a cell cultured in cell culture medium and isolating the polypeptide from the culture medium. In one embodiment, the polypeptide is a processed polypeptide comprising at least two amino acid chains.
- 15 [0041] In one embodiment, the invention pertains to a nucleic acid molecule of the invention which is present in a vector.
- [0042] In one embodiment, the vector further comprises a nucleotide sequence encoding an enzyme which cleaves at least one of the intracellular processing sites.
- [0043] In another aspect, the invention pertains to a processed polypeptide
- 20 comprising at least two amino acid chains which polypeptide is encoded by the nucleic acid molecule of the invention.
- [0044] In one embodiment, the invention pertains to a host cell comprising the vector of the invention, wherein the host cell expresses an enzyme which cleaves the polypeptide linker.
- 25 [0045] In one embodiment, the enzyme is endogenous to the cell. In another embodiment, the enzyme is exogenous to the cell.
- [0046] In another aspect, the invention pertains to a method for producing a polypeptide comprising culturing the host cell of the invention in culture such that a mature polypeptide comprising two amino acid chains is produced.
- 30 [0047] In one aspect, the invention pertains to a processed, heterodimeric polypeptide comprising two polypeptide chains, wherein said processed, heterodimeric polypeptide is made by expressing the vector of of the invention in a cell cultured in cell culture medium and isolating the mature, heterodimeric polypeptide from the culture medium.
- 35 [0048] In embodiment, the invention pertains to a composition comprising a processed polypeptide of the invention and a pharmaceutically acceptable carrier.
- [0049] In another embodiment, the invention pertains to a composition comprising the nucleic acid molecule of the invention a pharmaceutically acceptable carrier.

5 [0050] In one embodiment, the invention pertains to a method for treating or preventing a disease or disorder in a subject, comprising administering a composition of the invention to subject.

[0051] In one embodiment, the disease or disorder is selected from the group consisting of a clotting disorder, a neurological disorder, an inflammatory disorder, an autoimmune disorder, and a neoplastic disorder.

[0052] In another embodiment, the disease or disorder is a disorder affecting hemostasis. In another embodiment, the composition promotes clot formation.

[0053] In one aspect, the instant invention is directed to a polypeptide, wherein the polypeptide comprises (i) at least one biologically active moiety, (ii) an Fc region encoded in a single contiguous genetic sequence, and (iii) an scFc linker moiety, wherein the scFc linker moiety comprises at least one intracellular processing site or enzymatic cleavage site which results in cleavage and substantial removal of the scFc linker.

[0054] In another aspect, the invention pertains to a nucleic acid molecule encoding a polypeptide, wherein the polypeptide comprises (i) at least one biologically active moiety, (ii) an Fc region encoded in a single contiguous genetic sequence, and (iii) an scFc linker moiety, wherein the scFc linker moiety comprises at least one intracellular processing site or enzymatic cleavage site which results in cleavage and substantial removal of the scFc linker.

[0055] In another embodiment, the invention pertains to a mature, heterodimeric polypeptide comprising two polypeptide chains, wherein said mature, heterodimeric polypeptide is made by expressing the vector of containing a nucleic molecule of thie invention in a cell cultured in cell culture medium and isolating the mature, heterodimeric polypeptide from the culture medium.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and B are illustrations of exemplary constructs of the invention with protease processing sites upstream and downstream of a polypeptide linker which links the Fc moieties.

[0056] Figure 2 illustrates a Factor Vila Fc heterodimer in which the FVII light chain-Fc and heavy chain-Fc are expressed as a single chain and secreted as an activated protein dimer following processing of the linker by proteases. The figure

5 includes a Western blot of a protein A pulldown of cells transiently transfected with FVII-024 +/- PC5

[0057] Figure 3A illustrates propeptidase cleavage of processing sites in FFX-Fc proteins for the removal of a polypeptide linker connecting both Fc moieties in an cscFc. Figure 3B illustrates that full cleavage of processing sites in FFX-044
10 was achieved with PACE, but not Kex2, PC5 or PC7, however, PACE cleavage results in extra cleavage of the protein (see band below B,D). Figure 3 panel C illustrates that optimal processing by PC5 was observed with the cleavable linkers incorporated in FFX-052 and -053.

[0058] Figure 4 panel A shows a schematic that illustrates a FVIII cleavable scFc molecule and the processed form of the molecule. Figure 4 Panel B shows an SDS-
15 PAGE gel of the purified FVIII-049 protein (after cotransfection with PC5) that demonstrates the full processing of the molecule.

[0059] Figure 5 panel A shows a schematic that illustrates the FVII cleavable scFc (FVII-064) construct and the processed form of the molecule. In this construct the
20 biologically active moiety comprises FVII as a single chain. Figure 5 Panel B shows an SDS-PAGE gel of the purified FVII-064 protein (after cotransfection with PC5) that demonstrates the full processing of the molecule.

[0060] Figure 6 panel A illustrates the FVIIFc -027 construct, which comprises a FVII cscFc fusion with a targeting moiety, MB9. Figure 6 Panel B shows an SDS-
25 PAGE gel of the purified FVII-027 protein after cotransfection with PC5 that demonstrates the full processing of the molecule.

[0061] Figure 7 illustrates several additional constructs that were made in which FVII heavy and light chains were expressed separately or as a single chain.

[0062] Figure 8 illustrates Western Blot analysis of FVIIFc species following
30 transient transfection of HEK 293 cells and protein A pulldown of the molecules illustrated in Figure 7.

[0063] Figure 9 illustrates a heterodimeric scFc construct and a monomeric Fc construct. These constructs were made and Western blots were performed. The data show intracellular activation is more efficient in the context of the heterodimer (FVII-
35 024) than the monomer (FVII-025), and required cotransfection of PC5 for full processing. These FVII-024 data are the same as shown in Figure 2, but show a direct comparison between FVII-024 and FVII-025.

5 **[0064]** Figure 10 shows an scFc molecule comprising IFN- β as the biologically active moiety. The Figure also shows a Western blot which illustrates partial cleavage of the processable linker in the absence of PC5 cotransfection, but full cleavage with cotransfection.

10 **[0065]** Figure 11 shows Western blot analysis (Fc western) of FVIIIFc species following transient transfection of HEK 293 cells and protein A pulldown.

15 **DETAILED DESCRIPTION OF THE INVENTION**

20 **[0066]** The present invention advances the art by providing molecules, e.g., nucleic acid and polypeptide molecules, comprising (i) at least one biologically active moiety (e.g., an antigen binding site or binding domain, receptor binding portion of a ligand, ligand binding portion of a receptor, or moiety that modulates clotting) and (ii) at least one Fc region (i.e., single-chain Fc ("scFc") region) comprising a cleavable scFc (cscFc) linker. However, in contrast to scFc linkers of the prior art scFc molecules, the cscFc linkers of the instant invention which link the Fc moieties forming the scFc region are adjacent to at least one enzymatic cleavage site (e.g., that can be cleaved by an intracellular processing enzyme), resulting in a dimeric molecule comprising a two chain Fc region in which the cscFc linker is cleaved or substantially removed. This cleavage step can occur before the polypeptide is secreted by a cell, prior to administration to a subject, or in vivo after administration.

25 **[0067]** In one embodiment, the polypeptide is represented by the formula:

30 **A-F1-P1- L-P2-B-F2 (I)**

 in linear sequence from the amino to carboxy terminus wherein A is a biologically active moiety, F1 is a first Fc moiety or domain, P1 is an enzymatic cleavage site, L is an cscFc linker, P2 is an enzymatic cleavage site, B is a biologically active moiety, F2 is a second Fc moiety or domain and "-" represents a peptide bond.

35 Formula (I) comprises at least an A or B and optionally both. A and B, if both present, can be the same or different. A and B can each also be subunits or chains of a molecule, such that when both present and associated with each other, they form a functional and active molecule. Formula (I) comprises at least a P1 or P2 and

5 optionally both. PI and P2, if both present, can be the same or different. Formula (I) comprises at least a F1 and F2. F1 and F2, if both present, can be the same or different. Exemplary polypeptide are also shown by the schematic in Figure 1A):
 [0068] Exemplary polypeptides according to formula I include: A-F1-P1- L-P2-F2;
 F1-P1- L-P2-B-F2; A-F1-P1- L- F2; F1-P1- L- B-F2; A-F1- L-P2-F2; and F1- L-P2-
 10 B-F2.

[0069] In one embodiment, F1 and F2 each comprise a CH₂ and CH₃ moiety. In another embodiment, F1 and F2 dimerize to form an Fc region and where A and B are optionally present and are biologically active moieties.

[0070] In one embodiment, P 1 and P2 are both present and are recognized by the
 15 same or by different enzymes. In one embodiment, at least one of PI or P2 is an intracellular processing site which comprises a cluster of basic amino acid residues that are recognized by arginine kex2/furin enzymes. Such enzymes cleave immediately C-terminal to an arginine residue. In one embodiment, at least one of PI or P2 intracellular processing site is an enzymatic cleavage site, which is recognized
 20 by thrombin. In another embodiment, at least one site is a cleavage site which is cleaved in vivo, for example at a cleavage site recognized, e.g., by thrombin or Factor IXa or XIa. Exemplary FXIa cleavage sites include, e.g., TQSFNDFTR (SEQ ID NO:7) and SVSQTSLTR (SEQ ID NO:8). Exemplary thrombin cleavage sites include: DFLAEGGGVR (SEQ ID NO:9), TTKIKPR (SEQ ID NO: 10), and a
 25 sequence comprising or consisting of ALRPR (e.g., ALRPRVVGGA (SEQ ID NO: 11)). Other cleavage sites can readily be selected by one of skill in the art, based upon the teachings herein.

[0071] The subject polypeptides comprise at least one biologically active moiety (represented as A and B in Formula I). Such biologically active moieties may be
 30 fused to either or both Fc moieties present in the Fc region of a molecule of the invention. Such fusions can be made to the C-terminus or N-terminus or to both of an Fc moiety. Such fusions can be direct (e.g., by a peptide bond) by peptide linker (e.g., a polypeptide spacer which imparts flexibility to the molecule), chemical conjugation, or other art recognized methods.

[0072] In one embodiment, a cell expressing a construct encoding a polypeptide of
 35 the invention endogenously expresses an enzyme which processes the cscFc linker (L) resulting in a multimeric molecule comprising at least two polypeptide chains, e.g., a dimeric molecule comprising two polypeptide chains. In another embodiment, a cell

5 expressing a construct encoding a polypeptide of the invention expresses a heterologous enzyme (e.g. recombinantly expresses an enzyme) which processes, i.e., cleaves the cscFc linker at the cleavage site.

[0073] Expression of the polypeptides of the invention from a single contiguous genetic construct has numerous advantages over conventional protein expression
10 methods which involve the co-expression of two genes (one encoding a polypeptide chain comprising a first Fc domain and a separate, second gene encoding a polypeptide chain comprising a second Fc domain with disulfide bonds linking the two polypeptide chains). The potential problems associated with such conventional constructs include significant heterogeneity within the population of resulting
15 molecules, such that the desired molecule must be purified away from undesired molecules, inevitably resulting in a decline in total yield of the desired molecule. For example, misfolded Fc fusion proteins can be difficult to separate from properly folded, bivalent, Fc proteins since the only difference between the two is often a heterogeneous misfolding event. The subject polypeptides cannot undergo scrambling
20 of the protein domains because this construction does not fix the molecules in close proximity to each other during the folding process. In addition, removal of the extraneous linker sequence may also relieve any steric stress or hindrance that was present in the covalently linked form. However, cleavage of the cscFc linker allows for biologically active moieties fused to the amino terminus of each Fc moiety to have
25 a free N-terminus. This is particularly valuable in the case of biologically active moieties which are catalytically active, such as serine protease domains which require a free N-terminus to be catalytically active, or moieties or which might be sterically hindered by the presence of an uncleaved polypeptide linker.

[0074] Exemplary constructs of the invention are illustrated in the accompanying
30 Figures and sequence listing. In one embodiment, the invention pertains to a polypeptide having the structure as set forth in the Figures. In another embodiment, the invention pertains to a polypeptide having the sequence set forth in the accompanying sequence listing or to a nucleic acid molecule encoding such a polypeptide. In one embodiment, the invention pertains to a mature form of a
35 polypeptide having the sequence set forth in the accompanying sequence listing. It will be understood that these constructs and nucleic acid molecules encoding them can be used to improve hemostasis in a subject.

5 [0075] In order to provide a clear understanding of the specification and claims, the following definitions are provided below.

I. Definitions

10 [0076] As used herein, the term "protein" or "polypeptide" refers to a polymer of two or more of natural amino acids or non-natural amino acids.

[0077] The term "amino acid" includes alanine (Ala or A); arginine (Arg or R); asparagine (Asn or N); aspartic acid (Asp or D); cysteine (Cys or C); glutamine (Gln or Q); glutamic acid (Glu or E); glycine (Gly or G); histidine (His or H); isoleucine (Ile or I); leucine (Leu or L); lysine (Lys or K); methionine (Met or M); phenylalanine (Phe or F); proline (Pro or P); serine (Ser or S); threonine (Thr or T); tryptophan (Trp or W); tyrosine (Tyr or Y); and valine (Val or V). Non-traditional amino acids are also within the scope of the invention and include norleucine, ornithine, norvaline, homoserine, and other amino acid residue analogues such as those described in Ellman *et al.* Meth. Enzym. 202:301-336 (1991). To generate such non-naturally occurring amino acid residues, the procedures of Noren *et al.* Science 244: 182 (1989) and Ellman *et al.*, supra, can be used. Briefly, these procedures involve chemically activating a suppressor tRNA with a non-naturally occurring amino acid residue followed by in vitro transcription and translation of the RNA. Introduction of the non-traditional amino acid can also be achieved using peptide chemistries known in the art.

[0078] An "amino acid substitution" refers to the replacement of at least one existing amino acid residue in a predetermined amino acid sequence (an amino acid sequence of a starting polypeptide) with a second, different "replacement" amino acid residue. An "amino acid insertion" refers to the incorporation of at least one additional amino acid into a predetermined amino acid sequence. While the insertion will usually consist of the insertion of one or two amino acid residues, the present larger "peptide insertions", can be made, e.g. insertion of about three to about five or even up to about ten, fifteen, or twenty amino acid residues. The inserted residue(s) may be naturally occurring or non-naturally occurring as disclosed above. An "amino acid deletion" refers to the removal of at least one amino acid residue from a predetermined amino acid sequence.

[0079] Polypeptides may be either monomers or multimers. For example, in one embodiment, a protein of the invention is a dimer. In one embodiment, the

5 dimers of the invention are homodimers, comprising two identical monomeric subunits or polypeptides (e.g., two identical Fc moieties or two identical biologically active moieties). In another embodiment, the dimers of the invention are heterodimers, comprising two non-identical monomeric subunits or polypeptides (e.g., comprising two different biologically active moieties, one biologically active moiety
10 only, and/or an Fc region comprising non-identical Fc moieties which dimerize to form a heteromeric Fc region). Polypeptide dimmers may comprise two polypeptide chains or may consist of one polypeptide chains.

[0080] As used herein, the term "scFc polypeptide" refers to a polypeptide comprising a single-chain Fc (scFc) region. The polypeptides of the invention
15 comprise cscFc linkers (L of formula I) linking the Fc moieties of the scFc region. The cscFc linker is interposed between the Fc moieties that comprise the scFc region and is flanked by at least one enzymatic cleavage site, e.g., an intracellular enzymatic processing site. As used herein, the term scFc polypeptide refers to a polypeptide comprising a single-chain Fc (scFc) region. Fc moieties of the polypeptide can be
20 linked either directly or indirectly. If the cscFc linker connects two Fc moieties contiguously in the linear polypeptide sequence, it is a "direct" linkage. In contrast, the cscFc linkers may link the first Fc moiety to a different moiety (e.g., a binding moiety, a targeting moiety, or a functional moiety) which is, in turn, linked to the second Fc moiety, thereby forming an indirect linkage.

25 [0081] In one embodiment a polypeptide of the invention comprises additional modifications. Exemplary modifications are described in more detail below. For example, in one embodiment a polypeptide may be modified to add a functional moiety (e.g., PEG, a drug, or a label).

[0082] A "biologically active moiety" refers to a molecule, portion, fragment,
30 derivative or component of a molecule capable of one or more of performing a function, an action or a reaction in a biological context. A biologically active moiety may comprise a complete protein or biologically active portion thereof. For example, the term "biologically active moiety" includes active and functional molecules, binding domains of molecules which bind to components of a biological system (e.g.,
35 proteins in sera or on the surface of cells or in cellular matrix) and which binding results in a biological effect (e.g., as measured by a change in the active moiety and/or the component to which it binds (e.g., a cleavage of the active moiety and/or the component to which it binds, the transmission of a signal, or the augmentation or

5 inhibition of a biological response in a cell or in a subject)). Exemplary biologically active moieties may comprise natural molecules, e.g., a component of the clotting cascade, an antigen binding site or fragment of an antibody molecule (e.g., F(ab) or scFv) (e.g., to impart, induce or block a biological response), a ligand binding portion of a receptor or a receptor binding portion of a ligand, or a catalytic domain. In one
10 embodiment, a biologically active moiety comprises the mature form of a protein. In another embodiment, a biologically active moiety comprises a full length protein or a portion of a full length protein which retains biological activity.

[0083] As used herein, the term "biologically active moiety" includes, for example, a first moiety which may not have activity when present alone in monomeric form, but
15 which has a biological activity when paired with a second moiety in the context of a construct of the invention. In some such embodiments, the first moiety may be represented by A (or B) in Formula I and the second moiety may be represented by B (or A). When A and B and then associate in the polypeptide, they form a functional molecule (examples include e.g., the light and heavy chains of FVII or e.g., the
20 subunits of FSH).

[0084] The term "biologically active moiety" includes moieties which require enzymatic activity in order to be fully biologically active. For example, clotting factors, whether in their zymogen form or in their fully activated form (e.g., FVII, FVIIa, FFX, FFXa, FX or FXa), are embraced by the term "biologically active
25 moiety".

[0085] The term "ligand binding domain" as used herein refers to a native receptor (e.g., cell surface receptor) or a region or derivative thereof retaining at least a qualitative ligand binding ability, and preferably the biological activity of the corresponding native receptor. The term "receptor binding domain" as used herein
30 refers to a native ligand or region or derivative thereof retaining at least a qualitative receptor binding ability, and preferably the biological activity of the corresponding native ligand.

[0086] In one embodiment, the polypeptides of the invention comprise at least one biologically active moiety which binds to a molecule targeted for reduction or
35 elimination, e.g., a cell surface antigen or a soluble antigen. In one embodiment, the biologically active moiety comprises or consists of an antigen binding site (e.g., comprising a variable heavy chain sequence and variable light chain sequence or six CDRs from an antibody placed into alternative framework regions (e.g., human

5 framework regions optionally comprising one or more amino acid substitutions). In another preferred embodiment, a biologically active moiety comprises a biologically active portion of a component of the clotting cascade.

[0087] The term "specificity" includes the number of potential binding sites which specifically bind (e.g., immunoreact with) a given target. A polypeptide may be
10 monospecific and contain one or more binding sites which specifically bind the same target (e.g., the same epitope) or the polypeptide may be multispecific and contain two or more binding sites which specifically bind different regions of the same target (e.g., different epitopes) or different targets.

[0088] As used herein the term "valency" refers to the number of biologically active
15 moieties (e.g., binding domains) in a polypeptide or protein. When a polypeptide comprises more than one biologically active moiety, each binding domain may specifically bind the same or different molecules (e.g., may bind to different ligands or different antigens, or different epitopes on the same antigen). In one embodiment, the polypeptides of the invention are monovalent. In another embodiment, the
20 polypeptides of the invention are multivalent (e.g., bivalent).

[0089] As used herein, the term "polypeptide linkers" refers to a peptide or polypeptide sequence (e.g., a synthetic peptide or polypeptide sequence) which connects two domains in a linear amino acid sequence of a polypeptide chain. Preferred linkers include, e.g., gly-ser polypeptide linkers. The polypeptides of
25 invention are encoded by nucleic acid molecules comprising a nucleotide sequence that encodes polypeptide linkers linking the two Fc moieties which make up the construct, either directly or indirectly. These linkers are referred to herein as "cscFc linkers". Rather than linking two Fc moieties contiguously in the linear polypeptide sequence, the cscFc linker may, for example, link the first Fc moiety to a different
30 moiety (e.g., a biologically active moiety, targeting moiety, or functional moiety) which is, in turn, linked to the second Fc moiety. These cscFc linker (L) result in the formation of a single chain genetic construct. However, the polypeptides also comprise enzymatic cleavage sites which result in the cscFc linker being cleaved and, in one embodiment, substantially excised (e.g., during processing by a cell). Thus,
35 the processed molecule is a dimeric molecule comprising at least two amino acid chains and substantially lacking extraneous linker amino acid sequences. In some embodiments, all or substantially all of the linker is excised, while in some

5 embodiments, a portion of the cleavage site may remain, e.g., four arginines of the RRRR cleavage site. In another embodiment, the linker is cleaved at one site.

 [0090] In another embodiment, another type of polypeptide linker, herein referred to as a "spacer" may be used to connect different moieties, e.g., a biologically active moiety to an Fc moiety. This type of polypeptide linkers may provide flexibility to
10 the polypeptide molecule. Spacers are not typically cleaved; however such cleavage may be desirable. Exemplary positions of spacers are shown in the accompanying drawings.

 [0091] As used herein, the term "gly-ser polypeptide linker" refers to a polypeptide linker that consists of glycine and serine residues. An exemplary gly/ser polypeptide
15 linker comprises the amino acid sequence $(\text{Gly}_4 \text{Ser})_n$ (SEQ ID NO:4). Another exemplary gly/ser polypeptide linker comprises the amino acid sequence $\text{S}(\text{Gly}_4 \text{Ser})_n$.

 [0092] In one embodiment, $n=1$. In one embodiment, $n=2$. In another embodiment, $n=3$, i.e., $(\text{Gly}_4 \text{Ser})_3$. In another embodiment, $n=4$, i.e., $(\text{Gly}_4 \text{Ser})_4$ (SEQ ID NO:6). In another embodiment, $n=5$. In yet another embodiment, $n=6$. In another
20 embodiment, $n=7$. In yet another embodiment, $n=8$. In another embodiment, $n=9$. In yet another embodiment, $n=10$. Another exemplary gly/ser polypeptide linker comprises the amino acid sequence $\text{Ser}(\text{Gly}_4 \text{Ser})_n$ (SEQ ID NO:26). In one embodiment, $n=1$. In one embodiment, $n=2$. In a preferred embodiment, $n=3$. In another embodiment, $n=4$. In another embodiment, $n=5$. In yet another embodiment,
25 $n=6$.

 [0093] A polypeptide or amino acid sequence "derived from" a designated polypeptide or protein refers to the origin of the polypeptide. Preferably, the polypeptide or amino acid sequence which is derived from a particular sequence has an amino acid sequence that is essentially identical to that sequence or a portion
30 thereof, wherein the portion consists of at least 10-20 amino acids, preferably at least 20-30 amino acids, more preferably at least 30-50 amino acids, or which is otherwise identifiable to one of ordinary skill in the art as having its origin in the sequence.

 [0094] Polypeptides derived from another peptide may have one or more mutations relative to the starting polypeptide, e.g., one or more amino acid residues which have
35 been substituted with another amino acid residue or which has one or more amino acid residue insertions or deletions. Preferably, the polypeptide comprises an amino acid sequence which is not naturally occurring. Such variants necessarily have less than 100% sequence identity or similarity with the starting antibody. In a preferred

5 embodiment, the variant will have an amino acid sequence from about 75% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of the starting polypeptide, more preferably from about 80% to less than 100%, more preferably from about 85% to less than 100%, more preferably from about 90% to less than 100% (e.g., 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%) and most
10 preferably from about 95% to less than 100%, e.g., over the length of the variant molecule. In one embodiment, there is one amino acid difference between a starting polypeptide sequence and the sequence derived therefrom. Identity or similarity with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical (*i. e.* same residue) with the starting amino
15 acid residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity.

[0095] Preferred polypeptides of the invention comprise an amino acid sequence (e.g., at least one Fc moiety or domain) derived from a human immunoglobulin sequence. However, polypeptides may comprise one or more amino acids from
20 another mammalian species. For example, a primate Fc domain or binding site may be included in the subject polypeptides. Alternatively, one or more amino acids derived from a non-human species may be present in a polypeptide. Preferred polypeptides of the invention are not immunogenic.

[0096] It will also be understood by one of ordinary skill in the art that the
25 polypeptides of the invention may be altered such that they vary in amino acid sequence from the naturally occurring or native polypeptides from which they were derived, while retaining the desirable activity of the native polypeptides. For example, nucleotide or amino acid substitutions leading to conservative substitutions or changes at "non-essential" amino acid residues may be made. An isolated nucleic
30 acid molecule encoding a non-natural variant of a polypeptide derived from an immunoglobulin (e.g., an Fc domain, moiety, or antigen binding site) can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of the immunoglobulin such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein.
35 Mutations may be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis.

[0097] The polypeptides of the invention may comprise conservative amino acid substitutions at one or more amino acid residues, e.g., at essential or non-essential

5 amino acid residues. Thus, a nonessential amino acid residue in a polypeptide may be replaced with another amino acid residue from the same side chain family. In another embodiment, a string of amino acids can be replaced with a structurally similar string that differs in order and/or composition of side chain family members. Alternatively, in another embodiment, mutations may be introduced randomly along all or part of a
10 coding sequence, such as by saturation mutagenesis, and the resultant mutants can be incorporated into polypeptides of the invention and screened for their ability to bind to the desired target.

[0098] In the context of polypeptides, a "linear sequence" or a "sequence" is the order of amino acids in a polypeptide in an amino to carboxyl terminal direction in which
15 residues that flank or are adjacent to each other in the sequence are contiguous in the primary structure of the polypeptide, i.e., are linked via a peptide bond.

[0099] As used herein, the terms "linked," "fused", or "fusion" refer to linkage via a peptide bond, chemical conjugation or other means. The terms "genetically fused," "genetically linked" or "genetic fusion" are used interchangeably and refer to the co-
20 linear, covalent linkage or attachment of two or more proteins, polypeptides, or fragments thereof via their individual peptide backbones, through genetic expression of a single polynucleotide molecule encoding those proteins, polypeptides, or fragments. Such genetic fusion results in the expression of a single contiguous genetic sequence. In one embodiment, moieties of an scFc polypeptide are genetically fused.
25 Preferred genetic fusions are in frame, i.e., two or more open reading frames (ORFs) are fused to form a continuous longer ORF, in a manner that maintains the correct reading frame of the original ORFs. Thus, the resulting recombinant fusion protein is a single polypeptide containing two or more protein segments that correspond to polypeptides encoded by the original ORFs (which segments are not normally so
30 joined in nature). In this case, the single polypeptide is cleaved during processing to yield dimeric molecules comprising two polypeptide chains.

[00100] As used herein, the term "Fc region" shall be defined as the portion of a polypeptide which corresponds to the Fc region of native immunoglobulin, i.e., as formed by the dimeric association of the respective Fc domains) of its two heavy
35 chains. A native Fc region is homodimeric and comprises two polypeptide chains. 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 or moieties genetically linked within a single polypeptide chain (i.e.,

5 encoded in a single contiguous genetic sequence) wherein the Fc domains or moieties of the single polypeptide chain dimerize to form an Fc region.

[00101] As used herein, the term "Fc domain" refers to the portion of a single immunoglobulin 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
10 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.

[00102] As used herein, the term "Fc domain portion" or "Fc moiety" includes an amino acid sequence of an Fc domain or derived from an Fc domain. In certain
15 embodiments, an Fc moiety comprises at least one of: a hinge (e.g., upper, middle, and/or lower hinge region) domain, a CH2 domain, a CH3 domain, a CH4 domain, or a variant, portion, or fragment thereof. In other embodiments, an Fc moiety comprises a complete Fc domain (i.e., a hinge domain, a CH2 domain, and a CH3 domain). In one embodiment, a Fc moiety comprises a hinge domain (or portion
20 thereof) fused to a CH3 domain (or portion thereof). In another embodiment, an Fc moiety comprises a CH2 domain (or portion thereof) fused to a CH3 domain (or portion thereof). In another embodiment, an Fc moiety consists of a CH3 domain or portion thereof. In another embodiment, an Fc moiety consists of a hinge domain (or portion thereof) and a CH3 domain (or portion thereof). In another embodiment, a Fc
25 moiety consists of a CH2 domain (or portion thereof) and a CH3 domain. In another embodiment, a Fc moiety consists of a hinge domain (or portion thereof) and a CH2 domain (or portion thereof). In one embodiment, an Fc moiety lacks at least a portion of a CH2 domain (e.g., all or part of a CH2 domain).

[00103] In one embodiment, an Fc moiety of the invention comprises at least
30 the portion of an Fc molecule known in the art to be required for FcRn binding, referred to herein as a neonatal receptor (FcRn) binding partner. The skilled artisan will understand that portions of an immunoglobulin constant region for use in the chimeric protein of the invention can include mutants or analogs thereof, or can include chemically modified immunoglobulin constant regions (e.g. pegylated), or
35 fragments thereof (see, e.g., Aslam and Dent 1998, Bioconjugation: Protein Coupling Techniques For the Biomedical Sciences Macmilan Reference, London). In one instance, a mutant can provide for enhanced binding of an FcRn binding partner for the FcRn. Also contemplated for use in the chimeric protein of the invention are

- 5 peptide mimetics of at least a portion of an immunoglobulin constant region, e.g., a peptide mimetic of an Fc fragment or a peptide mimetic of an FcRn binding partner. In one embodiment, the peptide mimetic is identified using phage display or via chemical library screening (see, e.g., McCafferty et al. 1990, Nature 348:552, Kang et al. 1991, Proc. Natl. Acad. Sci. USA 88:4363; EP 0 589 877 B1). In another
- 10 embodiment, an Fc region of the invention (an scFc region) comprises at least the portion of an Fc molecule known in the art to be required for FcγR binding.

- [00104] In one embodiment, an Fc region of the invention (an scFc region) comprises at least the portion of an Fc molecule known in the art to be required for Protein A binding. In one embodiment, an Fc region of the invention (an scFc region)
- 15 comprises at least the portion of an Fc molecule known in the art to be required for protein G binding.

- [00105] As set forth herein, it will be understood by one of ordinary skill in the art that an Fc domain may also be modified such that it varies in other effector functions from the native Fc domain of a naturally occurring immunoglobulin
- 20 molecule. In certain exemplary embodiments, the Fc moiety retains an effector function (e.g., FcγR binding).

- [00106] The Fc domains or moieties of a polypeptide of the invention may be derived from different immunoglobulin molecules. For example, an Fc domain or moiety of a polypeptide may comprise a CH2 and/or CH3 domain derived from an
- 25 IgG1 molecule and a hinge region derived from an IgG3 molecule. In another example, an Fc domain or moiety can comprise a chimeric hinge region derived, in part, from an IgG1 molecule and, in part, from an IgG3 molecule. In another example, an Fc domain or moiety can comprise a chimeric hinge derived, in part, from an IgG1 molecule and, in part, from an IgG4 molecule.

- 30 [00107] Amino acid positions in a heavy chain constant region, including amino acid positions in the CH1, hinge, CH2, and CH3 domains, are numbered herein according to the EU index numbering system (see Kabat *et al.*, in "Sequences of Proteins of Immunological Interest", U.S. Dept. Health and Human Services, 5th edition, 1991). In contrast, amino acid positions in a light chain constant region (*e.g.*
- 35 CL domains) are numbered herein according to the Kabat index numbering system (see Kabat *et al.*, *ibid.*).

5 **[00108]** As used herein, the term "**v_H** domain" includes the amino terminal variable domain of an immunoglobulin heavy chain, and the term "**v_L** domain" includes the amino terminal variable domain of an immunoglobulin light chain according to the Kabat index numbering system.

10 **[00109]** As used herein, the term "CHI domain" includes the first (most amino terminal) constant region domain of an immunoglobulin heavy chain that extends, *e.g.*, from about EU positions 118-215. The CHI domain is adjacent to the **v_H** domain and amino terminal to the hinge region of an immunoglobulin heavy chain molecule, and does not form a part of the Fc region of an immunoglobulin heavy chain. In one embodiment, a polypeptide of the invention comprises a CHI domain
15 derived from an immunoglobulin heavy chain molecule (*e.g.*, a human IgG1 or IgG4 molecule).

20 **[00110]** As used herein, the term "hinge region" includes the portion of a heavy chain molecule that joins the CHI domain to the CH2 domain. This hinge region comprises approximately 25 residues and is flexible, thus allowing the two N-terminal antigen binding regions to move independently. Hinge regions can be subdivided into three distinct domains: upper, middle, and lower hinge domains (Roux et al. J. Immunol. 1998, 161:4083).

25 **[00111]** As used herein, the term "CH2 domain" includes the portion of a heavy chain immunoglobulin molecule that extends, *e.g.*, from about EU positions 231-340. The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. In one embodiment, a polypeptide of the invention comprises a CH2 domain derived from an IgG1 molecule (*e.g.* a human IgG1 molecule). In another embodiment, a polypeptide of the invention
30 comprises a CH2 domain derived from an IgG4 molecule (*e.g.*, a human IgG4 molecule). In an exemplary embodiment, a polypeptide of the invention comprises a CH2 domain (EU positions 231-340), or a portion thereof.

35 **[00112]** As used herein, the term "CH3 domain" includes the portion of a heavy chain immunoglobulin molecule that extends approximately 110 residues from N-terminus of the CH2 domain, *e.g.*, from about position 341-446b (EU numbering system). The CH3 domain typically forms the C-terminal portion of the antibody. In some immunoglobulins, however, additional domains may extend from CH3 domain to form the C-terminal portion of the molecule (*e.g.* the CH4 domain in the μ chain of

5 IgM and the ϵ chain of IgE). In one embodiment, a polypeptide of the invention comprises a CH3 domain derived from an IgG1 molecule (*e.g.*, a human IgG1 molecule). In another embodiment, a polypeptide of the invention comprises a CH3 domain derived from an IgG4 molecule (*e.g.*, a human IgG4 molecule).

[00113] As used herein, the term "CL domain" includes the first (most amino
10 terminal) constant region domain of an immunoglobulin light chain that extends, *e.g.* from about Kabat position 107A-216. The CL domain is adjacent to the ν L domain. In one embodiment, a polypeptide of the invention comprises a CL domain derived from a kappa light chain (*e.g.*, a human kappa light chain).

[00114] Fc moieties for use in the polypeptides of the invention can be
15 modified at art-recognized positions to alter, *e.g.*, increase or decrease effector function. As used herein, the term "effector function" refers to the functional ability of the Fc region or portion thereof to bind proteins and/or cells of the immune system and mediate various biological effects. Effector functions may be antigen-dependent or antigen-independent. A decrease in effector function refers to a decrease in one or
20 more effector functions, while maintaining the antigen binding activity of the variable region of the antibody (or fragment thereof). Increase or decreases in effector function, *e.g.*, Fc binding to an Fc receptor or complement protein, can be expressed in terms of fold change (*e.g.*, changed by 1-fold, 2-fold, and the like) and can be calculated based on, *e.g.*, the percent changes in binding activity determined using
25 assays the are well-known in the art.

[00115] Fc moieties for use in the polypeptides of the invention can be modified at art-recognized positions to alter, *e.g.*, increase or decrease half life. 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
30 quantity administered to a subject to be cleared from the circulation and/or other tissues in the animal.

[00116] As used herein, the term "antigen binding site" or "antigen binding domain" includes a site that specifically binds (immunoreacts with) an antigen such as a cell surface or soluble antigen). In one embodiment, the binding site includes an
35 immunoglobulin heavy chain and light chain variable region and the binding site formed by these variable regions determines the specificity of the antibody. An antigen binding site is formed by variable regions (VH and VL domains) that vary from one polypeptide to another.

5 **[00117]** In certain embodiments, the polypeptides of the invention comprise at least two antigen binding domains (*e.g.*, within the same polypeptide (*e.g.*, at both the N- and C-terminus of a single polypeptide) or linked to each component binding polypeptide of a multimeric binding protein of the invention) that provide for the association of the polypeptide with the selected antigen. The antigen binding domains
10 need not be derived from the same immunoglobulin molecule. In this regard, the variable region may or may not be derived from any type of animal that can be induced to mount a humoral response and generate immunoglobulins against the desired antigen. As such, the variable region may be, for example, of mammalian origin *e.g.*, may be human, murine, non-human primate (such as cynomolgus
15 monkeys, macaques, etc.), lupine, camelid (*e.g.*, from camels, llamas and related species).

[00118] The term "antibody variant" or "modified antibody" includes an antibody which does not occur in nature and which has an amino acid sequence or amino acid side chain chemistry which differs from that of a naturally-derived
20 antibody by at least one amino acid or amino acid modification as described herein or a non-naturally occurring molecule comprising at least one antigen-binding site. As used herein, the term "antibody variant" includes synthetic forms of antibodies which are altered such that they are not naturally occurring, *e.g.*, antibodies that comprise at least two heavy chain portions but not two complete heavy chains (such as, domain
25 deleted antibodies or minibodies); multispecific forms of antibodies (*e.g.*, bispecific, trispecific, etc.) altered to bind to two or more different antigens or to different epitopes on a single antigen); heavy chain molecules joined to scFv molecules; single-chain antibodies; diabodies; triabodies; and antibodies with altered effector function and the like.

30 **[00119]** As used herein the term "scFv molecule" includes binding molecules which consist of one light chain variable domain (VL) or portion thereof, and one heavy chain variable domain (VH) or portion thereof, wherein each variable domain (or portion thereof) is derived from the same or different antibodies. scFv molecules preferably comprise an scFv linker interposed between the VH domain and the VL
35 domain. ScFv molecules are known in the art and are described, *e.g.*, in US patent 5,892,019, Ho et al. 1989. *Gene* 77:51; Bird et al. 1988 *Science* 242:423; Pantoliano et al. 1991. *Biochemistry* 30:10117; Milenic et al. 1991. *Cancer Research* 51:6363; Takkinen et al. 1991. *Protein Engineering* 4:837.

5 **[00120]** A "scFv linker" as used herein refers to a moiety interposed between the VL and VH domains of the scFv. scFv linkers preferably maintain the scFv molecule in a antigen binding conformation. In one embodiment, a scFv linker comprises or consists of an scFv linker peptide. In certain embodiments, a scFv linker peptide comprises or consists of a gly-ser polypeptide linker. In other embodiments, a
10 scFv linker comprises a disulfide bond.

[00121] The term "glycosylation" refers to the covalent linking of one or more carbohydrates to a polypeptide. Typically, glycosylation is a posttranslational event which can occur within the intracellular milieu of a cell or extract therefrom. The term glycosylation includes, for example, N-linked glycosylation (where one or more
15 sugars are linked to an asparagine residue) and/or O-linked glycosylation (where one or more sugars are linked to an amino acid residue having a hydroxyl group (*e.g.*, serine or threonine). In one embodiment, a molecule of the invention is glycosylated. In another embodiment, a molecule of the invention is aglycosylated. In yet another embodiment, a molecule of the invention has reduced glycosylation as compared to
20 that in a wild type Fc region.

[00122] As used herein the term "disulfide bond" includes the covalent bond formed between two sulfur atoms.

[00123] As used herein the term "moiety" refers to a component part or constituent of a chimeric polypeptide.

25 **[00124]** The term "functional moiety" includes moieties which, preferably, add a desirable function to the polypeptide. Preferably, the function is added without significantly altering an intrinsic desirable activity of the polypeptide, *e.g.*, clotting activity, solubility, or half life of the molecule. A polypeptide of the invention may comprise one or more functional moieties, which may be the same or different.

30 Examples of useful functional moieties include, but are not limited to, a detectable moiety, an functional moiety, a drug moiety, an affinity moiety, and a blocking moiety (*e.g.*, which sterically blocks binding to an enzyme or which blocks the ability to bind to a particular receptor). Functional moieties may be linked to polypeptides using methods known in the art, *e.g.*, via cleavable or non-cleavable linking moieties.

35

[00125] As used herein, the term "prodrug" refers to a precursor or derivative form of a pharmaceutically active agent that is less active, reactive or prone to side effects as compared to the parent drug and is capable of being enzymatically activated

5 or otherwise converted into a more active form *in vivo*. In one embodiment, a prodrug of the invention is a polypeptide which comprises an uncleaved or unprocessed scFc region which, upon administration or prior to administration to a subject is cleaved to form a dimeric molecule comprising at least two polypeptide chains.

10 [00126] The term "vector" or "expression vector" is used herein to mean vectors used in accordance with the present invention as a vehicle for introducing into and expressing a desired polynucleotide in a cell. As known to those skilled in the art, such vectors may easily be selected from the group consisting of plasmids, phages, viruses and retroviruses. In general, vectors compatible with the instant invention will
15 comprise a selection marker, appropriate restriction sites to facilitate cloning of the desired gene and the ability to enter and/or replicate in eukaryotic or prokaryotic cells.

[00127] The constructs of the invention can be expressed in a single plasmid, avoiding the need for multiple plasmids. Numerous expression vector systems may be employed. For example, one class of vector utilizes DNA elements which are
20 derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MOMLV) or SV40 virus. Additionally, cells which have integrated the DNA into their chromosomes may be selected by introducing one or more markers which allow selection of transfected host cells. The marker may provide for prototrophy to an
25 auxotrophic host, biocide resistance (e.g., antibiotics) or resistance to heavy metals such as copper. The selectable marker gene can either be directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation. In one embodiment, an inducible expression system can be employed. Additional elements may also be needed for optimal synthesis of mRNA. These elements may
30 include signal sequences, splice signals, as well as transcriptional promoters, enhancers, and termination signals. In one embodiment, a secretion signal, e.g., any one of several well characterized bacterial leader peptides (e.g., pelB, phoA, or ompA), can be fused in-frame to the N terminus of a polypeptide of the invention to obtain optimal secretion of the polypeptide. (Lei *et al.* (1988), *Nature*, 331:543; Better
35 *et al.* (1988) *Science*, 240:1041; Mullinax *et al.*, (1990). *PNAS*, 87:8095).

[00128] The term "host cell" refers to a cell that has been transformed with a vector constructed using recombinant DNA techniques and encoding at least one heterologous gene. In descriptions of processes for isolation of proteins from

5 recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of protein unless it is clearly specified otherwise. In other words, recovery of protein from the "cells" may mean either from spun down whole cells, or from the cell culture containing both the medium and the suspended cells. The host cell line used for protein expression is most preferably of mammalian origin; those skilled in the art are credited with ability to preferentially determine particular host cell lines which are best suited for the desired gene product to be expressed therein. Exemplary host cell lines include, but are not limited to, DG44 and DUXB1 1 (Chinese Hamster Ovary lines, DHFR minus), HELA (human cervical carcinoma), CVI (monkey kidney line), COS (a derivative of CVI with SV40 T antigen), R1610 (Chinese hamster fibroblast) BALBC/3T3 (mouse fibroblast), HAK (hamster kidney line), SP2/0 (mouse myeloma), P3x63-Ag3.653 (mouse myeloma), BFA-lclBPT (bovine endothelial cells), RAJI (human lymphocyte) and 293 (human kidney). Host cell lines are typically available from commercial services, the American Tissue Culture Collection or from published literature. The polypeptides of the invention can also be expressed in non-mammalian cells such as bacteria or yeast or plant cells. In this regard it will be appreciated that various unicellular non-mammalian microorganisms such as bacteria can also be transformed; *i.e.* those capable of being grown in cultures or fermentation. Bacteria, which are susceptible to transformation, include members of the enterobacteriaceae, such as strains of *Escherichia coli* or 25 *Salmonella*; *Bacillaceae*, such as *Bacillus subtilis*; *Pneumococcus*; *Streptococcus*, and *Haemophilus influenzae*. It will further be appreciated that, when expressed in bacteria, the polypeptides typically become part of inclusion bodies. The polypeptides must be isolated, purified and then assembled into functional molecules.

[00129] In addition to prokaryotes, eukaryotic microbes may also be used. 30 *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among eukaryotic microorganisms although a number of other strains are commonly available including *Pichia pastoris*. For expression in *Saccharomyces*, the plasmid YRp7, for example, (Stinchcomb *et al.*, (1979), *Nature*, 282:39; Kingsman *et al.*, (1979), *Gene*, 7:141; Tschemper *et al.*, (1980), *Gene*, 10:157) is commonly used. This plasmid 35 already contains the TRP1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, (1977), *Genetics*, 85:12). The presence of the *trp1* lesion as a characteristic of the

5 yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

[00130] As used herein the term "endogenous" refers to molecules (e.g. nucleic acid and/or protein molecules) that are naturally present in a cell. In contrast, the term "exogenous" or "heterologous" refers to such molecules that are not normally found in a
10 given context, e.g., in a cell or in a polypeptide. For example, an exogenous or heterologous molecule may be introduced into a cell and is present after manipulation of the cell, (e.g., by transfection or other forms of genetic engineering) or a heterologous amino acid sequence may be present in a protein in which it is not naturally found

[00131] As used herein, the term "cleavage site" or "enzymatic cleavage site"
15 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 is one that is activated during the clotting cascade, such that cleavage of such sites occurs at the site of clot formation. Exemplary such sites include those recognized by thrombin, Factor XIa or Factor Xa. Exemplary FXIa
20 cleavage sites include, e.g, TQSFNDFTR and SVSQTSKLTR. Exemplary thrombin cleavage sites include, e.g, DFLAEGGGVR, TTKIKPR, LVPRG (SEQ ID NO:35) and a sequence comprising or consisting of ALRPR (e.g. ALRPRVVGGA). Other useful cleavage sites are known in the art.

[00132] As used herein, the term "processing site" or "intracellular processing
25 site" refers to a type of enzymatic cleavage site in a polypeptide which is the 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.

30 [00133] One example of processing sites includes sites 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 cleaves many proteins on the carboxyterminal side of the sequence motif Arg-[any residue]-(Lys or Arg)-Arg. As used herein the "furin" family of
35 enzymes includes, e.g., furin, PC2, PC1/PC3, PC4, PACE4, PC5/PC6, and LPC/PC7/PC8/SPC7. Other useful processing sites are known in the art.

[00134] A "chimeric protein" or "fusion protein", as used herein, refers to any protein comprised of a first amino acid sequence derived from a first source, bonded,

5 covalently or non-covalently, to a second amino acid sequence derived from a second source, wherein the first and second source are not the same. A first source and a second source that are not the same can include two different biological entities, or two different proteins from the same biological entity, or a biological entity and a non-biological entity. A chimeric protein can include for example, a protein derived
10 from at least 2 different biological sources. A biological source can include any non-synthetically produced nucleic acid or amino acid sequence (e.g. a genomic or cDNA sequence, a plasmid or viral vector, a native virion or a mutant or analog, as further described herein, of any of the above). A synthetic source can include a protein or nucleic acid sequence produced chemically and not by a biological system (e.g. solid
15 phase synthesis of amino acid sequences). A chimeric protein can also include a protein derived from at least 2 different synthetic sources or a protein derived from at least one biological source and at least one synthetic source. A chimeric protein may also comprise a first amino acid sequence derived from a first source, covalently or non-covalently linked to a nucleic acid, derived from any source or a small organic or
20 inorganic molecule derived from any source. The chimeric protein may comprise a linker molecule between the first and second amino acid sequence or between the first amino acid sequence and the nucleic acid, or between the first amino acid sequence and the small organic or inorganic molecule. Examples of chimeric molecules include the scFc molecules of the invention.

25 **[00135]** As used herein, the term "clotting factor," refers to molecules, or analogs thereof, naturally occurring or recombinantly produced which prevent or decrease the duration of a bleeding episode in a subject. In other words, it means molecules which in their active form have pro-clotting activity, i.e., are responsible for the conversion of fibrinogen into a mesh of insoluble fibrin causing the blood to
30 coagulate or clot.

[00136] "Clotting activity", as used herein, means the ability to participate in a cascade of biochemical reactions that culminates in the formation of a fibrin clot and/or reduces the severity, duration or frequency of hemorrhage or bleeding episode.

[00137] "Hemostasis", as used herein, means the stopping or slowing of
35 bleeding or hemorrhage; or the stopping or slowing of blood flow through a blood vessel or body part.

5 **[00138]** "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
10 "Christmas disease") and hemophilia C (factor XI deficiency, mild bleeding tendency), 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 is a defect or deficiency in GPIb. GPIb, the receptor for vWF, can be defective and lead to lack of
15 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.

[00139] As used herein, the term "targeting moiety" refers to a molecule,
20 fragment thereof or a component of a polypeptide which localizes or directs the polypeptides of the invention to a desired site or cell. In one embodiment, a construct of the invention comprises a "targeting moiety" which enhances the activity of the polypeptide, e.g., by localizing the molecule to a desired site. Such a moiety may be, e.g., an antibody or variant thereof (e.g., an scFv) or a peptide. In another
25 embodiment, such a targeting moiety may be a polypeptide, a receptor binding portion of a ligand, or a ligand binding portion of a receptor which is linked to a polypeptide of the invention and binds to the desired target, e.g., on a cell or tissue. The targeting moiety may be genetically fused to a construct, chemically conjugated to the construct or linked to the construct via a peptide bond, e.g., a polypeptide spacer. For example,
30 targeting moieties may be attached to a construct of the invention by formation of a bond between the targeting moiety and an Fc moiety of a construct, where the targeting moiety comprises a first functional group and the Fc moiety comprises a second functional group, and where the first and second functional groups are capable of reacting with each other to form a chemical bond (see, e.g., U.S. patent 7381408).
35 In one embodiment, of the invention a targeting moiety binds to platelets as described in more detail herein.

[00140] As used herein, the phrase "subject that would benefit from administration of a polypeptide" includes subjects, such as mammalian subjects, that

- 5 would benefit from administration of polypeptides of the invention, e.g., for treatment of a disorder. For example, in one embodiment, the subject may benefit from reduction or elimination of a soluble or particulate molecule from the circulation or serum (e.g., a toxin or pathogen) or from reduction or elimination of a population of cells expressing the target (e.g., tumor cells). In another embodiment, a subject may
- 10 benefit from the biological activity of the imparted by the binding portion of the construct, e.g., as in the case of a polypeptide comprising a clotting factor. As discussed above, the polypeptide can be used in unconjugated form or can be chemically conjugated or linked, e.g., to a functional moiety, to form a modified polypeptide for administering to said subject.
- 15 **[00141]** 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. In another embodiment, administration of
- 20 a composition made according to the invention results in the prophylaxis of one or more symptoms associated with a disease or condition.

II. Molecules Comprisin2 Sin2le-Chain Fc ("scFc") Re2ions

- [00142]** In one embodiment, the invention provides for nucleic acid molecules encoding polypeptides comprising at least one genetically fused Fc region or portion thereof within a single polypeptide chain (i.e., polypeptides comprising a single-chain Fc (scFc) region). A nucleic acid molecule of the invention encodes a polypeptide in which the Fc moieties are genetically fused in a contiguous linear sequence of amino acids via a cscFc linker.
- 30 **[00143]** The invention also provides polypeptides specified by such nucleic acid molecules. In one embodiment, the invention provides unprocessed polypeptides in which at least two Fc moieties or domains (e.g., 2, 3, 4, 5, 6, or more Fc moieties or domains) within the same linear polypeptide chain that are capable of folding (e.g., intramolecularly or intermolecularly folding) to form one functional scFc region
- 35 which is linked by an Fc polypeptide linker. For example, in one preferred embodiment, a polypeptide of the invention is capable of binding, via its scFc region, to at least one Fc receptor (e.g., an FcRn, an FcγR receptor (e.g., FcγRIII), or a

5 complement protein (e.g. Clq)) in order to improve half life or trigger an immune effector function (e.g., antibody-dependent cytotoxicity (ADCC), phagocytosis, or complement-dependent cytotoxicity (CDCC) and/or to improve manufacturability).

[00144] In one embodiment, the invention pertains to processed (e.g., mature) polypeptides in which the at least one cleavage site adjacent to a cscFc polypeptide
10 linker has been cleaved such that the molecule is no longer a single polypeptide chain. The resulting processed polypeptide is comprised of at least two polypeptide chains (owing to cleavage at the enzymatic cleavage site(s) P1 and/or P2).

[00145] In one embodiment, such processed polypeptides comprise a biologically active moiety linked to the second Fc moiety (i.e., the second Fc moiety
15 when counting from the amino terminus to the carboxy terminus prior to cleavage of the polypeptide linker) which has a free amino terminus after cleavage of the polypeptide linker.

[00146] In one embodiment, a biologically active moiety attached to the N-terminus of the second Fc moiety comprises an antigen binding site (e.g., an scFv
20 molecule). In another embodiment, a biologically active moiety attached to the N-terminus of the second Fc moiety is catalytically active, e.g., has enzymatic activity. In another embodiment, a biologically active moiety attached to the N-terminus of the second Fc moiety is secreted by a cell as a zymogen requiring further enzymatic processing of the biologically active moiety in order to be fully activated, e.g., FVII,
25 IX, orX).

[00147] In one embodiment, the invention pertains to clotting factors which are secreted from cells in active or activated form without the need for further activation during processing. For example, Factor VII is generally produced recombinantly as a zymogen and require activation during manufacturing to produce the active form for
30 administration. In one embodiment, a polypeptide of the invention is secreted from the cell in which it is expressed in active form to improve manufacturability. As is set forth in more detail below, such clotting factors can be produced by expressing the light chain of a clotting factor and the heavy chain of a clotting factor separately in the context of an cscFc molecule. Activation of such a construct is delayed until late in
35 the secretory pathway during processing, e.g., when the protein colocalizes with active processing enzymes in the trans-Golgi apparatus.

5 [00148] A variety of polypeptides of alternative designs are within the scope of the invention. For example, in one embodiment, a nucleic acid molecule of the invention specifies a polypeptide represented by the formula:

A-F1-P1- L-P2-B-F2 (I)

[00149] in linear sequence from the amino to carboxy terminus wherein A is a
 10 biologically active moiety, F1 is a first Fc moiety or domain, PI is an enzymatic cleavage site, L is an cscFc linker, P2 is an enzymatic cleavage site B is a biologically active moiety, F2 is a second Fc moiety or domain and "-" represents a peptide bond. Formula (I) comprises at least an A or B and optionally both. A and B, if both present, can be the same or different. Formula (I) comprises at least a PI or P2 and optionally
 15 both. PI and P2, if both present, can be the same or different. Formula (I) comprises at least a F1 and F2. F1 and F2, if both present, can be the same or different.

[00150] Exemplary polypeptides according to formula I include: A-F1-P1- L- P2-F2; F1-PI- L-P2-B-F2; A-F1-P1- L- F2; F1-PI- L- B-F2; A-F1- L-P2-F2; and F1- L-P2-B-F2.

20 [00151] In one embodiment, F1 and F2 each comprise a CH2 and CH3 moiety.

A. Fc Moieties or Domains

[00152] Fc moieties useful as F1 and F2 for producing the polypeptides of the present invention may be obtained from a number of different sources. In preferred
 25 embodiments, an Fc moiety of the polypeptide is derived from a human immunoglobulin. It is understood, however, that the Fc moiety may be derived from an immunoglobulin 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 polypeptide Fc domain or portion thereof may be
 30 derived from any immunoglobulin class, including IgM, IgG, IgD, IgA and IgE, and any immunoglobulin isotype, including IgG1, IgG2, IgG3 and IgG4. In a preferred embodiment, the human isotype IgG (*e.g.*, IgG1) is used.

[00153] A variety of Fc moiety gene sequences (*e.g.* human constant region gene sequences) are available in the form of publicly accessible deposits. Constant
 35 region domains comprising an Fc moiety 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 Fc moiety sequences (*e.g.* hinge,

- 5 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.
- 10 **[00154]** Fc moiety sequences can be cloned, *e.g.*, using the polymerase chain reaction and primers which are selected to amplify the domain of interest. To clone an Fc moiety sequence 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.
- 15 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
- 20 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.
- 30 **[00155]** The polypeptides of the invention may comprise two or more Fc moieties (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more Fc moieties). These two or more Fc moieties can form a Fc region. In one embodiment, the Fc moieties may be of different types. In one embodiment, at least one Fc moiety present in the polypeptide comprises a hinge domain or portion thereof. In another embodiment, the polypeptide
- 35 of the invention comprises at least one Fc moiety which comprises at least one CH2 domain or portion thereof. In another embodiment, the polypeptide of the invention comprises at least one Fc moiety which comprises at least one CH3 domain or portion thereof. In another embodiment, the polypeptide of the invention comprises at least

5 one Fc moiety which comprises at least one CH4 domain or portion thereof. In another embodiment, the polypeptide of the invention comprises at least one Fc moiety which 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 another embodiment, the polypeptide of the invention comprises at least one Fc moiety which
10 comprises at least one CH2 domain or portion thereof and at least one CH3 domain or portion thereof (e.g, in the CH2-CH3 orientation). In another embodiment, the polypeptide of the invention comprises at least one Fc moiety comprising at least one hinge domain or portion thereof, at least one CH2 domain or portion thereof, and least one CH3 domain or portion thereof, for example in the orientation hinge-CH2-CH3,
15 hinge-CH3-CH2, or CH2-CH3-hinge.

[00156] In certain embodiments, the polypeptide comprises at least one complete Fc region derived from one or more immunoglobulin heavy chains (*e.g.* , an Fc domain including hinge, CH2, and CH3 domains, although these need not be derived from the same antibody). In other embodiments, the polypeptide comprises at
20 least two complete Fc regions derived from one or more immunoglobulin heavy chains. In preferred embodiments, the complete Fc moiety is derived from a human IgG immunoglobulin heavy chain (*e.g.*, human IgG1).

[00157] In another embodiment, a polypeptide of the invention comprises at least one Fc moiety comprising a complete CH3 domain (about amino acids 341-438
25 of an antibody Fc region according to EU numbering). In another embodiment, a polypeptide of the invention comprises at least one Fc moiety comprising a complete CH2 domain (about amino acids 231-340 of an antibody Fc region according to EU numbering). In another embodiment, a polypeptide of the invention comprises at least one Fc moiety comprising at least a CH3 domain, and at least one of a hinge region
30 (about amino acids 216-230 of an antibody Fc region according to EU numbering), and a CH2 domain. In one embodiment, a polypeptide of the invention comprises at least one Fc moiety comprising a hinge and a CH3 domain. In another embodiment, a polypeptide of the invention comprises at least one Fc moiety comprising a hinge, a CH₂, and a CH₃ domain. In one embodiment, an Fc moiety comprises or consists of
35 amino acids corresponding to EU numbers 221 to 447.

[00158] In preferred embodiments, the Fc moiety is derived from a human IgG immunoglobulin heavy chain (*e.g.*, human IgG1).

- 5 **[00159]** In another embodiment, a polypeptide of the invention comprises at least one Fc moiety comprising an FcRn binding partner. An FcRn binding partner is a molecule or portion thereof that can be specifically bound by the FcRn receptor with consequent active transport by the FcRn receptor of the FcRn binding partner. Specifically bound refers to two molecules forming a complex that is relatively stable
- 10 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 more preferably higher than 10^8 M^{-1} . If necessary, non-specific binding can be reduced without
- 15 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.
- 20 **[00160]** 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 immunoglobulin classes such as IgA, IgM, IgD, and IgE) at relatively low pH, actively transports the IgG transcellularly in a luminal to serosal
- 25 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
- 30 epithelium, vaginal surfaces, and biliary tree surfaces.
- [00161]** FcRn binding partners of 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
- 35 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

5 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 immunoglobulins or immunoglobulin 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.

[00162] The Fc region 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 γ_1) 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 wildtype proline substituted by alanine at position number 238. As an example, one 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 wildtype amino acids at the positions specified above. Mutations may be introduced singly into Fc giving rise to more than one hundred FcRn binding partners distinct from native Fc. Additionally, combinations of two, three, or more of these individual mutations may be introduced together, giving rise to hundreds more FcRn binding partners. Moreover, one of the FcRn binding partners of a construct of the invention may be mutated and the other FcRn binding partner not

5 mutated at all, or they both may be mutated but with different mutations. Any of the mutations described herein, including N297A, may be used to modify Fc, regardless of the biologically active molecule (e.g., EPO, IFN, Factor VII, Factor IX, T20).

[00163] Certain of the above mutations may confer new functionality upon the FcRn binding partner. For example, one embodiment incorporates N297A, removing
10 a highly conserved N-glycosylation site. The effect of this mutation is to reduce immunogenicity, thereby enhancing circulating half life of the FcRn binding partner, and to render the FcRn binding partner 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
15 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. Mutations believed to impart an increased affinity for FcRn include T256A, T307A,
20 E380A, and N434A (Shields et al. 2001, J. Biol. Chem. 276:6591).

[00164] 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
25 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.

30 [00165] In one embodiment, the FcRn binding partner is a polypeptide including the sequence PKNSSMISNTP (SEQ ID NO: 12) and optionally further including a sequence selected from HQSLGTQ (SEQ ID NO: 13), HQNLSDGK (SEQ ID NO: 14), HQNISDGK (SEQ ID NO: 24), or VISSHLGQ (SEQ ID NO: 25) (U.S. Pat. No. 5,739,277).

35 [00166] 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

5 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.

10 [00167] The constant region domains or portions thereof making up an Fc moiety of a polypeptide of the invention may be derived from different immunoglobulin molecules. For example, a polypeptide of the invention may comprise a CH2 domain or portion thereof derived from an IgG1 molecule and a CH3 region or portion thereof derived from an IgG3 molecule. In another example, a
15 polypeptide can comprise an Fc moiety comprising a hinge domain derived, in part, from an IgG1 molecule and, in part, from an IgG3 molecule. As set forth herein, it will be understood by one of ordinary skill in the art that an Fc moiety may be altered such that it varies in amino acid sequence from a naturally occurring antibody molecule.

20 [00168] In another embodiment, a polypeptide of the invention comprises an scFc region comprising one or more truncated Fc moieties that are nonetheless sufficient to confer Fc receptor (FcR) binding properties to the Fc region. For example, the portion of an Fc domain that binds to FcRn (i.e., the FcRn binding portion) comprises from about amino acids 282-438 of IgG1, EU numbering (with the
25 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 moiety of a polypeptide 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 IgG1, IgG2, IgG3 and IgG4. In
30 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.

[00169] In one embodiment, a polypeptide of the invention lacks one or more constant region domains of a complete Fc region, i.e., they are partially or entirely
35 deleted. In a certain embodiments polypeptides of the invention will lack an entire CH2 domain (ACH2 constructs). Those skilled in the art will appreciate that such constructs may be preferred due to the regulatory properties of the CH2 domain on the

5 catabolic rate of the antibody. In certain embodiments, polypeptides of the invention comprise CH2 domain-deleted Fc regions derived from a vector (e.g., from IDEC Pharmaceuticals, San Diego) encoding an IgGi human constant region domain (see, e.g., WO 02/060955A2 and WO02/096948A2). This exemplary vector is engineered to delete the CH2 domain and provide a synthetic vector expressing a domain-deleted
10 IgGi constant region. It will be noted that these exemplary constructs are preferably engineered to fuse a binding CH3 domain directly to a hinge region of the respective Fc domain.

[00170] In other constructs it may be desirable to provide a spacer moiety between one or more constituent Fc moieties. For example, a spacer moiety may be
15 placed between a hinge region and a CH2 domain and/or between a CH2 and a CH3 domains. For example, compatible constructs could be expressed wherein the CH2 domain has been deleted and the remaining CH3 domain (synthetic or unsynthetic) is joined to the hinge region with a 5 - 20 amino acid spacer. Such a spacer may be added, for instance, to ensure that the regulatory elements of the constant region
20 domain remain free and accessible or that the hinge region remains flexible. Preferably, any linker peptide compatible with the instant invention will be relatively non-immunogenic and not prevent proper folding of the scFc region.

[00171] In certain embodiments, the polypeptides of the invention may comprise a dimeric Fc region comprising Fc moieties of the same, or substantially the
25 same, sequence composition (herein termed a "homodimeric Fc region"). In other embodiments, the polypeptides of the invention may comprise a dimeric Fc region comprising at least two Fc moieties which are of different sequence composition (*i. e.*, herein termed a "heterodimeric Fc region"). In one exemplary embodiment, the heterodimeric Fc region comprises an amino acid substitution in a first Fc moiety
30 (*e.g.*, an amino acid substitution of Asparagine at EU position 297), but not in a second Fc moiety.

[00172] In certain embodiments, the Fc region is hemi-glycosylated. For example, the heteromeric scFc region may comprise a first, glycosylated, Fc moiety (e.g., a glycosylated CH2 region) and a second, aglycosylated, Fc moiety (e.g., an
35 aglycosylated CH2 region), wherein a linker is interposed between the glycosylated and aglycosylated Fc moieties. In other embodiments, the Fc region is fully glycosylated, *i.e.*, all of the Fc moieties are glycosylated. In still further

5 embodiments, the Fc region may be aglycosylated, i.e., none of the Fc moieties are glycosylated.

 [00173] In certain embodiments, an Fc moiety employed in a polypeptide of the invention is altered, e.g., by amino acid mutation (e.g., addition, deletion, or substitution). As used herein, the term "variant Fc moiety" refers to an Fc moiety
10 having at least one amino acid substitution as compared to the wild-type Fc from which the Fc moiety is derived. For example, wherein the Fc moiety is derived from a human IgG1 antibody, a variant comprises at least one amino acid mutation (e.g., substitution) as compared to a wild type amino acid at the corresponding position of the human IgG1 Fc region.

15 [00174] The amino acid substitution(s) of an Fc variant may be located at a position within the Fc moiety referred to as corresponding to the position number that that residue would be given in an Fc region in an antibody (as set forth using the EU numbering convention). One of skill in the art can readily generate alignments to determine what the EU number corresponding to a position in an Fc moiety would be.

20 [00175] In one embodiment, the Fc variant comprises a substitution at an amino acid position located in a hinge domain or portion thereof. In another embodiment, the Fc variant comprises a substitution at an amino acid position located in a CH2 domain or portion thereof. In another embodiment, the Fc variant comprises a substitution at an amino acid position located in a CH3 domain or portion thereof. In
25 another embodiment, the Fc variant comprises a substitution at an amino acid position located in a CH4 domain or portion thereof.

 [00176] In certain embodiments, the polypeptides of the invention comprise an Fc variant comprising more than one amino acid substitution. The polypeptides of the invention may comprise, for example, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid
30 substitutions. Preferably, the amino acid substitutions are spatially positioned from each other by an interval of at least 1 amino acid position or more, for example, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid positions or more. More preferably, the engineered amino acids are spatially positioned apart from each other by an interval of at least 5, 10, 15, 20, embodiments, the polypeptides of the invention comprise an
35 Fc variant comprising more than one amino acid substitution. The polypeptides of the invention may comprise, for example, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid substitutions. Preferably, the amino acid substitutions are spatially positioned from each other by an interval of at least 1 amino acid position or more, for example, at

5 least 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid positions or more. More preferably, the engineered amino acids are spatially positioned apart from each other by an interval of at least 5, 10, 15, 20, or 25 amino acid positions or more.

[00177] 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.*,
 10 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

15 [00178] The polypeptides 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,
 20 WO96/14339A1, WO98/05787A1, WO98/23289A1, WO99/5 1642A1, WO99/58572A1, WO00/09560A2, WO00/32767A1, WO00/42072A2, WO02/44215A2, WO02/060919A2, WO03/074569A2, WO04/016750A2, WO04/029207A2, WO04/035752A2, WO04/06335 1A2, WO04/074455A2, WO04/099249A2, WO05/040217A2, WO04/044859, WO05/070963A1 ,
 25 WO05/077981A2, WO05/092925A2, WO05/123780A2, WO06/019447A1, WO06/047350A2, and WO06/085967A2; US Patent Publication Nos. US2007/023 1329, US2007/023 1329, US2007/0237765, US2007/0237766, US2007/0237767, US2007/0243 188, US20070248603, US20070286859, US20080057056 ; or US Patents 5,648,260; 5,739,277; 5,834,250; 5,869,046;
 30 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; 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
 35 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.

5 [00179] In certain embodiments, a polypeptide of the invention comprises an amino acid substitution to an Fc moiety which alters the antigen-independent effector functions of the antibody, in particular the circulating half-life of the antibody.

[00180] Such polypeptides exhibit either increased or decreased binding to FcRn when compared to polypeptides lacking these substitutions and, therefore, have
 10 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
 15 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
 20 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 polypeptides of the invention exhibit reduced transport across the
 25 epithelium of kidney glomeruli from the vasculature. In another embodiment, the polypeptides of the invention exhibit reduced transport across the blood brain barrier (BBB) from the brain, into the vascular space. In one embodiment, a polypeptide with altered FcRn binding comprises at least one Fc moiety (e.g. one or two Fc moieties) having one or more amino acid substitutions within the "FcRn binding
 30 loop" of an Fc moiety. The FcRn binding loop is comprised of amino acid residues 280-299 (according to EU numbering) of a wild-type, full-length, Fc moiety. In other embodiments, a polypeptide of the invention having altered FcRn binding affinity comprises at least one Fc moiety (e.g. one or two Fc moieties) having one or more amino acid substitutions within the 15 A_{FcRn} "contact zone." As used herein, the
 35 term 15 A_{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 preferred embodiments, a polypeptide of the invention having altered FcRn binding affinity

5 comprises at least one Fc moiety (e.g., one or two Fc moieties) 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
10 Publication No. WO05/047327 which is incorporated by reference herein.

[00181] A polypeptide of the invention may also comprise an art recognized amino acid substitution which alters the glycosylation of the polypeptide. For example, the scFc region of the binding polypeptide may comprise an Fc moiety having a mutation leading to reduced glycosylation (*e.g.*, N- or O-linked
15 glycosylation) or may comprise an altered glycoform of the wild-type Fc moiety (*e.g.*, a low fucose or fucose-free glycan).

[00182] In other embodiments, a polypeptide of the invention comprises at least one Fc moiety having engineered cysteine residue or analog thereof which is located at the solvent-exposed surface. Preferably the engineered cysteine residue or
20 analog thereof does not interfere with an effector function conferred by the scFc region. More preferably, the alteration does not interfere with the ability of the scFc region to bind to Fc receptors (e.g., FcγRI, FcγRII, or FcγRIII) or complement proteins (e.g., C1q), or to trigger immune effector function (e.g., antibody-dependent cytotoxicity (ADCC), phagocytosis, or complement-dependent cytotoxicity (CDCC)).

[00183] In one embodiment, an unprocessed polypeptide of the invention may comprise a genetically fused Fc region (i.e., scFc region) having two or more of its constituent Fc moieties independently selected from the Fc moieties described herein. In one embodiment, the Fc moieties of a dimeric Fc region are the same. In another
25 embodiment, at least two of the Fc moieties are different. For example, the Fc moieties of the polypeptides 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 moieties of the polypeptides of
30 the invention may differ in sequence at one or more amino acid positions. For example, at least two of the Fc moieties may differ at about 5 amino acid positions

5 (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).

B. Polypeptide Linkers

[00184] The genetic constructs of the instant invention encode polypeptides
10 comprising two or more Fc domains or moieties linked via a cscFc linker to form an Fc region comprised in a single polypeptide chain. The cscFc linker is flanked by at least one enzymatic cleavage site, e.g., a site for processing by an intracellular enzyme. Cleavage of the polypeptide at the at least one enzymatic cleavage site results in a polypeptide which comprises at least two polypeptide chains. These
15 linkers are referred to herein as "cscFc linkers" and the cscFc linker is interposed between the two Fc moieties of a polypeptide which comprises it. If the cscFc linker connects two Fc moieties contiguously in the linear polypeptide sequence, it is a "direct" linkage. In contrast, the cscFc linkers may link the first Fc moiety to a binding moiety which is, in turn, linked to the second Fc moiety, thereby forming an
20 indirect linkage.

[00185] As is set forth above, other polypeptide linkers may optionally be used in a construct of the invention, e.g., to connect a biologically active moiety to an Fc moiety. These polypeptide linkers are referred to here as spacers. Some exemplary locations of spacers that can be used in connection with the invention include, e.g.,
25 polypeptides comprising GlySer amino acids such as those set forth in the accompanying figures and described in more detail below.

[00186] In one embodiment, the polypeptide linker is synthetic, i.e., non-naturally occurring. In one embodiment, a polypeptide linker includes peptides (or polypeptides) (which may or may not be naturally occurring) which comprise an
30 amino acid sequence that links or genetically fuses a first linear sequence of amino acids to a second linear sequence of amino acids to which it is not naturally linked or genetically fused in nature. For example, in one embodiment the polypeptide linker may comprise non-naturally occurring polypeptides which are modified forms of naturally occurring polypeptides (e.g., comprising a mutation such as an addition,
35 substitution or deletion). In another embodiment, the polypeptide linker may comprise non-naturally occurring amino acids. In another embodiment, the polypeptide linker may comprise naturally occurring amino acids occurring in a linear

5 sequence that does not occur in nature. In still another embodiment, the polypeptide linker may comprise a naturally occurring polypeptide sequence.

[00187] In certain embodiments, a polypeptide linker can be used to fuse identical Fc moieties, thereby forming a homomeric scFc region. In other embodiments, a polypeptide linker can be used to fuse different Fc moieties (*e.g.* a
10 wild-type Fc moiety and an Fc moiety variant), thereby forming a heteromeric scFc region.

[00188] In another embodiment, a polypeptide linker comprises or consists of a gly-ser linker. In one embodiment, a cscFc linker comprises at least a portion of an immunoglobulin hinge and a gly-ser linker. As used herein, the term "gly-ser linker"
15 refers to a peptide that consists of glycine and serine residues. An exemplary gly-ser linker comprises an amino acid sequence of the formula $(\text{Gly}_4\text{Ser})_n$ (SEQ ID NO: 4), wherein n is a positive integer (*e.g.* , 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10). A preferred gly-ser linker is $(\text{Gly}_4\text{Ser})_2$ (SEQ ID NO:29) , $(\text{Gly}_4\text{Ser})_4$ (SEQ ID NO:6) , or $(\text{Gly}_4\text{Ser})_6$ (SEQ ID NO: 5) Another exemplary gly-ser linker is GGGSSGGGSG (SEQ ID NO:
20 30). In certain embodiments, said gly-ser linker may be inserted between two other sequences of the polypeptide linker (*e.g.* , any of the polypeptide linker sequences described herein). In other embodiments, a gly-ser linker is attached at one or both ends of another sequence of the polypeptide linker (*e.g.* , any of the polypeptide linker sequences described herein). In yet other embodiments, two or more gly-ser linkers
25 are incorporated in series in a polypeptide linker. In one embodiment, a polypeptide linker of the invention comprises at least a portion of an upper hinge region (*e.g.*, derived from an IgG1 , IgG2, IgG3, or IgG4 molecule), at least a portion of a middle hinge region (*e.g.*, derived from an IgG1 , IgG2, IgG3, or IgG4 molecule) and a series of gly-ser amino acid residues (*e.g.*, a gly-ser linker such as $(\text{Gly}_4\text{Ser})_n$ (SEQ ID
30 NO:4)).

[00189] Polypeptide linkers of the invention are at least one amino acid in length and can be of varying lengths. In one embodiment, a polypeptide linker of the invention is from about 1 to about 50 amino acids in length. As used in this context, the term "about" indicates +/- two amino acid residues. Since linker length must be a
35 positive interger, the length of from about 1 to about 50 amino acids in length, means a length of from 1-3 to 48-52 amino acids in length. In another embodiment, a polypeptide linker of the invention is from about 10-20 amino acids in length. In another embodiment, a polypeptide linker of the invention is from about 15 to about

5 50 amino acids in length. In another embodiment, a polypeptide linker of the invention is from about 20 to about 45 amino acids in length. In another embodiment, a polypeptide linker of the invention is from about 15 to about 35 or about 20 to about 30 amino acids in length. In another embodiment, a polypeptide linker of the invention is from about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19,
10 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, or 60 amino acids in length.

[00190] In one embodiment, a peptide linker of the invention is 20 or 30 amino acids in length.

[00191] Polypeptide linkers can be introduced into polypeptide sequences using techniques known in the art. Modifications can be confirmed by DNA sequence
15 analysis. Plasmid DNA can be used to transform host cells for stable production of the polypeptides produced.

20 C. Enzymatic Cleavage Sites

[00192] In one embodiment, one or more enzymatic cleavage site(s) flanks, i.e., is adjacent to (upstream or downstream of) a cscFc linker (L) of an unprocessed polypeptide of the invention forming a cleavable scFc linker. For example, in one embodiment of a construct encoding a polypeptide of the invention, a cleavage site is
25 fused at one or both ends of an cscFc linker (L). For example, in one embodiment, the polypeptide is represented by the formula:

A-F1-P1- L-P2-B-F2 (I)

[00193] in linear sequence from the amino to carboxy terminus wherein A is a biologically active moiety, F1 is a first Fc moiety or domain, PI is an enzymatic
30 cleavage site, L is an cscFc linker, P2 is an enzymatic cleavage site B is a biologically active moiety, F2 is a second Fc moiety or domain and "-" represents a peptide bond. Formula (I) comprises at least an A or B and optionally both. A and B, if both present, can be the same or different. Formula (I) comprises at least one of PI or P2 and optionally both. PI and P2, if both present, can be the same or different. Formula (I)
35 comprises at least a F1 and F2. F1 and F2, if both present, can be the same or different. In one embodiment, P 1 and P2 are both present and are recognized by the same or by different enzymes.

5 **[00194]** In one embodiment, one or both of the enzymatic cleavage sites is an intracellular processing site recognized by a member of the furin family of enzymes, e.g., furin, PC2, PC1/PC3, PC4, PACE4, PC5/PC6, and LPC/PC7/PC8/SPC7.

Exemplary cleavage sites for this family of enzymes include an amino acid sequence comprising the motif Arg-Xaa-Lys/Arg-Arg(SEQ ID NO:34). Other cleavage sites
10 are known in the art.

[00195] In another embodiment, a Factor XIa or Xa cleavage site may be incorporated into a construct of the invention. Exemplary FXIa cleavage sites include, e.g., TQSFNDFTR and SVSQTSLKTR. Exemplary thrombin cleavage sites include, e.g., DFLAEGGGVR, TTKIKPR, LVPRG (SEQ ID NO:35) and a sequence
15 comprising or consisting of ALRPR (e.g. ALRPRVVGGA)).

[00196] In one embodiment, an scFc linker is cleaved at one site (e.g., when PI is present). In another embodiment, an scFc linker is cleaved at two sites (e.g., when PI and P2 are present). In one embodiment, some portion of the linker may remain after cleavage at the at least one enzymatic cleavage site. In order to minimize the
20 presence of extraneous amino acid sequences, two cleavage sites may be included in a polypeptide of the invention. In some embodiments, all or substantially all of the linker is excised, while in some embodiments, a portion of the cleavage site may remain, e.g., four arginines of the RRRR cleavage site.

25 **D. Biologically Active Moieties**

[00197] The polypeptides of the invention comprise at least one biologically active moiety. Such a moiety can be biologically active as a single chain, or may require association with another polypeptide chain (e.g., by dimerization), or may need to be enzymatically cleaved to impart biological activity.

30 **[00198]** The polypeptides of the invention can comprise only one biologically active moiety (creating a molecule which is monovalent with regard to the biologically active moiety, but which is multimeric (e.g., dimeric) with regard to the number of polypeptide chains present after processing or cleavage). Examples of such molecules are shown in US 7404956, which is incorporated in its entirety by
35 reference.

[00199] In one embodiment, a biologically active moiety is operably linked (e.g. via a polypeptide linker) or directly by a peptide bond to the C-terminus and/or N terminus of an Fc moiety. In certain embodiments, the polypeptides of the

5 invention may comprise two or more biologically active moieties. In one embodiment, the biologically active moieties are identical. In another embodiment, the biologically active moieties are different or are each separate subunits or chains of one functional molecule.

[00200] In certain aspects, a polypeptide of the invention comprises more than
10 one biologically active moiety and is multivalent, i.e., has at least one biologically active moiety having a first biological activity and at least one second biologically active moiety having a second biological activity. In certain embodiments, at least one biologically active moiety of the invention is an antigen binding region of an antibody or an antigen binding fragment thereof (e.g. an antibody or antigen binding
15 fragment described *supra*).

[00201] In other embodiments, two or more same or different biologically active moieties are linked to each other (*e.g.*, via a polypeptide linker) in series, and the tandem array of biologically active moieties is operably linked (*e.g.*, chemically conjugated or genetically fused (*e.g.*, either directly or via a polypeptide linker)) to
20 either the C-terminus or the N-terminus of a single genetically-fused Fc region (i.e., a single scFc region) or a tandem array of genetically-fused Fc regions (i.e., tandem scFc regions). In other embodiments, the tandem array of biologically active moieties is operably linked to both the C-terminus and the N-terminus of a single genetically-fused Fc region or a tandem array of genetically-fused Fc regions.

25 [00202] In one embodiment, a polypeptide of the invention comprises at least one of a biologically active moiety that is an antigen binding site (*e.g.*, an antigen binding site of an antibody, antibody variant, or antibody fragment), a receptor binding portion of ligand, or a ligand binding portion of a receptor.

[00203] In one embodiment, the biologically active moiety modulates cellular
30 activation or inhibition (*e.g.*, by binding to a cell surface receptor and resulting in transmission of an activating or inhibitory signal). In one embodiment, the biologically active moiety is capable of initiating transduction of a signal which results in death of the cell (*e.g.*, by a cell signal induced pathway, by complement fixation or exposure to a payload (*e.g.*, a toxic payload) present on the binding
35 molecule), or which modulates a disease or disorder in a subject (*e.g.*, by mediating or promoting cell killing, by promoting lysis of a fibrin clot or promoting clot formation, or by modulating the amount of a substance which is bioavailable (*e.g.*, by enhancing or reducing the amount of a ligand such as TNF α in the subject)). In another

5 embodiment, the polypeptides of the invention have at least one binding site specific for an antigen targeted for reduction or elimination, *e.g.*, a cell surface antigen or a soluble antigen, together with at least one genetically-fused Fc region (*i.e.*, scFc region).

[00204] In another embodiment, binding of a biologically active moiety of the
10 invention to a target molecule (*e.g.* antigen) results in the reduction or elimination of the target molecule or a cell expressing the target molecule, *e.g.*, from a tissue or from circulation. In another embodiment, the a biologically active moiety has at least one binding site specific for a target molecule that can be used to detect the presence of the target molecule (*e.g.*, to detect a contaminant or diagnose a condition or disorder).
15 Exemplary biologically active moieties are discussed further below.

i. Antigen Binding Portions

[00205] In certain embodiments, a polypeptide of the invention comprises at least one biologically active moiety which is a binding site, *e.g.*, an antigen binding portion of an antibody.

20 [00206] In other embodiments, a binding site of a polypeptide of the invention may comprise an antigen binding portion of an antibody. The term "antigen-binding portion" refers to a polypeptide fragment of an immunoglobulin, antibody, or antibody variant which binds antigen or competes with intact antibody (*i.e.* , with the intact antibody from which they were derived) for antigen binding (*i.e.*, specific
25 binding). For example, said antigen binding portions can be derived from any of the antibodies or antibody variants known in the art. Antigen binding portions can be produced by recombinant or biochemical methods that are well known in the art. Exemplary antigen-binding fragments include VH and VL regions, Fv, Fab, Fab', and (Fab')₂.

30 [00207] In exemplary embodiments, a genetic construct encoding a polypeptide of the invention comprises a nucleotide sequence encoding at least one antigen binding fragment that is operably linked (*e.g.* , chemically conjugated or genetically-fused (*e.g.* , directly fused or fused via a polypeptide linker)) to the C-terminus and/or N-terminus of a genetically-fused Fc region (*i.e.*, a scFc region). In one exemplary
35 embodiment, an immature polypeptide of the invention comprises an antigen binding fragment (*e.g.* a Fab) which is operably linked to the N-terminus (or C-terminus) of at least one genetically-fused Fc region via a hinge domain or portion thereof (*e.g.* , an

5 IgG1 hinge or portion thereof, *e.g.*, a human IgG1 hinge). An exemplary hinge domain portion comprises the sequence DKTHTCPPCPAPELLGG (SEQ ID NO: 28).

[00208] In other embodiments, a biologically active moiety of the invention may comprise a binding site from a single chain binding molecule (*e.g.*, a single chain variable region or scFv). Techniques described for the production of single chain
 10 antibodies (U.S. Pat. No. 4,694,778; Bird, *Science* 242:423-442 (1988); Huston *et al.*, *Proc. Natl. Acad. Sci. USA* 85:5879-5883 (1988); and Ward *et al.*, *Nature* 334:544-554 (1989)) can be adapted to produce single chain molecules. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain antibody. Techniques for the
 15 assembly of functional Fv fragments in *E. coli* may also be used (Skerra *et al.*, *Science* 242:1038-1041 (1988)).

[00209] Single chain variable region sequences comprise a single polypeptide having one or more antigen binding sites, *e.g.*, a VL domain linked by a flexible linker to a VH domain. The VL and/or VH domains may be derived from any of the
 20 antibodies or antibody variants described *supra*. ScFv molecules can be constructed in a VH-linker-VL orientation or VL-linker-VH orientation. The flexible linker that links the VL and VH domains that make up the antigen binding site preferably comprises from about 10 to about 50 amino acid residues. In one embodiment, the polypeptide linker is a gly-ser polypeptide linker. An exemplary gly-ser polypeptide
 25 linker is of the formula (Gly4Ser)*n*, wherein *n* is a positive integer (*e.g.*, 1, 2, 3, 4, 5, or 6). Other polypeptide linkers are known in the art. Antibodies having single chain variable region sequences (*e.g.* single chain Fv antibodies) and methods of making said single chain antibodies are well-known in the art (see *e.g.*, Ho *et al.* 1989. *Gene* 77:51; Bird *et al.* 1988 *Science* 242:423; Pantoliano *et al.* 1991. *Biochemistry*
 30 30:10117; Milenic *et al.* 1991. *Cancer Research* 51:6363; Takkinen *et al.* 1991. *Protein Engineering* 4:837).

[00210] In certain embodiments, a scFv molecule employed in a polypeptide of the invention is a stabilized scFv molecule. In one embodiment, the stabilized cFv molecule may comprise a scFv linker interposed between a VH domain and a VL
 35 domain, wherein the VH and VL domains are linked by a disulfide bond between an amino acid in the VH and an amino acid in the VL domain. In other embodiments, the stabilized scFv molecule may comprise a scFv linker having an optimized length or composition. In yet other embodiments, the stabilized scFv molecule may comprise a

5 **VH** or **VL** domain having at least one stabilizing amino acid substitution(s). In yet another embodiment, a stabilized scFv molecule may have at least two of the above listed stabilizing features.

[00211] Stabilized scFv molecules have improved protein stability or impart improved protein stability to the polypeptide to which they are operably linked.

10 Preferred scFv linkers of the invention improve the thermal stability of a polypeptide of the invention by at least about 2°C or 3°C as compared to a conventional polypeptide. Comparisons can be made, for example, between the scFv molecules of the invention. In certain preferred embodiments, the stabilized scFv molecule comprises a (Gly₄Ser)₄ scFv linker and a disulfide bond which links **VH** amino acid 44
15 and **VL** amino acid 100. Other exemplary stabilized scFv molecules which may be employed in the polypeptides of the invention are described in US Provisional Patent Application No. 60/873,996, filed on December 8, 2006 or US Patent Application No. 11/725,970, filed on March 19, 2007, each of which is incorporated herein by reference in its entirety.

20 [00212] In certain exemplary embodiments, the polypeptides of the invention comprise at least one scFv molecule that is operably linked (*e.g.*, chemically conjugated or genetically-fused (*e.g.*, directly fused or fused via a polypeptide linker) to the C-terminus and/or N-terminus of a genetically-fused Fc region (*i.e.*, a scFc region). In one exemplary embodiment, a polypeptide of the invention comprises at
25 least one scFv molecule (*e.g.*, one or more stabilized scFv molecules) which are operably linked to the N-terminus (or C-terminus) of at least one genetically-fused Fc region via a gly/ser linker.

[00213] Polypeptides of the invention may comprise a variable region or portion thereof (*e.g.*, a VL and/or VH domain) derived from an antibody using art recognized
30 protocols or may be obtained from an art-recognized antibody using standard molecular biology techniques.

[00214] Those skilled in the art will also appreciate that DNA encoding antibody variable domains may also be derived from antibody libraries expressed in phage, yeast, or bacteria using methods known in the art. Exemplary methods are set forth, for
35 example, in EP 368 684 B1; U.S. Pat. No. 5,969,108; Hoogenboom *et al.*, (2000) *Immunol. Today* 21:371; Nagy *et al.* (2002) *Nat. Med.* 8:801; Huie *et al.* (2001), *PNAS*, 98:2682; Lui *et al.* (2002), *J. Mol. Biol.* 315:1063, each of which is incorporated herein by reference. Several publications (*e.g.*, Marks *et al.* (1992), *Bio/Technology* 10:779-

5 783) have described the production of high affinity human antibodies by chain shuffling, as well as combinatorial infection and *in vivo* recombination as a strategy for constructing large phage libraries. In another embodiment, ribosomal display can be used to replace bacteriophage as the display platform (see, *e.g.*, Hanes, *et al.* (1998), *PNAS* 95:14130; Hanes and Pluckthun. (1999), *Curr. Top. Microbiol. Immunol.* 10 243:107; He and Taussig. (1997), *Nuc. Acids Res.*, 25:5132; Hanes *etal.* (2000), *Nat. Biotechnol.* 18:1287; Wilson *etal.* (2001), *PNAS*, 98:3750; or Irving *etal.* (2001) *J. Immunol. Methods* 248:31).

[00215] Moreover, variable region sequences useful for producing the biologically active moieties of the present invention may be obtained from a number 15 of different sources. For example, as discussed above, a variety of human gene sequences are available in the form of publicly accessible deposits. Many sequences of antibodies and antibody-encoding genes have been published and suitable variable region sequences (*e.g.* VL and VH sequences) can be chemically synthesized from these sequences using art recognized techniques.

20 [00216] Further, a biologically active moiety of the invention may comprise a variable domain or CDR derived from a fully murine, fully human, chimeric, humanized, deimmunized, non-human primate or primatized antibody.

[00217] Exemplary antibodies from which binding sites can be derived for use in the molecules of the invention are known in the art. For example, antibodies 25 currently approved by the FDA can be used to derive binding sites.

[00218] In one embodiment, a polypeptide of the invention binds to a molecule which is useful in treating cancer.

[00219] In still other embodiments, a biologically active moiety of the invention binds to a molecule which is useful in treating an autoimmune or inflammatory 30 disease or disorder.

[00220] For example, a polypeptide may bind to an antigen present on an immune cell (*e.g.*, a B or T cell) or an autoantigen responsible for an autoimmune disease or disorder. The antigen associated with an autoimmune or inflammatory disorder may be a tumor-associated antigen described *supra*. Thus, a tumor 35 associated antigen may also be an autoimmune or inflammatory associated disorder. As used herein, the term "autoimmune disease or disorder" refers to disorders or conditions in a subject wherein the immune system attacks the body's own cells, causing tissue destruction. Autoimmune diseases include general autoimmune

5 diseases, *i.e.*, in which the autoimmune reaction takes place simultaneously in a number of tissues, or organ specific autoimmune diseases, *i.e.*, in which the autoimmune reaction targets a single organ. Examples of autoimmune diseases that can be diagnosed, prevented or treated by the methods and compositions of the present invention include, but are not limited to, Crohn's disease; Inflammatory bowel
10 disease (IBD); systemic lupus erythematosus; ulcerative colitis; rheumatoid arthritis; Goodpasture's syndrome; Grave's disease; Hashimoto's thyroiditis; pemphigus vulgaris; myasthenia gravis; scleroderma; autoimmune hemolytic anemia; autoimmune thrombocytopenic purpura; polymyositis and dermatomyositis; pernicious anemia; Sjogren's syndrome; ankylosing spondylitis; vasculitis; type I
15 diabetes mellitus; neurological disorders, multiple sclerosis, and secondary diseases caused as a result of autoimmune diseases.

[00221] In other embodiments, a biologically active moiety of the invention that binds to a target molecule associated with an inflammatory disease or disorder. As used herein the term "inflammatory disease or disorder" includes diseases or
20 disorders which are caused, at least in part, or exacerbated by inflammation, *e.g.*, increased blood flow, edema, activation of immune cells (*e.g.*, proliferation, cytokine production, or enhanced phagocytosis). For example, a polypeptide of the invention may bind to an inflammatory factor (*e.g.*, a matrix metalloproteinase (MMP), TNF α , an interleukin, a plasma protein, a cytokine, a lipid metabolite, a protease, a toxic
25 radical, a mitochondrial protein, an apoptotic protein, an adhesion molecule, *etc.*) involved or present in an area in aberrant amounts, *e.g.*, in amounts which may be advantageous to alter, *e.g.*, to benefit the subject. The inflammatory process is the response of living tissue to damage. The cause of inflammation may be due to physical damage, chemical substances, micro-organisms, tissue necrosis, cancer or
30 other agents. Acute inflammation is short-lasting, *e.g.*, lasting only a few days. If it is longer lasting however, then it may be referred to as chronic inflammation.

[00222] Inflammatory disorders include acute inflammatory disorders, chronic inflammatory disorders, and recurrent inflammatory disorders. Acute inflammatory disorders are generally of relatively short duration, and last for from about a few
35 minutes to about one to two days, although they may last several weeks. The main characteristics of acute inflammatory disorders include increased blood flow, exudation of fluid and plasma proteins (edema) and emigration of leukocytes, such as neutrophils. Chronic inflammatory disorders, generally, are of longer duration, *e.g.*,

5 weeks to months to years or even longer, and are associated histologically with the presence of lymphocytes and macrophages and with proliferation of blood vessels and connective tissue. Recurrent inflammatory disorders include disorders which recur after a period of time or which have periodic episodes. Examples of recurrent inflammatory disorders include asthma and multiple sclerosis. Some disorders may

10 fall within one or more categories. Inflammatory disorders are generally characterized by heat, redness, swelling, pain and loss of function. Examples of causes of inflammatory disorders include, but are not limited to, microbial infections (*e.g.*, bacterial, viral and fungal infections), physical agents (*e.g.*, burns, radiation, and trauma), chemical agents (*e.g.*, toxins and caustic substances), tissue necrosis and

15 various types of immunologic reactions. Examples of inflammatory disorders include, but are not limited to, osteoarthritis, rheumatoid arthritis, acute and chronic infections (bacterial, viral and fungal); acute and chronic bronchitis, sinusitis, and other respiratory infections, including the common cold; acute and chronic gastroenteritis and colitis; acute and chronic cystitis and urethritis; acute respiratory distress

20 syndrome; cystic fibrosis; acute and chronic dermatitis; acute and chronic conjunctivitis; acute and chronic serositis (pericarditis, peritonitis, synovitis, pleuritis and tendinitis); uremic pericarditis; acute and chronic cholecystitis; acute and chronic vaginitis; acute and chronic uveitis; drug reactions; and burns (thermal, chemical, and electrical).

25 **[00223]** In yet other embodiments, a biologically active moiety of the invention binds to a molecule which is useful in treating a neurological disease or disorder. For example, a polypeptide may bind to an antigen present on a neural cell (*e.g.*, a neuron, a glial cell, or a). In certain embodiments, the antigen associated with a neurological disorder may be an autoimmune or inflammatory disorder described *supra*. As used

30 herein, the term "neurological disease or disorder" includes disorders or conditions in a subject wherein the nervous system either degenerates (*e.g.*, neurodegenerative disorders, as well as disorders where the nervous system fails to develop properly or fails to regenerate following injury, *e.g.*, spinal cord injury. Examples of neurological disorders that can be diagnosed, prevented or treated by the methods and

35 compositions of the present invention include, but are not limited to, Multiple Sclerosis, Huntington's Disease, Alzheimer's Disease, Parkinson's Disease, neuropathic pain, traumatic brain injury, Guillain-Barre syndrome and chronic inflammatory demyelinating polyneuropathy (CIDP).

5 **[00224]** In other aspects, the biologically active moieties of the invention may
comprise antigen binding sites, or portions thereof, derived from modified forms of
antibodies. Exemplary such forms include, e.g., minibodies, diabodies, triabodies,
nanobodies, camelids, Dabs, tetravalent antibodies, intradiabodies (e.g., Jendreyko et
al. 2003. J. Biol. Chem. 278:47813), fusion proteins (e.g., antibody cytokine fusion
10 proteins, proteins fused to at least a portion of an Fc receptor), and bispecific
antibodies. Other modified antibodies are described, for example in U.S. Pat. No.
4,745,055; EP 256,654; Faulkner et al., Nature 298:286 (1982); EP 120,694; EP
125,023; Morrison, J. Immun. 123:793 (1979); Kohler et al., Proc. Natl. Acad. Sci.
USA 77:2197 (1980); Raso et al., Cancer Res. 41:2073 (1981); Morrison et al., Ann.
15 Rev. Immunol. 2:239 (1984); Morrison, Science 229:1202 (1985); Morrison et al.,
Proc. Natl. Acad. Sci. USA 81:6851 (1984); EP 255,694; EP 266,663; and WO
88/03559. Reassorted immunoglobulin chains also are known. See, for example, U.S.
Pat. No. 4,444,878; WO 88/03565; and EP 68,763 and references cited therein.

20 **[00225]** In another embodiment, a biologically active moiety of the invention
comprises an antigen binding site or region which is a diabody or an antigen binding
site derived therefrom. Diabodies are dimeric, tetravalent molecules each having a
polypeptide similar to scFv molecules, but usually having a short (e.g., less than 10
and preferably 1-5) amino acid residue linker connecting both variable domains, such
that the V_L and V_H domains on the same polypeptide chain cannot interact. Instead,
25 the V_L and V_H domain of one polypeptide chain interact with the V_H and V_L domain
(respectively) on a second polypeptide chain (see, for example, WO 02/02781). In
one embodiment, an immature polypeptide of the invention comprises a diabody
which is operably linked to the N-terminus and/or C-terminus of at least one
genetically-fused Fc region (i.e., scFc region).

30 **[00226]** In certain embodiments, a biologically active moiety of the invention
comprises a single domain binding molecule (e.g. a single domain antibody) linked to
an scFc. Exemplary single domain molecules include an isolated heavy chain
variable domain (V_H) of an antibody, i.e., a heavy chain variable domain, without a
light chain variable domain, and an isolated light chain variable domain (V_L) of an
35 antibody, i.e., a light chain variable domain, without a heavy chain variable domain.,
Exemplary single-domain antibodies employed in the molecules of the invention
include, for example, the Camelid heavy chain variable domain (about 118 to 136
amino acid residues) as described in Hamers-Casterman, et al., Nature 363:446-448

5 (1993), and Dumoulin, et al., Protein Science 11:500-515 (2002). Other exemplary single domain antibodies include single VH or VL domains, also known as Dabs® (Domantis Ltd., Cambridge, UK). Yet other single domain antibodies include shark antibodies (e.g., shark Ig-NARs). Shark Ig-NARs comprise a homodimer of one variable domain (V-NAR) and five C-like constant domains (C-NAR), wherein
 10 diversity is concentrated in an elongated CDR3 region varying from 5 to 23 residues in length. In camelid species (e.g., llamas), the heavy chain variable region, referred to as VHH, forms the entire antigen-binding domain. The main differences between camelid VHH variable regions and those derived from conventional antibodies (VH) include (a) more hydrophobic amino acids in the light chain contact surface of VH as
 15 compared to the corresponding region in VHH, (b) a longer CDR3 in VHH, and (c) the frequent occurrence of a disulfide bond between CDR1 and CDR3 in VHH. Methods for making single domain molecules are described in US Patent Nos 6.005,079 and 6,765,087, both of which are incorporated herein by reference. Exemplary single domain antibodies comprising VHH domains include Nanobodies®
 20 (Ablynx NV, Ghent, Belgium).

[00227] In one embodiment, biologically active moiety comprises an antigen binding portion of an antibody that binds to a TNF receptor family member. The nucleotide and amino acid sequences of several TNF receptors family members are known in the art and include at least 29 human genes: TNFRSF1A (TNFR1, also
 25 known as DR1, CD120a, TNF-R-I p55, TNF-R, TNFR1, TNFAR, TNF-R55, p55TNFR, p55R, or TNFR60, GenBank GI No. 4507575; see also US 5,395,760)), TNFRSF1B (CD120b, also known as p75, TNF-R, TNF-R-II, TNFR80, TNFR2, TNF-R75, TNFBR, or p75TNFR; GenBank GI No. 4507577), TNFRSF3 (Lymphotoxin Beta Receptor (**LtβR**), also known as TNFR2-RP, CD18, TNFR-RP, TNFCR, or
 30 TNF-R-III; GI Nos. 4505038 and 20072212), TNFRSF4 (OX40, also known as ACT35, TXGP1L, or CD134 antigen; GI Nos. 4507579 and 8926702), TNFRSF5 (CD40, also known as p50 or Bp50; GI Nos. 4507581 and 23312371), TNFRSF6 (FAS, also known as FAS-R, DcR-2, DR2, CD95, APO-1, or APT1; GenBank GI Nos. 4507583, 23510421, 23510423, 23510425, 23510427, 23510429, 23510431, and
 35 23510434)), TNFRSF6B (DcR3, DR3; GenBank GI Nos. 4507569, 23200021, 23200023, 23200025, 23200027, 23200029, 23200031, 23200033, 23200035, 23200037, and 23200039), TNFRSF7 (CD27, also known as Tp55 or S152; GenBank GI No. 4507587), TNFRSF8 (CD30, also known as Ki-1, or D1S166E; GenBank GI

- 5 Nos. 4507589 and 23510437), TNFRSF9 (4-1-BB, also known as CD137 or ILA; GI Nos. 5730095 and 728738), TNFRSF10A (TRAIL-R1, also known as DR4 or Apo2; GenBank GI No. 21361086), TNFRSF10B (TRAIL-R2, also known as DR5, KILLER, TRICK2A, or TRICKB; GenBank GI Nos. 22547116 and 22547119), TNFRSF10C (TRAIL-R3, also known as DcR1, LIT, or TRID; GenBank GI No. 22547121), TNFRSF10D (TRAIL-R4, also known as DcR2 or TRUNDD),
- 10 TNFRSF11A (RANK; GenBank GI No. 4507565; see US Patent Nos. 6,562,948; 6,537,763; 6,528,482; 6,479,635; 6,271,349; 6,017,729), TNFRSF11B (Osteoprotegerin (OPG), also known as OCIF or TR1; GI Nos. 38530116, 22547122 and 33878056), TNFRSF12 (Translocating chain-Association Membrane Protein (TRAMP), also known as DR3, WSL-1, LARD, WSL-LR, DDR3, TR3, APO-3, Fnl4, or TWEAKR; GenBank GI No. 7706186; US Patent Application Publication No. 2004/0033225A1), TNFRSF12L (DR3L), TNFRSF13B (TACI; GI No. 6912694), TNFRSF13C (BAFFR; GI No. 16445027), TNFRSF14 (Herpes Virus Entry Mediator (HVEM), also known as ATAR, TR2, LIGHTR, or HVEA; GenBank
- 20 GI Nos. 23200041, 12803895, and 3878821), TNFRSF16 (Low-Affinity Nerve Growth Factor Receptor (LNGFR), also known as Neurotrophin Receptor or p75(NTR); GenBank GI Nos. 128156 and 4505393), TNFRSF17 (BCM, also known as BCMA; GI No. 23238192), TNFRSF18 (AITR, also known as GTR; GenBank GI Nos. 4759246, 23238194 and 23238197), TNFRSF19 (Troy/Trade, also known as
- 25 TAJ; GenBank GI Nos. 23238202 and 23238204), TNFRSF20 (RELT, also known as FLJ14993; GI Nos. 21361873 and 23238200), TNFRSF21 (DR6), TNFRSF22 (SOBa, also known as Tnfrh2 or 2810028K06Rik), and TNFRSF23 (mSOB, also known as Tnfrhl). Other TNF family members include EDAR1 (Ectodysplasin A Receptor, also known as Downless (DL), ED3, ED5, ED1R, EDA3, EDA1R, EDA-
- 30 AIR; GenBank GI No. 11641231; US Patent No. 6,355,782), XEDAR (also known as EDA-A2R; GenBank GI No. 11140823); and CD39 (GI Nos. 2135580 and 765256).

ii. Non-Immunoglobulin Binding Molecules

- [00228] In certain other embodiments, a biologically active moiety of the invention comprises one or more binding sites derived from a non-immunoglobulin
- 35 binding molecule. As used herein, the term "non-immunoglobulin binding molecules" are binding molecules whose binding sites comprise a portion (e.g., a scaffold or framework) which is derived from a polypeptide other than an

5 immunoglobulin, but which may be engineered (e.g., mutagenized) to confer a desired binding specificity.

[00229] Other examples of biologically active moieties not derived from antibody molecules include receptor binding sites and ligand binding sites which are discussed in more detail infra.

10 [00230] Non-immunoglobulin biologically active moieties can comprise binding site portions that are derived from a member of the immunoglobulin superfamily that is not an immunoglobulin (e.g. a T-cell receptor or a cell-adhesion protein (e.g., CTLA-4, N-CAM, telokin)). Such binding molecules comprise a binding site portion which retains the conformation of an immunoglobulin fold and is
15 capable of specifically binding an IGF1-R epitope. In other embodiments, non-immunoglobulin binding molecules of the invention also comprise a binding site with a protein topology that is not based on the immunoglobulin fold (e.g. such as ankyrin repeat proteins or fibronectins) but which nonetheless are capable of specifically binding to a target (e.g. an IGF-1R epitope).

20 [00231] Non-immunoglobulin biologically active moieties may be identified by selection or isolation of a target-binding variant from a library of binding molecules having artificially diversified binding sites. Diversified libraries can be generated using completely random approaches (e.g., error-prone PCR, exon shuffling, or directed evolution) or aided by art-recognized design strategies. For example, amino
25 acid positions that are usually involved when the binding site interacts with its cognate target molecule can be randomized by insertion of degenerate codons, trinucleotides, random peptides, or entire loops at corresponding positions within the nucleic acid which encodes the binding site (see e.g., U.S. Pub. No. 20040132028). The location of the amino acid positions can be identified by investigation of the
30 crystal structure of the binding site in complex with the target molecule. Candidate positions for randomization include loops, flat surfaces, helices, and binding cavities of the binding site. In certain embodiments, amino acids within the binding site that are likely candidates for diversification can be identified by their homology with the immunoglobulin fold. For example, residues within the CDR-like loops of
35 fibronectin may be randomized to generate a library of fibronectin binding molecules (see, e.g., Koide et al., J. Mol. Biol., 284: 1141-1151 (1998)). Other portions of the binding site which may be randomized include flat surfaces. Following randomization, the diversified library may then be subjected to a selection or

5 screening procedure to obtain binding molecules with the desired binding characteristics, e.g. specific binding to an IGF-IR epitope described *supra*. For example, selection can be achieved by art-recognized methods such as phage display, yeast display, or ribosome display.

[00232] In one embodiment, a biologically active moiety is derived from a
10 fibronectin binding molecule. Fibronectin binding molecules (e.g., molecules comprising the Fibronectin type I, II, or III domains) display CDR-like loops which, in contrast to immunoglobulins, do not rely on intra-chain disulfide bonds. Methods for making fibronectin polypeptides are described, for example, in WO 01/64942 and in US Patent Nos. 6,673,901, 6,703,199, 7,078,490, and 7,119,171, which are
15 incorporated herein by reference. In one exemplary embodiment, the fibronectin polypeptide is as AdNectin® (Adnexus Therapeutics, Waltham, MA).

[00233] In another embodiment, a biologically active moiety of the invention comprises a binding site from an Affibody® (Abeam, Cambridge, MA). Affibodies are derived from the immunoglobulin binding domains of staphylococcal Protein A
20 (SPA) (see e.g., Nord et al., Nat. Biotechnol., 15: 772-777 (1997)). Affibody binding sites employed in the invention may be synthesized by mutagenizing an SPA-related protein (e.g., Protein Z) derived from a domain of SPA (e.g., domain B) and selecting for mutant SPA-related polypeptides having binding affinity for an IGF-IR epitope. Other methods for making affibody binding sites are described in US Patents
25 6,740,734 and 6,602,977 and in WO 00/63243, each of which is incorporated herein by reference.

[00234] In another embodiment, a biologically active moiety of the invention comprises a binding site from an Anticalin® (Pieris AG, Friesing, Germany).
Anticalins (also known as lipocalins) are members of a diverse β -barrel protein
30 family whose function is to bind target molecules in their barrel/loop region. Lipocalin binding sites may be engineered to bind an IGF-IR epitope by randomizing loop sequences connecting the strands of the barrel (see e.g., Schlehuber et al., Drug Discov. Today, 10: 23-33 (2005); Beste et al., PNAS, 96: 1898-1903 (1999)).
Anticalin binding sites employed in the binding molecules of the invention may be
35 obtainable starting from polypeptides of the lipocalin family which are mutated in four segments that correspond to the sequence positions of the linear polypeptide sequence comprising amino acid positions 28 to 45, 58 to 69, 86 to 99 and 114 to 129

5 of the Bilin-binding protein (BBP) of *Pieris brassica*. Other methods for making anticalin binding sites are described in W099/16873 and WO 05/019254, each of which is incorporated herein by reference.

[00235] In another embodiment, a biologically active moiety of the invention comprises a binding site from a cysteine-rich polypeptide. Cysteine-rich domains
10 employed in the practice of the present invention typically do not form a α -helix, a β sheet, or a β -barrel structure. Typically, the disulfide bonds promote folding of the domain into a three-dimensional structure. Usually, cysteine-rich domains have at least two disulfide bonds, more typically at least three disulfide bonds. An exemplary cysteine-rich polypeptide is an A domain protein. A-domains (sometimes called
15 "complement-type repeats") contain about 30-50 or 30-65 amino acids. In some embodiments, the domains comprise about 35-45 amino acids and in some cases about 40 amino acids. Within the 30-50 amino acids, there are about 6 cysteine residues. Of the six cysteines, disulfide bonds typically are found between the following cysteines: C1 and C3, C2 and C5, C4 and C6. The A domain constitutes a
20 ligand binding moiety. The cysteine residues of the domain are disulfide linked to form a compact, stable, functionally independent moiety. Clusters of these repeats make up a ligand binding domain, and differential clustering can impart specificity with respect to the ligand binding. Exemplary proteins containing A-domains include, e.g., complement components (e.g., C6, C7, C8, C9, and Factor I), serine
25 proteases (e.g., enteropeptidase, matriptase, and corin), transmembrane proteins (e.g., ST7, LRP3, LRP5 and LRP6) and endocytic receptors (e.g., Sortilin-related receptor, LDL-receptor, VLDLR, LRP1, LRP2, and ApoER2). Methods for making A domain proteins of a desired binding specificity are disclosed, for example, in WO 02/088171 and WO 04/044011, each of which is incorporated herein by reference.

30 [00236] In other embodiments, a biologically active moiety of the invention comprises a binding site from a repeat protein. Repeat proteins are proteins that contain consecutive copies of small (e.g., about 20 to about 40 amino acid residues) structural units or repeats that stack together to form contiguous domains. Repeat proteins can be modified to suit a particular target binding site by adjusting the
35 number of repeats in the protein. Exemplary repeat proteins include Designed Ankyrin Repeat Proteins (i.e., a DARPin[®], Molecular Partners, Zurich, Switzerland) (see e.g., Binz et al., Nat. Biotechnol., 22: 575-582 (2004)) or leucine-rich repeat proteins (i.e., LRRPs) (see e.g., Pancer et al., Nature, 430: 174-180 (2004)). All so far

5 determined tertiary structures of ankyrin repeat units share a characteristic composed of a β -hairpin followed by two antiparallel α -helices and ending with a loop connecting the repeat unit with the next one. Domains built of ankyrin repeat units are formed by stacking the repeat units to an extended and curved structure. LRRP binding sites from part of the adaptive immune system of sea lampreys and other
 10 jawless fishes and resemble antibodies in that they are formed by recombination of a suite of leucine-rich repeat genes during lymphocyte maturation. Methods for making DARpin or LRRP binding sites are described in WO 02/20565 and WO 06/083275, each of which is incorporated herein by reference.

[00237] Other non-immunoglobulin binding sites which may be employed in
 15 molecules of the invention include binding sites derived from Src homology domains (e.g. SH2 or SH3 domains), PDZ domains, beta-lactamase, high affinity protease inhibitors, or small disulfide binding protein scaffolds such as scorpion toxins. Methods for making binding sites derived from these molecules have been disclosed in the art, see e.g., Silverman et al., Nat. Biotechnol., 23(12): 1493-4 (2005); Panni et
 20 al, J. Biol. Chem., 277: 21666-21674 (2002), Schneider et al., Nat. Biotechnol., 17: 170-175 (1999); Legendre et al., Protein Sci., 11:1506-1518 (2002); Stoop et al., Nat. Biotechnol., 21: 1063-1068 (2003); and Vita et al., PNAS, 92: 6404-6408 (1995). Yet other binding sites may be derived from a binding domain selected from the group consisting of an EGF-like domain, a Kringle-domain, a PAN domain, a Gla domain, a
 25 SRCR domain, a Kunitz/Bovine pancreatic trypsin Inhibitor domain, a Kazal-type serine protease inhibitor domain, a Trefoil (P-type) domain, a von Willebrand factor type C domain, an Anaphylatoxin-like domain, a CUB domain, a thyroglobulin type I repeat, LDL-receptor class A domain, a Sushi domain, a Link domain, a
 30 Thrombospondin type I domain, an Immunoglobulin-like domain, a C-type lectin domain, a MAM domain, a von Willebrand factor type A domain, a Somatomedin B domain, a WAP-type four disulfide core domain, a F5/8 type C domain, a Hemopexin domain, a Laminin-type EGF-like domain, a C2 domain, a CTLA-4 domain, and other such domains known to those of ordinary skill in the art, as well as derivatives and/or variants thereof. Additional non-immunoglobulin polypeptides include Avimers®
 35 (Avidia, Inc., Mountain View, CA -see International PCT Publication No. WO 06/055689 and US Patent Pub 2006/0234299), Telobodies® (Biotech Studio, Cambridge, MA), Evibodies® (Evogenix, Sydney, Australia -see US Patent No. 7,166,697), and Microbodies® (Nascacell Technologies, Munich, Germany).

5 **iii. Binding Portions of Receptors or Ligands**

[00238] In other aspects, a polypeptide of the invention comprises a ligand binding site of a receptor and/or a receptor binding portion of a ligand which is operably linked to at least one genetically-fused Fc region.

[00239] In certain embodiments, transmembrane regions or lipid or
10 phospholipid anchor recognition sequences of the ligand binding receptor are preferably inactivated or deleted prior to fusion. DNA encoding the ligand or ligand binding partner is cleaved by a restriction enzyme at or proximal to the 5' and 3' ends of the DNA encoding the desired ORF segment. The resultant DNA fragment can be readily inserted (e.g., ligated in-frame) into DNA encoding a genetically-fused Fc
15 region. The precise site at which the fusion is made may be selected empirically to optimize the secretion or binding characteristics of the soluble fusion protein. DNA encoding the fusion protein can then subcloned into an appropriate expression vector than can be transfected into a host cell for expression.

[00240] Exemplary binding portions of receptors or ligands that can be present
20 in a polypeptide of the invention are set forth below:

 a. Cytokines and Cytokine Receptors

[00241] Cytokines have pleiotropic effects on the proliferation, differentiation, and functional activation of lymphocytes. Various cytokines, or receptor binding portions thereof, can be utilized in the fusion proteins of the invention as biologically
25 active molecules, binding sites and/or domains. Exemplary cytokines include the interleukins (*e.g.* IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12, IL-13, and IL-18), the colony stimulating factors (CSFs) (*e.g.* granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), and monocyte macrophage CSF (M-CSF)), tumor necrosis factor (TNF) alpha and beta, cytotoxic T lymphocyte antigen 4
30 (CTLA-4), and interferons such as interferon- α , β , or γ (US Patent Nos. 4,925,793 and 4,929,554).

[00242] Cytokine receptors typically consist of a ligand-specific alpha chain and a common beta chain. Exemplary cytokine receptors include those for GM-CSF, IL-3 (US Patent No. 5,639,605), IL-4 (US Patent No. 5,599,905), IL-5 (US Patent No.
35 5,453,491), IL10 receptor, IFN γ (EP0240975), and the TNF family of receptors (*e.g.*, TNFa (*e.g.* TNFR-1 (EP 417, 563), TNFR-2 (EP 417,014) lymphotoxin beta receptor).

 b. Adhesion Proteins

5 **[00243]** Adhesion molecules are membrane-bound proteins that allow cells to interact with one another. Various adhesion proteins, including leukocyte homing receptors and cellular adhesion molecules, or receptor binding portions thereof, can be incorporated in a fusion protein of the invention as biologically active molecules, binding sites and/or domains. Leukocyte homing receptors are expressed on
 10 leukocyte cell surfaces during inflammation and include the β -1 integrins (*e.g.* VLA-1, 2, 3, 4, 5, and 6) which mediate binding to extracellular matrix components, and the P2-integrins (*e.g.* LFA-1, LPAM-1, CR3, and CR4) which bind cellular adhesion molecules (CAMs) on vascular endothelium. Exemplary CAMs include ICAM-1, ICAM-2, VCAM-1, and MAdCAM-1. Other CAMs include those of the selectin
 15 family including E-selectin, L-selectin, and P-selectin.

c. Chemokines

[00244] Chemokines, chemotactic proteins which stimulate the migration of leucocytes towards a site of infection, can also be incorporated into a fusion protein of the invention. Exemplary chemokines include Macrophage inflammatory proteins
 20 (MIP-1- α and MIP-1- β), neutrophil chemotactic factor, and RANTES (regulated on activation normally T-cell expressed and secreted).

d. Hormones

[00245] Exemplary growth hormones for use as biologically active moieties in the fusion proteins of the invention include renin, human growth hormone (HGH; US
 25 Patent No. 5,834,598), N-methionyl human growth hormone; bovine growth hormone; growth hormone releasing factor; parathyroid hormone (PTH); thyroid stimulating hormone (TSH); thyroxine; proinsulin and insulin (US Patent Nos. 5,157,021 and 6,576,608); follicle stimulating hormone (FSH); calcitonin, luteinizing hormone (LH), leptin, glucagons; bombesin; somatropin; mullerian-inhibiting
 30 substance; relaxin and prorelaxin; gonadotropin-associated peptide; prolactin; placental lactogen; OB protein; or mullerian-inhibiting substance.

e. Receptors and Ligands

[00246] In one embodiment, a polypeptide of the invention combines the binding site(s) of the ligand or receptor (*e.g.* the extracellular domain (ECD) of a
 35 receptor) with at least one genetically-fused Fc region (*i.e.*, scFc region). In certain embodiments, the binding site or domain of the ligand-binding portion of a receptor may be derived from a receptor bound by an antibody or antibody variant described *supra*. In other embodiments, the ligand binding portion of a receptor is derived from

5 a receptor selected from the group consisting of a receptor of the Immunoglobulin (Ig) superfamily (e.g., a soluble T-cell receptor, e.g., mTCR® (Medigene AG, Munich, Germany), a receptor of the TNF receptor superfamily described *supra* (e.g., a soluble TNF α receptor of an immunoadhesin), a receptor of the Glial Cell-Derived Neurotrophic Factor (GDNF) receptor family (e.g., GFR α 3), a receptor of the G-protein coupled receptor (GPCR) superfamily, a receptor of the Tyrosine Kinase (TK) receptor superfamily, a receptor of the Ligand-Gated (LG) superfamily, a receptor of the chemokine receptor superfamily, IL-1/Toll-like Receptor (TLR) superfamily, and a cytokine receptor superfamily.

In other embodiments, the binding site or domain of the receptor-binding portion of a ligand may be derived from a ligand bound by an antibody or antibody variant described *supra*. For example, the ligand may bind a receptor selected from the group consisting of a receptor of the Immunoglobulin (Ig) superfamily, a receptor of the TNF receptor superfamily, a receptor of the G-protein coupled receptor (GPCR) superfamily, a receptor of the Tyrosine Kinase (TK) receptor superfamily, a receptor of the Ligand-Gated (LG) superfamily, a receptor of the chemokine receptor superfamily, IL-1/Toll-like Receptor (TLR) superfamily, and a cytokine receptor superfamily. In one exemplary embodiment, the binding site of the receptor-binding portion of a ligand is derived from a ligand belonging to the TNF ligand superfamily described *supra* (e.g., CD40L).

25 **[00247]** Growth factors or their receptors (or receptor binding or ligand binding portions thereof) may be incorporated in the fusion proteins of the invention. Exemplary growth factors include Vascular Endothelial Growth Factor (VEGF) and its isoforms (U.S. Pat. No. 5,194,596); Fibroblastic Growth Factors (FGF), including aFGF and bFGF; atrial natriuretic factor (ANF); hepatic growth factors (HGFs; US Patent Nos. 5,227,158 and 6,099,841), neurotrophic factors such as bone-derived neurotrophic factor (BDNF), glial cell derived neurotrophic factor ligands (e.g., GDNF, neurturin, artemin, and persephin), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF- β platelet-derived growth factor (PDGF) (U.S. Pat. Nos. 4,889,919, 4,845,075, 5,910,574, and 5,877,016); transforming growth factors (TGF) such as TGF- α and TGF- β (WO 90/14359), osteoinductive factors including bone morphogenetic protein (BMP); insulin-like growth factors-I and -II (IGF-I and IGF-II; US Patent Nos. 6,403,764 and 6,506,874);

5 Erythropoietin (EPO); Thrombopoietin (TPO; stem-cell factor (SCF), thrombopoietin (TPO, *c-Mpl* ligand), and the Wnt polypeptides (US Patent No. 6,159,462).

[00248] Exemplary growth factor receptors which may be used as biologically active moieties of the invention include EGF receptors; VEGF receptors (*e.g.* Flt1 or Flk1/KDR), PDGF receptors (WO 90/14425); HGF receptors (US Patent Nos. 10 5,648,273, and 5,686,292), and neurotrophic receptors including the low affinity receptor (LNGFR), also termed as p75^{NTR} or p75, which binds NGF, BDNF, and NT-3, and high affinity receptors that are members of the trk family of the receptor tyrosine kinases (*e.g.* trkA, trkB (EP 455,460), trkC (EP 522,530)).

f. Heterodimeric Receptors

15 [00249] In one embodiment, antagonists to cytokines that utilize an a specificity determining component which, when combined with the cytokine, binds to a first β signal transducing component to form a nonfunctional intermediate which then binds to a second β signal transducing component causing β -receptor dimerization and consequent signal transduction can be made using the methods of 20 the invention. Such molecules are described in the art (see *e.g.*, US Patent 6,927,044). In one example, a soluble specificity determining component of the receptor and the extracellular domain of the first β signal transducing component of the cytokine receptor are combined to form a heterodimer that binds the cytokine to form a nonfunctional complex. Exemplary cytokines that can be inhibited using such 25 heterodimeric receptors include: IL1, IL-2, IL-3, IL-4, IL-5, IL-3, IL-4, IL-5, IL-11, IL-15, GM-CSF, LIF, INF γ , and TGF β .

E. Clotting Factors

[00250] Exemplary clotting factors (blood coagulation factors) for use as biologically active moieties in the fusion proteins of the invention include the clotting 30 factors (*e.g.*, factors V, VII, VIII, IX, X, XI, XII and XIII, von Willebrand factor); tissue factor (U.S. Pat. Nos. 5,346,991, 5,349,991, 5,726,147, and 6,596,84); thrombin and prothrombin; fibrin and fibrinogen; plasmin and plasminogen; plasminogen activators, such as urokinase or human urine or tissue-type plasminogen activator (t-PA). Variants and biologically active portions of such clotting factors 35 may also be used as biologically active molecules. Exemplary molecules that can be made using the scFc regions of the invention are also set forth in, *e.g.*, U.S. Patent 7,404,956 and 7,348,004.

5 **[00251]** Factors VII, IX, and X are all structurally related in that in each the amino terminal end of the light chain is not amenable to the incorporation of additional moieties, due to the requirement for the propeptide sequence that provides a docking site for the vitamin K-dependent gamma-glutamyl carboxylase. Similarly, the amino terminal end of the heavy chain of these three clotting factors is not
10 amenable to the incorporation of additional moieties, with the exception of cleaveable moieties, i.e., moieties linked via a cleavage site or moieties which consist of a cleavage site, due to the requirement for a free N-terminus in order to form a catalytically active protease domain. Although factor VII is often shown to illustrate exemplary embodiments of the invention, the subject constructs may be made using
15 factor IX, or X. For example, one of skill in the art would understand that the FVII portion of a construct of the invention could be substituted with a FIX or FX portion.

[00252] Exemplary clotting factor constructs of the invention are set forth in the accompanying Figures. Although the Figures generally illustrate the clotting Factor as a single chain (in its zymogen form) it will be understood that the clotting
20 factor may also be present in its active form in a construct of the invention, e.g. as a two chain, disulfide bonded form.

[00253] In one embodiment, a clotting factor of the invention is expressed by a cell in active form. In another embodiment, a clotting factor is expressed in inactive form and is subsequently activated under appropriate conditions in vitro such that the
25 active form of the clotting factor is present in the construct. In another embodiment, a clotting factor of the invention comprises a clotting factor in inactive form and the clotting factor is activated in vivo after administration.

[00254] In one embodiment, a clotting factor of the invention is a mature form of Factor VII or a variant thereof. Factor VII (FVII, F7; also referred to as Factor 7, coagulation factor VII, serum factor VII, serum prothrombin conversion accelerator, SPCA, proconvertin and eptacog alpha) is a serine protease that is part of the
30 coagulation cascade. FVII includes a Gla domain, two EGF domains (EGF- 1 and EGF-2), and a serine protease domain (or peptidase SI domain) that is highly conserved among all members of the peptidase SI family of serine proteases, such as
35 for example with chymotrypsin. FVII occurs as a single chain zymogen, an activated zymogen-like two-chain polypeptide and a fully activated two-chain form. As used herein, a "zymogen-like" protein or polypeptide refers to a protein that has been activated by proteolytic cleavage, but still exhibits properties that are associated with

5 a zymogen, such as, for example, low or no activity, or a conformation that resembles the conformation of the zymogen form of the protein. For example, when it is not bound to tissue factor, the two-chain activated form of FVII is a zymogen-like protein; it retains a conformation similar to the uncleaved FVII zymogen, and, thus, exhibits very low activity. Upon binding to tissue factor, the two-chain activated form
10 of FVII undergoes conformational change and acquires its full activity as a coagulation factor.

[00255] Exemplary FVII variants include those with increased specific activity, e.g., mutations that increase the activity of FVII by increasing its enzymatic activity (K_{cat} or K_m). Such variants have been described in the art and include, e.g., mutant
15 forms of the molecule as described for example in Persson et al. 2001. PNAS 98:13583; Petrovan and Ruf. 2001. J. Biol. Chem. 276:6616; Persson et al. 2001 J. Biol. Chem. 276:29195; Soejima et al. 2001. J. Biol. Chem. 276:17229; Soejima et al. 2002. J. Biol. Chem. 247:49027. In one embodiment, a variant form of FVII includes the mutations Exemplary mutations include V158D-E296V-M298Q. In
20 another embodiment, a variant form of FVII includes a replacement of amino acids 608-619 (LQQSRKVGDSPN, corresponding to the 170-loop) from the FVII mature sequence with amino acids EASYPGK from the 170-loop of trypsin. High specific activity variants of FIX are also known in the art. For example, Simioni et al. (2009 N.E. Journal of Medicine 361:1671) describe an R338L mutation. Chang et al. (1988
25 JBC 273: 12089) and Pierri et al. (2009 Human Gene Therapy 20:479) describe an R338A mutation. Other mutations are known in the art and include those described, e.g., in Zogg and Brandstetter. 2009 Structure 17:1669; Sichler et al. 2003. J. Biol. Chem. 278:4121; and Sturzebecher et al. 1997. FEBS Lett 412:295. Another version of factor IX (the triple mutant V86A/E277A/R338A) with augmented
30 clotting activities has been described by Lin et al. 2010. Journal of Thrombosis and Haemostasis 8: 1773). The contents of these references are incorporated herein by this reference.

[00256] Full activation, which occurs upon conformational change from a zymogen-like form, occurs upon binding to its co-factor tissue factor. Also, mutations
35 can be introduced that result in the conformation change in the absence of tissue factor. Hence, reference to FVIIa includes both two-chain forms thereof, the zymogen-like form and the fully activated two-chain form.

5 **[00257]** In one embodiment, a clotting factor of the invention is a mature form of Factor VIII or a variant thereof. FVIII functions in the intrinsic pathway of blood coagulation as a cofactor to accelerate the activation of factor X by factor IXa, a reaction that occurs on a negatively charged phospholipid surface in the presence of calcium ions. FVIII is synthesized as a 2351 amino acid single-chain polypeptide

10 having the domain structure A1-A2-B-A3-C1-C2. Wehar, G. A. et al., *Nature* 312:337-342 (1984) and Toole, J. J. et al., *Nature* 312:342-347 (1984). The domain structure of FVIII is identical to that of the homologous coagulation factor, factor V (FV). Kane, W. H. et al., *PNAS (USA)* 83:6800-6804 (1986) and Jenny, R. J. et al., *PNAS (USA)* 84:4846-4850 (1987). The FVIII A-domains are 330 amino acids and

15 have 40% amino acid identity with each other and to the A-domain of FV and the plasma copper-binding protein ceruloplasmin. Takahashi, N. et al., *PNAS (USA)* 81:390-394 (1984). Each C-domain is 150 amino acids and exhibits 40% identity to the C-domains of FV, and to proteins that bind glycoconjugates and negatively charged phospholipids. Stubbs, J. D. et al., *PNAS (USA)* 87:8417-8421 (1990). The

20 FVIII B-domain is encoded by a single exon and exhibits little homology to any known protein including FV B-domain. Gitschier, J. et al., *Nature* 312:326-330 (1984) and Cripe, L. D. et al., *Biochemistry* 31:3777-3785 (1992).

[00258] FVIII is secreted into plasma as a heterodimer of a heavy chain (domains A1-A2-B) and a light chain (domains A3-C1-C2) associated through a

25 noncovalent divalent metal ion linkage between the A1- and A3-domains. In plasma, FVIII is stabilized by binding to von Willebrand factor. More specifically, the FVIII light chain is bound by noncovalent interactions to a primary binding site in the amino terminus of von Willebrand factor. Upon proteolytic activation by thrombin, FVIII is activated to a heterotrimer of 2 heavy chain fragments (A1, a 50 kDa fragment, and

30 A2, a 43 kDa fragment) and the light chain (A3-C1-C2, a 73 kDa chain). The active form of FVIII (FVIIIa) thus consists of an A1-subunit associated through the divalent metal ion linkage to a thrombin-cleaved A3-C1-C2 light chain and a free A2 subunit associated with the A1 domain through an ion association. Eaton, D. et al., *Biochemistry* 25: 505 (1986); Lollar, P. et al., *J. Biol. Chem.* 266: 12481 (1991); and

35 Fay, P. J. et al., *J. Biol. Chem.* 266: 8957 (1991). This FVIIIa heterotrimer is unstable and subject to rapid inactivation through dissociation of the A2 subunit under physiological conditions.

5 **[00259]** In one embodiment, a clotting factor comprises a B-domain deleted version of factor VIII. "B-domain" of Factor VIII, 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 Factor VIII. The other human Factor VIII domains are defined by the following

10 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-

15 domains, for porcine, mouse and canine Factor VIII are also known in the art. In one embodiment, the B domain of Factor VIII is deleted ("B-domain-deleted factor VIII" or "BDD FVIII"). An example of a BDD FVIII is REFACTO® (recombinant BDD FVIII with S743/Q1638 fusion), which is known in the art.

20 **[00260]** A "B-domain-deleted Factor VIII" 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, each of which is incorporated herein by reference in its entirety. In some embodiments, a B-domain-deleted Factor VIII sequence of the present invention comprises any one of the deletions disclosed at col.

25 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 version Factor VIII) (*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: 6, *i.e.*, SEQ ID NO:

30 2). In some embodiments, a B-domain-deleted Factor VIII 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

35 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

5 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 Factor VIII 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
10 chain, as disclosed in WO 91/09122, which is incorporated herein by reference in its
entirety. In some embodiments, a B-domain-deleted Factor VIII 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), incorporated herein
by reference in its entirety. A B-domain-deleted Factor VIII may also contain a
15 deletion of amino acids 771-1666 or amino acids 868-1562 of Factor VIII. Meulien
P., *et al. Protein Eng.* 2(4): 301-6 (1988), incorporated herein by reference in its
entirety. 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.*
20 *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)), each of
which is incorporated herein by reference in its entirety. Each of the foregoing
25 deletions may be made in any Factor VIII sequence.

[00261] In one embodiment, a clotting factor of the invention is a mature form
of Factor IX or a variant thereof. Factor IX circulates as a 415 amino acid, single
chain plasma zymogen (A. Vysotchin et al., *J. Biol. Chem.* 268, 8436 (1993)). The
zymogen of FIX is activated by FXIa or by the tissue factor/FVIIa complex. Specific
30 cleavages between arginine-alanine 145-146 and arginine-valine 180-181 result in a
light chain and a heavy chain linked by a single disulfide bond between cysteine 132
and cysteine 289 (S. Bajaj et al., *Biochemistry* 22, 4047 (1983)). The structural
organization of FFX is similar to that of the vitamin K-dependent blood clotting
proteins FVII, FX and protein C (B. Furie and B. Furie, *supra*). The approximately 45
35 amino acids of the amino terminus comprise the gamma-carboxy glutamic acid, or gla,
domain. This is followed by two epidermal growth factor homology domains (EOF),
an activation peptide and the catalytic "heavy chain" which is a member of the serine
protease family (A. Vysotchin et al., *J. Biol. Chem.* 268, 8436 (1993); S. Spitzer et al.,

- 5 Biochemical Journal 265, 219 (1990); H. Brandstetter et al., Proc. Natl. Acad. Sci. USA 92, 9796 (1995)).

[00262] In one embodiment, a clotting factor of the invention is a mature form of Factor X. Factor X is a vitamin-K dependent glycoprotein of a molecular weight of 58.5 kDa, which is secreted from liver cells into the plasma as a zymogen. Initially
10 factor X is produced as a prepropeptide with a signal peptide consisting in total of 488 amino acids. The signal peptide is cleaved off by signal peptidase during export into the endoplasmatic reticulum, the propeptide sequence is cleaved off after gamma carboxylation took place at the first 11 glutamic acid residues at the N-terminus of the mature N-terminal chain. A further processing step occurs by cleavage between
15 Arg182 and Ser183. This processing step also leads concomitantly to the deletion of the tripeptide Arg180-Lys181-Arg182. The resulting secreted factor X zymogen consists of an N-terminal light chain of 139 amino acids (M, 16,200) and a C-terminal heavy chain of 306 amino acids (M, 42,000) which are covalently linked via a disulfide bridge between Cys172 and Cys342. Further posttranslational processing
20 steps include the .beta.-hydroxylation of Asp103 as well as N- and O-type glycosylation.

[00263] It will be understood that in addition to wild type (WT) versions of these clotting factors or biologically active portions thereof, the present invention may also employ precursor truncated forms thereof that have activity, allelic variants and
25 species variants, variants encoded by splice variants, and other variants, including polypeptides that have at least 40%, 45%, 50%, 55%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the mature form of the clotting factor and which retain the ability to promote clot formation. For example, modified FVII polypeptides and variants thereof which retain at least one activity of a
30 FVII, such as TF binding, factor X binding, phospholipid binding, and/or coagulant activity of a FVII may be employed. By retaining activity, the activity can be altered, such as reduced or increased, as compared to a wild-type clotting factor so long as the level of activity retained is sufficient to yield a detectable effect. Exemplary sequences of clotting factors that can be used in the constructs of the invention are
35 found in the accompanying sequence listing.

[00264] Exemplary modified polypeptides include, but are not limited to, tissue-specific isoforms and allelic variants thereof, synthetic molecules prepared by translation of nucleic acids, proteins generated by chemical synthesis, such as

5 syntheses that include ligation of shorter polypeptides, through recombinant methods, proteins isolated from human and non-human tissue and cells, chimeric polypeptides and modified forms thereof.

[00265] Other variants of clotting factors include versions that are modified to alter activity. For example, high specific activity versions of clotting factors are
10 known in the art and can be used to make a polypeptide of the invention. Exemplary such high specific activity variants are described, e.g., in Persson et al. PNAS. 2001. 98:13583 and Soejima et al. Journal of Biological Chemistry. 2002. 277:49027. For example, in one embodiment, a high specific activity version of Factor VII, removes amino acids 311 to 322 of the FVII mature sequence (LQSRKVGDSPN,
15 corresponding to the 170- loop) and replaces them with amino acids EASYPGK from the 170-loop of trypsin. This substitution has been shown to confer high specific activity. An additional high specific activity version of Factor VII contains three point mutations in the heavy chain of FVII, V158D, E296V and M298Q.

[00266] The instant clotting factors may also consist of fragments or portions
20 of WT molecules that are of sufficient length or include appropriate regions to retain at least one activity (upon activation if needed) of a full-length mature polypeptide. Exemplary clotting factor variants are known in the art.

[00267] Exemplary clotting factors are those of mammalian, e.g., human, origin. The sequences of exemplary clotting factors are presented in the
25 accompanying sequence listing, e.g., alone or in the context of an clotting factor construct.

[00268] In one embodiment, more than one clotting factor may be present in a polypeptide of the invention. In another embodiment, a polypeptide of the invention comprises a light chain of a clotting factor genetically fused to one Fc moiety of a
30 construct of the invention and a heavy chain of a clotting factor genetically fused to the second Fc moiety of a construct of the invention, or vice versa.

[00269] Exemplary constructs comprising clotting factors as biologically active moieties are shown in the working examples. For example, in one embodiment, a clotting factor zymogen (e.g., factor VII heavy and light chain) is attached via an
35 optional linker (e.g., 6x(G₄S)) linker or directly to the amino terminus of a first Fc moiety (comprising CH1, CH2, and CH3 domains). The carboxy terminus of the first Fc moiety has a cscFc linker (e.g., a 6x(G₄S) linker or 4x(G₄S) linker) which comprises a first processing site (e.g., RRRRS processing site). The other end of the

5 cscFc linker comprises a second processing site (e.g., an RKRRKR or RRRR processing site) and is optionally linked via a spacer to a targeting moiety (e.g., a platelet targeting moiety) or is directly linked to a second Fc moiety. Where a targeting moiety is present, it may be linked to the second Fc moiety via a spacer (e.g., 6x (G₄S)). The FVII-027, FVII-064 and FIX-044 molecules described herein
10 are examples of such constructs.

[00270] Other exemplary constructs result in the secretion of an activated clotting factor rather than a zymogen following processing of the linker by proteases. For example, in one embodiment, the light chain of a clotting factor is fused via a spacer (e.g., a 4x(G₄S) linker) to the amino terminus of a first Fc moiety (comprising
15 CHI, CH2, and CH3 domains). The carboxy terminus of the first Fc moiety is linked to a linker (e.g., a 6x(G₄S) cscFc linker) which comprises a first processing site (e.g., RRRR) processing site). The other end of the linker comprises a second processing site, e.g., an RKRRKR processing site and is genetically fused to the heavy chain of the clotting factor (e.g., FVII heavy chain) which is in turn fused to the amino
20 terminus of the second Fc moiety via a second spacer (e.g., a 4x(G₄S) linker). An example of such a construct is the FVII-024 construct described herein and shown in Figure 2.

[00271] Additional constructs were made to vary the way in which the heavy and light chains of a clotting factor are expressed. As shown in Figure 7, the heavy
25 and light chain can be expressed as a single chain (FV-011 and FV-003) or separately (FV-010, FVII-013, or FVII-018).

[00272] An additional construct was made to test the ability of FVII to be expressed in activated form. The FVII-025 construct is set forth in Figure 9.

[00273] Another construct in which a different biologically active moiety,
30 IFN β , was employed is set forth in Figure 10 to show that not only clotting factors can be made using the methods of the invention. In making this construct, IFN β was linked to a first Fc moiety using a spacer molecule.

[00274] Failure to express the FVII heavy chain with a free N terminus led to generation of the constructs described in Figure 11. In these constructs, FVIIFc is
35 expressed as a heterodimer where one subunit comprises the FVII light chain and an Fc moiety, and the other subunit comprises the heavy chain preceded by an RKRRKR

- 5 processing site (FVII-019) or by a light chain C terminal fragment and an RKRRKR processing site (FVII-020).

F. Targeting Moieties

[00275] In one embodiment, the antigen binding portion targets the composition
 10 to a particular cell type or tissue. Such targeting moieties may comprise, e.g., an antigen binding site, a ligand binding portion of a receptor, or a receptor binding portion of a ligand. In another embodiment, a targeting moiety is a peptide.

[00276] In one embodiment, a clotting factor of the invention is targeted to
 15 platelets to enhance its efficacy by localizing the clotting factor to the site of coagulation using a "targeting moiety" which binds to a molecule expressed on platelets. Preferably the targeted molecules are not expressed on cells or tissues other than platelets, i.e., the targeting moieties specifically bind to platelets.

[00277] In one embodiment, receptors/conformations found on resting platelets
 20 are targeted. By doing so, sites for coagulation could be primed for enhanced efficacy. Targeting such molecule may also extend half life of the clotting factor and/or prevent clearance. Examples of such targets include, e.g., GpIb of the GpIb/V/IX complex, and GpVI and nonactive form of GPIIb/IIIa.

[00278] In one embodiment, receptors/conformations only found on activated
 25 platelets are targeted in order to localize the clotting factor to site of active coagulation. Examples of such targets include, e.g., the active form of GpIb/IIIa as well as CD62P.

[00279] In one embodiment, a polypeptide of the invention comprises a
 "targeting moiety" which has affinity for and binds to platelets. For example, in one
 embodiment, a targeting moiety binds to the GPIb complex, e.g. GPIb-alpha.
 30 Examples of such targeting moieties include the peptides PS4, OS1, and OS2 which bind to both active and nonactive platelets (Benard et al. 2008 Biochemistry 47:4674); In another embodiment, a targeting moiety binds to the active conformation of GPIIb/IIIa. Examples of such targeting moieties include SCE5 and MB9 variable regions which bind active platelets only (Schwarz et al. 2004 FASEB
 35 Journal express article 10.1096/fj.04-1513fje; Schwarz et al. 2006 Circulation Research. 99:25-33; U.S. Patent publication 20070218067). In another embodiment, a targeting moiety binds to both the active/nonactive conformation of GPIIb/IIIa. An example of such a targeting moiety is the variable region of the AP3 antibody

5 (Peterson et al. 2003. Hemostasis, Thrombosis, and Vascular Biology 101 :937; WO 20101 15866).

[00280] Other platelet targets or targeting moieties which bind to such targets could be readily selected by one of ordinary skill in the art.

10 [00281] The polypeptides of the invention can comprise one or more than one targeting moiety. Exemplary configurations are set forth in the accompanying Figures. Additionally, two or more targeting moieties may be linked to each other (*e.g.*, via a spacer) in series, and the tandem array operably linked to a construct of the invention. When two or more targeting moieties are present in a clotting factor of the invention, the moieties may be the same or different.

15 [00282] In one embodiment, a targeting moiety is fused to a polypeptide of the invention by a cleaveable linker or alternatively, in other embodiment, the polypeptide further comprises a cleavage site. Either of the cleavable linker or the cleavage site may be cleaved to remove the targeting moiety at the site of a clot. In another embodiment, a targeting moiety is attached via a spacer which is not
20 cleaveable and, therefore, is not cleaved at the site of a clot.

[00283] In one embodiment, the targeting moiety is located on the N- or C-terminus of factor VIII. In another embodiment, a targeting moiety is located on the C-terminus of FVII, FIX, FX, or the C-terminus of either or both chains of FVIIa, FFXa, or FXa. In one embodiment the targeting moiety may be positioned at the N or
25 C terminus of the second Fc moiety (F2), or the C-terminus of either or both Fc moieties (F1 and/or F2). The targeting moiety may be linked to the biologically active moiety or Fc moiety via a spacer.

[00284] In one embodiment, a targeting moiety is not fused to a construct via a peptide bond, but rather is chemically conjugated to the construct. For example,
30 targeting moieties may be attached to a construct of the invention by formation of a bond between the targeting moiety and an Fc moiety of a construct, where the targeting moiety comprises a first functional group and the Fc moiety comprises a second functional group, and where the first and second functional groups are capable of reacting with each other to form a chemical bond (see, *e.g.*, U.S. patent 7381408).

35 [00285] Exemplary formats of targeted clotting factors are also set forth in the accompanying Figures.

[00286] In one embodiment, a polypeptide of the invention comprises at least one of an antigen binding site (*e.g.*, an antigen binding site of an antibody, antibody

5 variant, or antibody fragment), a polypeptide, a receptor binding portion of ligand, or a ligand binding portion of a receptor which specifically binds to platelets, e.g., resting or activated platelets. Exemplary targeting moieties include scFv molecules or peptides which bind to molecules to be targeted. Examples of targeting moieties are found in the instant examples.

10 **[00287]** For example, in certain embodiments, a polypeptide of the invention comprises at least one antigen binding portion (e.g., binding site) of an antibody. In one embodiment, the antigen binding portion targets the composition to platelets. Such an antibody may bind to an epitope expressed by all platelets (e.g., activated and inactivated) or may bind to an epitope expressed specifically by activated platelets).

15 **[00288]** Exemplary antibodies from which binding sites can be derived or exemplary antibody binding sites for use in the polypeptide molecules of the invention are known in the art. As set forth above, antibodies known to bind to platelets can be used to derive binding sites, for example, the AP3 antibody or the MB9 scFv described in US 2007/0218067 or the variable region or an scFv molecule
20 comprising the variable region can be used as a targeting moiety in a construct of the invention. Other exemplary antibody binding sites include SCE5 which targets a confirmation found on activated platelets. Other useful antibodies can be readily selected from those known in the art.

[00289] In certain other embodiments, the polypeptides of the invention
25 comprise one or more binding sites derived from a non-immunoglobulin binding molecule. As used herein, the term "non-immunoglobulin binding molecules" are binding molecules whose binding sites comprise a portion (e.g., a scaffold or framework) which is derived from a polypeptide other than an immunoglobulin, but which may be engineered (e.g., mutagenized) to confer a desired binding specificity.

30 **[00290]** Other examples of binding molecules comprising binding sites not derived from antibody molecules include receptor binding sites and ligand binding sites which bind to platelets.

[00291] Non-immunoglobulin binding molecules may be identified by
selection or isolation of a target-binding variant from a library of binding molecules
35 having artificially diversified binding sites. Diversified libraries can be generated using completely random approaches (e.g., error-prone PCR, exon shuffling, or directed evolution) or aided by art-recognized design strategies. For example, amino acid positions that are usually involved when the binding site interacts with its

5 cognate target molecule can be randomized by insertion of degenerate codons, trinucleotides, random peptides, or entire loops at corresponding positions within the nucleic acid which encodes the binding site (see *e.g.*, U.S. Pub. No. 20040132028). The location of the amino acid positions can be identified by investigation of the crystal structure of the binding site in complex with the target molecule. Candidate
10 positions for randomization include loops, flat surfaces, helices, and binding cavities of the binding site. In certain embodiments, amino acids within the binding site that are likely candidates for diversification can be identified using techniques known in the art. Following randomization, the diversified library may then be subjected to a selection or screening procedure to obtain binding molecules with the desired binding
15 characteristics, *e.g.* specific binding platelets using methods known in the art. Selection can be achieved by art-recognized methods such as phage display, yeast display, or ribosome display. In one embodiment, molecules known in the art to bind to platelets may be employed in the constructs of the invention. For example, peptides which bind to GPIIb as described in the art (*e.g.*, PS4, OS1, or OS2) may be
20 used (Benard et al. 2008. *Biochemistry* 47:4674-4682).

III. Preparation of Polypeptides

[00292] A variety of methods are available for producing a polypeptide of the invention. In one embodiment, the invention relates to a nucleic acid construct
25 comprising a nucleic acid sequence encoding a polypeptide molecule of the invention. It will be understood that because of the degeneracy of the code, a variety of nucleic acid sequences will encode the amino acid sequence of the polypeptide. The desired polynucleotide can be produced by *de novo* solid-phase DNA synthesis or by PCR mutagenesis of an earlier prepared polynucleotide encoding the target polypeptide.

30 [00293] Nucleic acids encoding a biologically active molecule can be readily synthesized using recombinant techniques well known in the art. Alternatively, the peptides themselves can be chemically synthesized. Nucleic acids of the invention may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch,
35 Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. 1988, *Nucl. Acids Res.* 16:3209, methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports as described in Sarin et al. 1988, *Proc. Natl. Acad. Sci. USA*

5 85:7448. Additional methods of nucleic acid synthesis are known in the art. (see, e.g., U.S. Pat. Nos. 6,015,881; 6,281,331; 6,469,136).

[00294] Methods for linking desired biologically active moieties, whether derived from antibodies or other molecules, to cleavable scFc scaffolds are known in the art.

10 [00295] Oligonucleotide-mediated mutagenesis is one method for preparing a substitution, in-frame insertion, or alteration (*e.g.*, altered codon) to introduce a codon encoding an amino acid substitution (*e.g.*, into an Fc variant moiety). For example, the starting polypeptide DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a single-stranded DNA template. After hybridization, a DNA
15 polymerase is used to synthesize an entire second complementary strand of the template that incorporates the oligonucleotide primer. In one embodiment, genetic engineering, *e.g.*, primer-based PCR mutagenesis, is sufficient to incorporate an alteration, as defined herein, for producing a polynucleotide encoding a polypeptide of the invention.

20 [00296] For recombinant production, a polynucleotide sequence encoding the polypeptide is inserted into an appropriate expression vehicle, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence, or in the case of an RNA viral vector, the necessary elements for replication and translation.

25 [00297] In one embodiment, a nucleic acid molecule encoding the protein is inserted into the vector in proper reading frame. The expression vector is then transfected into a suitable target cell which will express the polypeptide. Transfection techniques known in the art include, but are not limited to, calcium phosphate precipitation (Wigler et al. 1978, Cell 14 : 725) and electroporation (Neumann et al.
30 1982, EMBO, J. 1 : 841). A variety of host-expression vector systems may be utilized to express the proteins described herein in eukaryotic cells. In one embodiment, the eukaryotic cell is an animal cell, including mammalian cells (*e.g.* 293 cells, PerC6, CHO, BHK, Cos, HeLa cells). When the protein is expressed in a eukaryotic cell the DNA encoding the protein may also code for a signal sequence that will permit the
35 protein to be secreted. One skilled in the art will understand that while the protein is translated the signal sequence is cleaved by the cell to form the mature protein. Various signal sequences are known in the art *e.g.*, native factor VII signal sequence, native factor IX signal sequence and the mouse IgK light chain signal sequence.

5 Alternatively, where a signal sequence is not included the protein can be recovered by lysing the cells.

[00298] The protein of the invention can be synthesized in a transgenic animal, such as a rodent, goat, sheep, pig, or cow. The term "transgenic animals" refers to non-human animals that have incorporated a foreign gene into their genome. Because
10 this gene is present in germline tissues, it is passed from parent to offspring. Exogenous genes are introduced into single-celled embryos (Brinster et al. 1985, Proc. Natl. Acad.Sci. USA 82 : 4438). Methods of producing transgenic animals are known in the art. including transgenics that produce immunoglobulin molecules (Wagner et al. 1981 , Proc. Natl. Acad. Sci. USA 78: 6376; McKnight et al. 1983, Cell
15 34 : 335 ; Brinster et al. 1983, Nature 306: 332; Ritchie et al. 1984, Nature 312: 517; Baldassarre et al. 2003, Theriogenology 59 : 831 ; Robl et al. 2003, Theriogenology 59: 107; Malassagne et al. 2003, Xenotransplantation 10 (3): 267).

[00299] The expression vectors can encode for tags that permit for easy purification or identification of the recombinantly produced protein. Examples
20 include, but are not limited to, vector pUR278 (Ruther et al. 1983, EMBO J. 2: 1791) in which the protein described herein coding sequence may be ligated into the vector in frame with the lac z coding region so that a hybrid protein is produced; pGEX vectors may be used to express proteins with a glutathione S-transferase (GST) tag. These proteins are usually soluble and can easily be purified from cells by adsorption
25 to glutathione-agarose beads followed by elution in the presence of free glutathione. The vectors include cleavage sites (e. g. PreCission Protease (Pharmacia, Peapack, N. J.)) for easy removal of the tag after purification.

[00300] For the purposes of this invention, numerous expression vector systems may be employed. These expression vectors are typically replicable in the host
30 organisms either as episomes or as an integral part of the host chromosomal DNA. Expression vectors may include expression control sequences including, but not limited to, promoters (*e.g.* , naturally-associated or heterologous promoters), enhancers, signal sequences, splice signals, enhancer elements, and transcription termination sequences. Preferably, the expression control sequences are eukaryotic
35 promoter systems in vectors capable of transforming or transfecting eukaryotic host cells. Expression vectors may also utilize DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MOMLV), cytomegalovirus

5 (CMV), or SV40 virus. Others involve the use of polycistronic systems with internal ribosome binding sites.

[00301] Commonly, expression vectors contain selection markers (*e.g.*, ampicillin-resistance, hygromycin-resistance, tetracycline resistance or neomycin resistance) to permit detection of those cells transformed with the desired DNA
10 sequences (see, *e.g.*, Itakura *et al.*, US Patent 4,704,362). Cells which have integrated the DNA into their chromosomes may be selected by introducing one or more markers which allow selection of transfected host cells. The marker may provide for prototrophy to an auxotrophic host, biocide resistance (*e.g.*, antibiotics) or resistance to heavy metals such as copper. The selectable marker gene can either be
15 directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation.

[00302] An exemplary expression vector is NEOSPLA (U.S. Patent No. 6,159,730). This vector contains the cytomegalovirus promoter/enhancer, the mouse beta globin major promoter, the SV40 origin of replication, the bovine growth
20 hormone polyadenylation sequence, neomycin phosphotransferase exon 1 and exon 2, the dihydrofolate reductase gene and leader sequence. This vector has been found to result in very high level expression of antibodies upon incorporation of variable and constant region genes, transfection in cells, followed by selection in G418 containing medium and methotrexate amplification. Vector systems are also taught in U.S. Pat.
25 Nos. 5,736,137 and 5,658,570, each of which is incorporated by reference in its entirety herein. This system provides for high expression levels, *e.g.*, > 30 pg/cell/day. Other exemplary vector systems are disclosed *e.g.*, in U.S. Patent No. 6,413,777.

[00303] In other embodiments the polypeptides of the invention of the instant
30 invention may be expressed using polycistronic constructs. In these expression systems, multiple gene products of interest such as multiple polypeptides of multimer binding protein may be produced from a single polycistronic construct. These systems advantageously use an internal ribosome entry site (IRES) to provide relatively high levels of polypeptides of the invention in eukaryotic host cells.
35 Compatible IRES sequences are disclosed in U.S. Pat. No. 6,193,980 which is also incorporated herein. Those skilled in the art will appreciate that such expression systems may be used to effectively produce the full range of polypeptides disclosed in the instant application.

5 **[00304]** More generally, once the vector or DNA sequence encoding a polypeptide has been prepared, the expression vector may be introduced into an appropriate host cell. That is, the host cells may be transformed. Introduction of the plasmid into the host cell can be accomplished by various techniques well known to those of skill in the art. These include, but are not limited to, transfection (including
10 electrophoresis and electroporation), protoplast fusion, calcium phosphate precipitation, cell fusion with enveloped DNA, microinjection, and infection with intact virus. See, Ridgway, A. A. G. *"Mammalian Expression Vectors"* Chapter 24.2, pp. 470-472 Vectors, Rodriguez and Denhardt, Eds. (Butterworths, Boston, Mass. 1988). Most preferably, plasmid introduction into the host is via electroporation. The
15 transformed cells are grown under conditions appropriate to the production of the light chains and heavy chains, and assayed for heavy and/or light chain protein synthesis. Exemplary assay techniques include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), or fluorescence-activated cell sorter analysis (FACS), immunohistochemistry and the like.

20 **[00305]** As used herein, the term "transformation" shall be used in a broad sense to refer to the introduction of DNA into a recipient host cell that changes the genotype and consequently results in a change in the recipient cell.

[00306] Along those same lines, "host cells" refers to cells that have been transformed with vectors constructed using recombinant DNA techniques and
25 encoding at least one heterologous gene. In descriptions of processes for isolation of polypeptides from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of polypeptide unless it is clearly specified otherwise. In other words, recovery of polypeptide from the "cells" may mean either from spun down whole cells, or from the cell culture containing both the medium and
30 the suspended cells.

[00307] The host cell line used for protein expression is most preferably of mammalian origin; those skilled in the art are credited with ability to preferentially determine particular host cell lines which are best suited for the desired gene product to be expressed therein. Exemplary host cell lines include, but are not limited to,
35 CHO, e.g., DG44 and DUXB1 1 (Chinese Hamster Ovary lines, DHFR minus), HELA (human cervical carcinoma), CV1 (monkey kidney line), COS (a derivative of CV1 with SV40 T antigen), R1610 (Chinese hamster fibroblast) BALBC/3T3 (mouse fibroblast), HAK (hamster kidney line), SP2/0 (mouse myeloma), P3.times.63-

5 Ag3.653 (mouse myeloma), BFA-lcIBPT (bovine endothelial cells), RAJI (human lymphocyte), PerC6, and 293 (human kidney). Host cell lines are typically available from commercial services, the American Tissue Culture Collection or from published literature.

[00308] In one embodiment, a host cell endogenously expresses an enzyme (or
10 the enzymes) necessary to cleave the polypeptide linker (L) during processing to form the mature polypeptide. During this processing, the polypeptide linker (L) is substantially removed to reduce the presence of extraneous amino acids. In another embodiment of the invention, a host cell is transformed to express one or more enzymes which are heterologous or exogenous to the cell such that processing of the
15 polypeptide linker (L) occurs or is improved.

[00309] In one embodiment an enzyme which may be endogenously or exogenously expressed by a cell is a member of the furin family of enzymes. Complete cDNA and amino acid sequences of human furin (i.e., PACE) were published in 1990. Van den Ouweland A M et al. (1990) Nucleic Acids Res. 18:664;
20 Erratum in: Nucleic Acids Res. 18:1332 (1990).

[00310] U.S. Pat. No. 5,460,950, issued to Barr et al., describes recombinant PACE and the coexpression of PACE with a substrate precursor polypeptide of a heterologous protein to improve expression of active, mature heterologous protein.

[00311] U.S. Pat. No. 5,935,815, issued to van de Ven et al., likewise describes
25 recombinant human furin (i.e., PACE) and the coexpression of furin with a substrate precursor polypeptide of a heterologous protein to improve expression of active, mature heterologous protein. Possible substrate precursors disclosed in this patent include a precursor of Factor IX. Other family members in the mammalian furin/subtilisin/Kex2p-like proprotein convertase (PC) family in addition to PACE are
30 reported to include PC1/PC3, PC2, PC4, PC5/6 (hereinafter referred to simply as PC5), PACE4, and LPC/PC7/PC8/SPC7. While these various members share certain conserved overall structural features, they differ in their tissue distribution, subcellular localization, cleavage specificities, and preferred substrates. For a review, see Nakayama K (1997) Biochem J. 327:625-35. Similar to PACE, these proprotein
35 convertases generally include, beginning from the amino terminus, a signal peptide, a propeptide (that may be autocatalytically cleaved), a subtilisin-like catalytic domain characterized by Asp, His, Ser, and Asn/Asp residues, and a Homo B domain that is also essential for catalytic activity and characterized by an Arg-Gly-Asp (ROD)

5 sequence. PACE, PACE4, and PC5 also include a Cys-rich domain, the function of which is unknown. In addition, PC5 has isoforms with and without a transmembrane domain; these different isoforms are known as PC5B and PC5A, respectively. Comparison between the amino acid sequence of the catalytic domain of PACE and the amino acid sequences of the catalytic domains of other members of this family of
10 proprotein convertases reveals the following degrees of identity: 70 percent for PC4; 65 percent for PACE4 and PC5; 61 percent for PC1/PC3; 54 percent for PC2; and 51 percent for LPC/PC7/PC8/SPC7. Nakayama K (1997) Biochem J. 327:625-35.

[00312] PACE and PACE4 have been reported to have partially overlapping but distinct substrates. In particular, PACE4, in striking contrast to PACE, has been
15 reported to be incapable of processing the precursor polypeptide of FIX. Wasley L C et al. (1993) J Biol Chem. 268:8458-65; Rehemtulla A et al. (1993) Biochemistry. 32:11586-90.

[00313] U.S. Pat. No. 5,840,529, issued to Seidah et al., discloses nucleotide and amino acid sequences for human PC7 and the notable ability of PC7, as compared
20 to other PC family members, to cleave HIV gpl60 to gpl20 and gp41.

[00314] Nucleotide and amino acid sequences of rodent PC5 were first described as PC5 by Lusson J et al. (1993) Proc Natl Acad Sci USA 90:6691-5 and as PC6 by Nakagawa T et al. (1993) J Biochem (Tokyo) 113:132-5. U.S. Pat. No. 6,380,171, issued to Day et al., discloses nucleotide and amino acid sequences for
25 human PC5A, the isoform without the transmembrane domain (see, e.g., US Patets 7,795,400 and 7,566,595).

[00315] Genes encoding the polypeptides of the invention can also be expressed in non-mammalian cells such as bacteria or yeast or plant cells. In this regard it will be appreciated that various unicellular non-mammalian microorganisms
30 such as bacteria can also be transformed; i.e., those capable of being grown in cultures or fermentation. Bacteria, which are susceptible to transformation, include members of the enterobacteriaceae, such as strains of Escherichia coli or Salmonella; Bacillaceae, such as Bacillus subtilis; Pneumococcus; Streptococcus, and Haemophilus influenzae. It will further be appreciated that, when expressed in
35 bacteria, the polypeptides typically become part of inclusion bodies. The polypeptides must be isolated, purified and then assembled into functional molecules.

[00316] In addition to prokaryotes, eukaryotic microbes may also be used. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used

5 among eukaryotic microorganisms although a number of other strains are commonly available.

For expression in *Saccharomyces*, the plasmid YRp7, for example, (Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)) is commonly used. This plasmid already contains the TRP1 gene which
10 provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, Genetics, 85:12 (1977)). The presence of the *trp1* lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

15 Other yeast hosts such *Pichia* may also be employed. Yeast expression vectors having expression control sequences (*e.g.*, promoters), an origin of replication, termination sequences and the like as desired. Typical promoters include 3-phosphoglycerate kinase and other glycolytic enzymes. Inducible yeast promoters include, among others, promoters from alcohol dehydrogenase, isocytochrome C, and
20 enzymes responsible for methanol, maltose, and galactose utilization.

[00317] Alternatively, polypeptide-coding nucleotide sequences can be incorporated in transgenes for introduction into the genome of a transgenic animal and subsequent expression in the milk of the transgenic animal (see, *e.g.*, Deboer *et al.*, US 5,741,957, Rosen, US 5,304,489, and Meade *et al.*, US 5,849,992). Suitable
25 transgenes include coding sequences for polypeptides in operable linkage with a promoter and enhancer from a mammary gland specific gene, such as casein or beta lactoglobulin.

[00318] *In vitro* production allows scale-up to give large amounts of the desired polypeptides. Techniques for mammalian cell cultivation under tissue culture
30 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
35 chromatography, chromatography over DEAE-cellulose or (immuno-)affinity chromatography, *e.g.*, after preferential biosynthesis of a synthetic hinge region polypeptide or prior to or subsequent to the HIC chromatography step described

5 herein. An affinity tag sequence (*e.g.* a His(6) tag) may optionally be attached or included within the polypeptide sequence to facilitate downstream purification.

[00319] In one embodiment, a host cell of the invention can comprise a genetic construct encoding a scFc polypeptide and one or more enzymes that can cleave a cscFc linker (L). The construct and the enzyme(s) can be expressed using a single
10 vector or two vectors. When the polypeptides are cloned on separate expression vectors, the vectors are co-transfected to obtain expression and assembly of intact whole proteins.

[00320] The subject methods result in a population of mature proteins that is substantially enriched for the desired heterodimeric two chain mature protein as
15 compared to prior art methods. In one embodiment, a mature polypeptide composition of the invention substantially lacks unprocessed (*i.e.*, single chain forms of the polypeptide). In one embodiment, the cell culture medium in which host cells expressing the polypeptides of the invention comprises a population of polypeptide which substantially lacks unprocessed (*i.e.*, single chain forms) of the polypeptide,
20 thereby simplifying purification. In another embodiment, the cell culture medium in which host cells expressing the polypeptides of the invention comprises a population of polypeptide which is enriched for active forms of a biologically active forms of a molecule. For example, in one embodiment, expression of a heterodimeric polypeptide of the invention using a cscFc linker allows for expression of active
25 forms of molecules, *e.g.*, clotting factors, without the need to activate them in an additional step.

[00321] Once expressed, the mature two chain protein can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity column chromatography, HPLC purification, gel electrophoresis
30 and the like (see generally Scopes, Protein Purification (Springer-Verlag, N.Y., (1982)). Substantially pure proteins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses.

[00322] *In vitro* production allows scale-up to give large amounts of the desired
35 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

5 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.

10 **IV. Purification of Polypeptides**

[00323] In one embodiment, the invention pertains to a method of purification of mature polypeptide molecules of the invention which are expressed as double-chain (i.e., dimeric) comprising an Fc region that is not genetically-fused Fc. Such molecules can be separated from unprocessed molecules comprising a genetically-fused Fc region as well as other contaminants. Methods for purification are known in
15 the art and include, e.g., size-exclusion chromatography, gel filtration column, SDS-PAGE, etc. The invention also pertains to purified populations of double-chain molecules.

20 **V. Labeling or Conjugation of Functional Moieties to Polypeptides**

[00324] The polypeptides of the present invention may be used in non-conjugated form or may be conjugated to at least one of a variety of functional moieties, e.g., to facilitate target detection or for imaging or therapy of the patient. The polypeptides of the invention can be labeled or conjugated either before or after
25 purification, when purification is performed. In particular, the polypeptides of the present invention may be conjugated (*e.g.* , via an engineered cysteine residue) to a functional moiety. Functional moieties are preferably attached to a portion of the polypeptide other than a binding site (*e.g.* , a polypeptide linker or an Fc moiety of a genetically-fused Fc region (i.e., a cscFc region)).

30 [00325] Exemplary functional moieties include cytotoxins (such as radioisotopes, cytotoxic drugs, or toxins) therapeutic agents, cytostatic agents, biological toxins, prodrugs, peptides, proteins, enzymes, viruses, lipids, and biological response modifiers,

35 **VI. Methods of Administering Polypeptides of the Invention**

[00326] Methods of preparing and administering polypeptides of the invention to a subject are well known to or are readily determined by those skilled in the art.

5 **[00327]** Compositions for administration to a subject include nucleic acid molecules which comprise a nucleotide sequence encoding a molecule of the invention whether processed or unprocessed (for gene therapy applications) as well as polypeptide molecules.

10 **[00328]** The route of administration of the polypeptides of the invention may be oral, parenteral, by inhalation or topical. The term parenteral as used herein includes intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, rectal or vaginal administration. The intravenous, intraarterial, subcutaneous and intramuscular forms of parenteral administration are generally preferred. While all these forms of administration are clearly contemplated as being within the scope of
15 the invention, a form for administration would be a solution for injection, in particular for intravenous or intraarterial injection or drip. Usually, a suitable pharmaceutical composition for injection may comprise a buffer (e.g. acetate, phosphate or citrate buffer), a surfactant (e.g. polysorbate), optionally a stabilizer agent (e.g. human albumin), etc. However, in other methods compatible with the teachings herein, the
20 polypeptides can be delivered directly to the site of the adverse cellular population thereby increasing the exposure of the diseased tissue to the therapeutic agent.

25 **[00329]** Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. In the subject invention, pharmaceutically acceptable carriers include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Other common parenteral vehicles include sodium phosphate solutions, Ringer's
30 dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present such as for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like.

35 **[00330]** More particularly, pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In such cases, the composition must be sterile and should be fluid to the

5 extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and will preferably be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity 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.

[00331] Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, 15 ascorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols, such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

20 [00332] In any case, sterile injectable solutions can be prepared by incorporating an active compound (*e.g.*, a polypeptide by itself or in combination with other active agents) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active 25 compound into a sterile vehicle, which 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, which yields a powder of an active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparations for injections are processed, filled into containers 30 such as ampoules, bags, bottles, syringes or vials, and sealed under aseptic conditions according to methods known in the art. Further, the preparations may be packaged and sold in the form of a kit such as those described in co-pending U.S.S.N. 09/259,337 and U.S.S.N. 09/259,338 each of which is incorporated herein by reference. Such articles of manufacture will preferably have labels or package inserts 35 indicating that the associated compositions are useful for treating a subject suffering from, or predisposed to autoimmune or neoplastic disorders.

5 [00333] Effective doses of the compositions of the present invention, for the treatment of conditions vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human but non-human mammals including transgenic mammals can also be treated. Treatment dosages may be titrated using routine methods known to those of skill in the art to optimize safety and efficacy.

[00334] In one embodiment, the dose of a biologically active moiety (e.g., comprising FIX) can range from about 25 to 100 IU/kg, e.g., 0.417 mg/kg to 1.67 mg/kg. In another embodiment, the dose of a biologically active moiety (e.g., comprising FVIII) can range from about 25 to 65 IU/kg, e.g., 0.003 125 mg/kg to 0.008 125 mg/kg. In another embodiment, the dose of a biologically active moiety (e.g., comprising FVII), can range from about 90 to 270 ug/kg or 0.090 to 0.270 mg/kg.

20 [00335] In another embodiment, the dosage can range, e.g., from about 1000 ug/kg to 0.1 ng/kg body weight. In one embodiment, the dosing range is lug/kg to 100 ug/kg. In another embodiment, doses can range from 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg (e.g., 0.02 mg/kg, 0.25 mg/kg, 0.5 mg/kg, 0.75 mg/kg, 1mg/kg, 2 mg/kg, etc.), of the host body weight. For example dosages can be 1 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg, preferably at least 1 mg/kg.

[00336] Doses intermediate in the above ranges are also intended to be within the scope of the invention. Subjects can be administered such doses daily, on alternative days, weekly or according to any other schedule determined by empirical analysis. An exemplary treatment entails administration in multiple dosages over a prolonged period, for example, of at least six months. In one embodiment, additional exemplary treatment regimes entail administration once per every two weeks or once a month or once every 3 to 6 months. Exemplary dosage schedules include 1-10 mg/kg or 15 mg/kg on consecutive days, 30 mg/kg on alternate days or 60 mg/kg weekly. In some methods, two or more polypeptides with different binding specificities are administered simultaneously, in which case the dosage of each polypeptide administered falls within the ranges indicated.

5 **[00337]** Polypeptides of the invention can be administered on multiple occasions. Intervals between single dosages can be weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of modified polypeptide or antigen in the patient. In some methods, dosage is adjusted to achieve a plasma modified polypeptide concentration of 1-1000 µg/ml and in some methods
10 25-300 µg/ml. Alternatively, polypeptides can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the polypeptide in the patient.

[00338] The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications,
15 compositions containing the polypeptides 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
20 dosages of from 5 to 25 mg being more commonly used for radioimmunoconjugates 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 preferably until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, the patient can be administered a prophylactic
25 regime.

[00339] Polypeptides of the invention can optionally be administered in combination with other agents that are effective in treating the disorder or condition in need of treatment (*e.g.*, prophylactic or therapeutic).

[00340] While the polypeptides of the invention may be administered as
30 described immediately above, it must be emphasized that, in other embodiments, polypeptides may be administered to otherwise healthy patients as a first line therapy. In such embodiments the polypeptides may be administered to patients having normal or average red marrow reserves and/or to patients that have not, and are not, undergoing. As used herein, the administration of polypeptides of the invention in
35 conjunction or combination with an adjunct therapy means the sequential, simultaneous, coextensive, concurrent, concomitant or contemporaneous administration or application of the therapy and the disclosed polypeptides. Those

5 skilled in the art will appreciate that the administration or application of the various components of the combined therapeutic regimen may be timed to enhance the overall effectiveness of the treatment. For example, chemotherapeutic or biologic agents could be administered in standard, well known courses of treatment in conjunction with the subject molecules. A skilled artisan (e.g. a physician) would be readily be
10 able to discern effective combined therapeutic regimens without undue experimentation based on the selected adjunct therapy and the teachings of the instant specification.

[00341] In this regard it will be appreciated that the combination of the polypeptide and the agent may be administered in any order and within any time
15 frame that provides a therapeutic benefit to the patient. That is, the agent and polypeptide may be administered in any order or concurrently. In selected embodiments the polypeptides of the present invention will be administered to patients that have previously undergone chemotherapy. In yet other embodiments, the polypeptides and the chemotherapeutic treatment will be administered substantially
20 simultaneously or concurrently. For example, the patient may be given the polypeptide while undergoing a course of chemotherapy. In preferred embodiments the polypeptide will be administered within 1 year of any agent or treatment. In other preferred embodiments the polypeptide will be administered within 10, 8, 6, 4, or 2 months of any agent or treatment. In still other preferred embodiments the
25 polypeptide will be administered within 4, 3, 2 or 1 week of any agent or treatment. In yet other embodiments the polypeptide will be administered within 5, 4, 3, 2 or 1 days of the selected agent or treatment. It will further be appreciated that the two agents or treatments may be administered to the patient within a matter of hours or minutes (i.e. substantially simultaneously).

30 [00342] In one embodiment, a polypeptide of the invention can be administered as a nucleic acid molecule. Nucleic acid molecules can be administered using techniques known in the art, including via vector, plasmid, liposome, DNA injection, electroporation, gene gun, intravenously injection or hepatic artery infusion. Vectors for use in gene therapy embodiments are known in the art.

35 [00343] It will further be appreciated that the molecules of the instant invention may be used in conjunction or combination with an agent or agents (e.g. to provide a combined therapeutic regimen). Exemplary agents with which a molecule of the invention may be combined include agents that represent the current standard of care

5 for a particular disorder being treated. Such agents may be chemical or biologic in nature. The term "biologic" or "biologic agent" refers to any pharmaceutically active agent made from living organisms and/or their products which is intended for use as a therapeutic.

[00344] The amount of agent to be used in combination with the polypeptides
10 of the instant invention may vary by subject or may be administered according to what is known in the art. See for example, Bruce A Chabner *et al.*, *Antineoplastic Agents*, in GOODMAN & OILMAN'S THE PHARMACOLOGICAL BASIS OF THERAPEUTICS 1233-1287 ((Joel G. Hardman *et al.*, eds., 9th ed. 1996). In another embodiment, an amount of such an agent consistent with the standard of care is administered.

15 [00345] As previously discussed, the polypeptides of the present invention, may be administered in a pharmaceutically effective amount for the *in vivo* treatment of mammalian disorders. In this regard, it will be appreciated that the molecule of the invention can be formulated to facilitate administration and promote stability of the active agent. Preferably, pharmaceutical compositions in accordance with the present
20 invention comprise a pharmaceutically acceptable, non-toxic, sterile carrier such as physiological saline, non-toxic buffers, preservatives and the like. For the purposes of the instant application, a pharmaceutically effective amount of a polypeptide of the invention, conjugated or unconjugated to a therapeutic agent, shall be held to mean an amount sufficient to achieve a benefit, e.g., to ameliorate symptoms of a disease or
25 disorder or to detect a substance or a cell. Of course, the pharmaceutical compositions of the present invention may be administered in single or multiple doses to provide for a pharmaceutically effective amount of the polypeptide.

[00346] The polypeptides of the invention have many uses as will be recognized by one skilled in the art, including, but not limited to methods of treating a
30 subject with a disease or condition. In keeping with the scope of the present disclosure, the molecule of the invention may be administered to a human or other animal in accordance with the aforementioned methods of treatment in an amount sufficient to produce a therapeutic or prophylactic effect. It will be understood that the type of disorders that can be treated depend upon the biologically active moiety
35 present in the polypeptide and the known biological effects of the biologically active moiety. Given the modular nature of the disclosed polypeptides, biologically active moieties can be selected by those of skill in the art and placed into a scFc scaffold with a cscFc linker according to the claimed invention.

5 **[00347]** In one embodiment, when the polypeptide comprises a clotting factor as a biologically active moiety, the invention relates to a method of treating a subject having a hemostatic disorder comprising administering a therapeutically effective amount of at least one polypeptides of the invention.

10 **[00348]** Polypeptides of the invention which comprise a clotting factor can be used to treat or prevent a hemostatic disorder by promoting the formation of a fibrin clot. The polypeptides of the invention can be used to treat hemostatic disorders, e.g., those known to be treatable with the particular clotting factor present in the polypeptide. The hemostatic disorders that may be treated by administration of the chimeric protein of the invention include, but are not limited to, hemophilia A,
15 hemophilia B, von Willebrand's disease, Factor XI deficiency (PTA deficiency), Factor XII deficiency, as well as deficiencies or structural abnormalities in fibrinogen, prothrombin, Factor V, Factor VII, Factor X, or Factor XIII.

20 **[00349]** In one embodiment, the hemostatic disorder is an inherited disorder. In one embodiment, the subject has hemophilia A, and the polypeptides comprises Factor VII or Factor VIII. In another embodiment, the subject has hemophilia A and the chimeric protein comprises Factor VII or Factor VIII. In another embodiment, the subject has hemophilia B and the chimeric protein comprises Factor IX or Factor FXa. In another embodiment, the subject has hemophilia B and the polypeptides comprises Factor VII or Factor VIII. In another embodiment, the subject has inhibitory
25 antibodies to Factor VII or Factor VIII and the polypeptides comprises Factor VII or Factor VIII. In yet another embodiment, the subject has inhibitory antibodies against Factor IX or Factor FXa and the polypeptides comprises Factor VII or Factor VIII.

30 **[00350]** The polypeptides of the invention can be used to prophylactically treat a subject with a hemostatic disorder. The polypeptides of the invention can be used to treat an acute bleeding episode in a subject with a hemostatic disorder.

35 **[00351]** In one embodiment, the hemostatic disorder is the result of a deficiency in a clotting factor, e.g., Factor IX, Factor VIII. In another embodiment, the hemostatic disorder can be the result of a defective clotting factor.

40 **[00352]** In another embodiment, the hemostatic disorder can be 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 autoimmune disease, or pregnancy. The acquired disorder can result from

5 old age or from medication to treat an underlying secondary disorder (e.g. cancer chemotherapy).

[00353] The invention also relates to methods of treating a subject that does not have a hemostatic disorder or a secondary disease or condition resulting in acquisition of a hemostatic disorder. The invention thus relates to a method of treating a subject
10 in need of a general hemostatic agent comprising administering a therapeutically effective amount of at least one polypeptide of the invention. For example, in one embodiment, the subject in need of a general hemostatic agent is undergoing, or is about to undergo, surgery. The polypeptides factor of the invention can be administered prior to or after surgery as a prophylactic. The polypeptides of the
15 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, or stem cell transplantation.

[00354] In another embodiment, the polypeptides of the invention can be used to treat a subject having an acute bleeding episode who does not have a hemostatic
20 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.

[00355] This invention is further illustrated by the following examples which
25 should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

5

EXAMPLES

[00356] Throughout the examples, the following materials and methods were used unless otherwise stated.

10 **Example 1: Cloning of pSYN-FVII-024**

[00357] The coding sequence of FVII was obtained by reverse transcription coupled to polymerase chain reaction from a human liver mRNA library (Ambion, Austin, Texas) using the following primers:

FVII-F1

15 GGG AATGTCAACAGGCAGGG (SEQ ID NO: 41)

FVII-R1

CTTGGCTTTCTCTCCACAGGC (SEQ ID NO:42)

[00358] A 50 µl reaction was carried out with 10 pmol of each primer using the Superscript One-step RT-PCR with Platinum Taq system (Invitrogen, Carlsbad,

20 Calif.) according to the manufacturer's standard protocol in a MJ thermocycler. The cycle used was 50° C for 30 minutes for the reverse transcription followed by denaturing at 94° C for 2 minutes and 30 cycles of (94° C 30 seconds, 53° C 30 seconds, 72° C 90 seconds) followed by 10 minutes at 72° C The expected sized band (-1400 bp) was gel-purified with a Gel Extraction kit (Qiagen, Valencia, Calif.) and
25 cloned in pCR2.1 TOPO using the TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif.) to produce the intermediate plasmid pSYN-FVII-001. To construct a plasmid for the expression of a two-chain FVII-Fc and Fc heterodimer, the FVII coding sequence was PCR-amplified using the following primers:

HindIII-Kozak-FVII-F

30 CGACAAGCTTGCCGCCACCATGGTCTCCCAGGCCCTCAGG (SEQ ID NO: 43)

BspEI-Fc-FVII-R

CGACTCCGGAGCTGGGCACGGTGGGCATGTGTGAGTTTTGTCGGGAAATG
GGGCTCGCAGG (SEQ ID NO: 44)

35

[00359] The forward primer HindIII-Kozak-FVII-F adds a HindIII restriction site followed by a Kozak sequence immediately upstream of the FVII coding region.

5 The reverse primer BspEI-Fc-FVII-R adds a fragment of the constant region of IgG1 (the Fc region) comprising amino acids 221-233 (EU numbering). This process also incorporates a BspEI restriction site at amino acids 231-233 using the degeneracy of the genetic code to preserve the correct amino acid sequence (EU numbering). A 50 ul reaction was carried out with 15 pmol of each primer and template pSYN-FVII-001 using Platinum Pfx DNA Polymerase system according to manufacturer's protocol in a MJ Thermocycler using the following cycles: 95° C 2 minutes; 30 cycles of (95° C 15 seconds, 49° C 30 seconds, 68° C 90 seconds); 68° C 10 minutes. Plasmid pSYN-FIX-027 (pBUD FIXFc/Fc) was digested with HindIII and BspEI and the expected sized band for the vector (approximately 5800 bp) was purified away from the FIX insert (expected size band approximately 1480 bp) with a Gel Extraction kit (Qiagen, Valencia, Calif.). Next, the PCR-amplified FVII sequence was subcloned into HindIII and EcoRI sites of the vector derived from pSYN-FFX-027 after removing the FFX insert. This generated pSYN-FVII-002 (pBUD FVIIFc/Fc). Next, A (GGGGS)₆x polypeptide linker was added between FVII and the Fc region coding sequences in pSYN-FVII-002 using the following primers:

FVII-linker-F:

CATCCCCAGCACGTACGTCC (SEQ ID NO: 45)

FVII-Linker-R:

GGGCATGTGTGAGTTTTGTCTGATCCCCCGCCACCGGAACCTCCACCGCCT

25 GATCCACCCCCACCTGATCCGCCGCCACCGGACCCACCTCCGCCGGAGCC
ACCGCCACCGGGAATGGGGCTCGCAGGAGG (SEQ ID NO: 46)

Fc-linker-F:

GACAAAACTCACACATGCCCACC (SEQ ID NO: 47)

Fc-linker-R:

30 GCAGAATTCTCATTTACCCGGAG (SEQ ID NO: 48)

[00360] Two 12 μl PCR reactions were carried out with either 12 pmol of FVII-linker-F and FVII-Linker-R (reaction 1) or Fc-linker-F and Fc-linker-R (reaction 2) using Expand High Fidelity System (Boehringer Mannheim, Indianapolis, Ind.) according to manufacturer's standard protocol in a MJ Thermocycler. The first and second reactions were carried out with 1 μg of pSYN-FVII-002 as template using the following cycle: 94 °C. 2 minutes; 14 cycles of (94°C. 30 seconds, 55°C. 30 seconds, 72°C. 2 minutes); 72°C. 10 minutes. The expected sized bands (532 bp for reaction 1

5 and 670 bp for reaction 2) were gel purified with a Gel Extraction kit (Qiagen, Valencia, Calif.), then combined in a PCR reaction with 25 pmol of FVII-linker-F and Fc-linker-R as before, but with 30 rounds of amplification. The expected sized band (1200 bp) was gel purified with a Gel Extraction kit (Qiagen, Valencia, Calif.) and digested with restriction enzymes KpnI and EcoRI. The expected sized band (920 bp) was gel purified as before and cloned into the KpnI/EcoRI sites of pSYN-FVII-002 to generate pSYN-FVII-003 (pBUD FVIIFc/6x(GGGGS) (SEQ ID NO: 36)/Fc).

Cloning of pSYN-FVII-024 to express a two-chain heterodimer

[00361] Plasmid (pSYN-FVII-024) was generated for the expression of a two-chain heterodimer where one chain consists of the FVII light chain (residues 1-152) followed by a (GGGGS)_{6x} (SEQ ID NO: 36) linker followed by the Fc region, while the other chain contains a FVII heavy chain (residues 153 to 406) followed by a (GGGGS)_{6x} (SEQ ID NO: 36) linker followed by the Fc region. The plasmid is designed to express the heterodimer as a single polypeptide where the C-terminus of the FVII heavy chain-linker-Fc chain is connected to the N-terminus of the heavy chain-linker-Fc chain by the following polypeptide sequence: RRRRS-(GGGGS)_{6x}-RKRRKR (SEQ ID NO: 50), where the RRRRS (SEQ ID NO: 38) and RKRRKR (SEQ ID NO: 39) sequences are proprotein convertase cleavage sites. Intracellular cleavage by proprotein convertases following the last Arg at each cleavage site can result in removal of the polypeptide linker. Consequently, cells will express a 2 chain heterodimer where the FVII light chain-linker-Fc chain has a RRRRS (SEQ ID NO: 38) sequence at the C-terminus, but the remainder of the linker and the RKRRKR (SEQ ID NO: 39) sequence have otherwise been removed. Construction of the pSYN-FVII-024 and several intermediate plasmids required the use of the following primers:

30 HindIII-Sall-BpEI-Fc-F
AGTCAAGCTTGTCTGACTCCGGAACCTCCTGGGCGGACC (SEQ ID NO: 51)
BamHI-linker(PACEI)-Fc-R
CATCGGATCCCCCGCCACCGGAACCTCCACCGCCTGATCCACCCCCACCT
GATCCGCCGCCACCGCTCCGGCGGCGCCGTTTACCCGGAGACAGGGAGAG
35 G (SEQ ID NO: 52)
HindIII-Kozak-FVII-F
CGACAAGCTTGCCGCCACCATGGTCTCCCAGGCCCTCAGG (SEQ ID NO: 43)
43)

5 BspEI-Fc-linker-FVIII-C-R

GAGTTCCGGAGCTGGGCACGGTGGGCATGTGTGAGTTTTGTCTGATCCCCC
 GCCACCGGAACCTCCACCGCCTGATCCACCCCCACCTGATCCGCCGCCAC
 CGGACCCACCTCCGCCGGAGCCACCGCCACCTCGGCCTTGGGGTTTGCTG
 G (SEQ ID NO: 53)

10 BamHI-2xlink-pace-HC-F

CAGTCTGGATCCGGCGGTGGAGGTTCCGGTGGGGGTGGATCAAGGAAGA
 GGAGGAAGAGGATTGTGGGGGGCAAGGTGTGCC (SEQ ID NO: 54)

Fc-EcoRI-R

ATGTCTGAATTCTCATTTACCCGGAGACAGGGAGAGG (SEQ ID NO: 55)

- 15 **[00362]** To generate the first intermediate plasmid, a PCR reaction was performed with 25 pmol of primers HindIII-Sall-BpEI-Fc-F and BamHI-linker(PACEI)-Fc-R and template pSYN-Fc-001 using Expand High Fidelity System (Boehringer Mannheim, Indianapolis, Ind.) according to manufacturer's standard protocol in a MJ Thermocycler. The following cycles were used: 95° C 2 minutes; 30
- 20 cycles of (95° C 30 seconds, 58° C 30 seconds, and 72° C 1 minute); 72° C 10 minutes. The correct sized band (approximately 730 bp) was gel purified as above and cloned into the HindIII/BamHI sites of pBUDCE4 vector (Invitrogen, Carlsbad, Calif.), generating pSYN-FVII-014. PCR amplification with primers HindIII-Sall-BpEI-Fc-F and BamHI-linker(PACEI)-Fc-R generated a DNA fragment encoding a
- 25 portion of the Fc region (Amino A X-Y) followed by an RRRRS (SEQ ID NO: 38) sequence and (GGGS)₂ (SEQ ID NO: 29) polypeptide linker. Primer HindIII-Sall-BpEI-Fc-F introduces a HindIII and Sail restriction site at the 5' end of the molecule, while primer BamHI-linker(PACEI)-Fc-R introduces a BamHI at the 3' end that overlaps the codons encoding the last 2 residues of the GGGS (SEQ ID NO: 56)
- 30 linker (residues GS with codons GGA TCC)

- [00363]** Next, another PCR reaction was performed as above with primers HindIII-Kozak-FVII-F and BspEI-Fc-linker-FVIII-C-R and template pSYN-FVII-002 using the same conditions described for cloning of pSYN-FVII-014, but with an annealing temperature of 57° C The expected sized band (approximately 700 bp) was
- 35 gel purified and cloned into the HindIII and BspEI sites of pSYN-FVII-014 to generate pSYN-FVII-023. Primers HindIII-Kozak-FVII-F and BspEI-Fc-linker-FVIII-C-R amplified a DNA fragment encoding the FVII light chain followed by a

5 (GGGGS)_{6x} (SEQ ID NO: 36) polypeptide linker and a portion of the Fc region up to amino acid 232 (EU numbering). Primer HindIII-Kozak-FVII-F introduces a HindIII restriction site at the 5' end of the molecule followed by a Kozak sequence while primer BspEI-Fc-linker-FVIIIC-R adds a BspEI site at the 3' end of the molecule.

[00364] In the final step a PCR reaction was carried out as above with primers
 10 BamHI-2xlink-pace-HC-F and Fc-EcoRI-R and template pSYN-FVII-003 with the following cycles: 95° C 2 minutes; 30 cycles of (95° C 30 seconds, 55° C 30 seconds, and 72° C 2 minute); 12 ° C 1 minutes. This PCR reaction generated a DNA molecule encoding a (GGGGS)_{2x} (SEQ ID NO: 29) polypeptide linker followed by a RKRRKR (SEQ ID NO: 39) sequence followed by the FVII heavy chain. Primers BamHI-
 15 2xlink-pace-HC-F and Fc-EcoRI-R introduce a BamHI site and an EcoRI site at the 5' and 3' end of the molecule, respectively. The expected sized band (approximately 1600 bp) was cloned into the BamHI and EcoRI sites of pSYN-FVII-023 to generate pSYN-FVII-024. The final structure of translation of pSYN-FVII-024 is illustrated in Figure IB.

20 **Example 2. Heterodimeric Constructs Comprising FIX-Fc**

Cloning of pSYN-FIX-044

[00365] Plasmid pSYN-FFX-044 was constructed for the expression of a 2-chain FFX-Fc and Fc heterodimer produced in the cell as a single chain protein where the FFX-Fc coding DNA region is linked to the second Fc coding region by a DNA
 25 fragment encoding the following polypeptide sequence: RRRRS-(GGGGS)_{4x}-RRRR (SEQ ID NO: 57), where the RRRR (SEQ ID NO: 40) sequence is a proprotein convertase cleavage site. Proprotein convertases then cleave 5' of the last Arg in the RRRRS (SEQ ID NO: 38) and RRRR (SEQ ID NO: 40) sequences intracellularly. Consequently, cells express a 2 chain FFX-Fc/Fc heterodimer where the FFX-Fc chain
 30 has a RRRRS (SEQ ID NO: 38) sequence at the C-terminus, but the remainder of the linker and the 3' RRRR (SEQ ID NO: 40) sequence have otherwise been removed. For this purpose, the synthesis of a DNA fragment (Genscript-FFX-044) was outsourced (Genscript, Piscataway, NJ). This fragment consisted of a DNA sequence encoding a portion of the Fc region from residues 231-447 (EU numbering) followed
 35 by a RRRRS (SEQ ID NO: 38) sequence followed by (GGGGS)_{4x} (SEQ ID NO: 6) polypeptide linker and a RRRR (SEQ ID NO: 40) sequence upstream of another portion of the Fc region (residues 221-230, EU numbering). The DNA region

- 5 encoding residues 231-233 of the Fc region at the 5' of the molecule overlaps a BspEI site, while the region encoding residues 236-238 of the Fc region at the 3' of the molecule include an RsrII restriction site. Genscript-FrX-044 was designed so that the RsrII site of the 5' Fc region and the BspEI site of the 3' Fc region were removed using the degeneracy of the genetic code to preserve the correct amino acid sequence.
- 10 Genscript-FrX-044 was cleaved with restriction enzymes BspEI and RsrII and cloned into the same sites of pSYN-FEX-029 to generate pSYN-FEX-044. The final structure of translation of pSYN-FEX-044 is illustrated in Figure 1B.

Example 3. Heterodimeric Constructs comprising FVII-Fc and MB9-Fc

Cloning of pSYN-FVII-027

- 15 **[00366]** Plasmid (pSYN-FVII-027) was generated for the expression FVII-Fc and MB9-Fc heterodimer, where MB9 is a scFv previously shown to bind to receptor GPIIb/IIIa on activated platelets. Protein from pSYN-FVII-027 is expressed in the cell as a single polypeptide where the C-terminus of the FVII-Fc subunit is linked to the N-terminus of the MB9-Fc subunit by a (GGGGS)₆ (SEQ ID NO: 36) polypeptide
- 20 linker. Furthermore, RRRRS (SEQ ID NO: 38) and RKRRKR (SEQ ID NO: 39) 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. Consequently, cells will express a 2 chain FVII-Fc/MB9-Fc heterodimer where the FVII-Fc chain has a RRRRS (SEQ ID NO: 38) sequence at the C-terminus,
- 25 but the remainder of the linker and the RKRRKR (SEQ ID NO: 39) sequence have otherwise been removed.

[00367] As a first step a series of intermediate plasmid were generated using the following primers:

HindIII-Sall-BspEI-Fc-F

- 30 AGTCAAGCTTGTCGACTCCGGAACCTCCTGGGCGGACC (SEQ ID NO: 51)

BamHI-linker-Fc-R

CATCGGATCCCCGCCACCGGAACCTCCACCGCCTGATCCACCCCCACCT
GATCCGCCGCCACCTTTACCCGGAGACAGGGAGAGG (SEQ ID NO: 58)

BclI-Fc-F

- 35 CAGTCTTGATCAGACAAAACCTCACACATGCCACC (SEQ ID NO: 59)

scFc-EcoRI-R

ACTGACGAATTCTCATTACCCGGAGACAGGGAG (SEQ ID NO: 60)

HindIII-Kozak-FVII-F:

5 CGACAAGCTTGCCGCCACCATGGTCTCCCAGGCCCTCAGG (SEQ ID NO:
43)
FVII-HC-BspEI-R:
AGGAGTTCCGGAGCTGGGACGGTGGGCATGTGTGAGTTTTGTCCGGATCC
CCCGCCACCGGAACCTCCACCGCCTGATCCACCCCCACCTGATCCGCCGC
10 CACCGGACCCACCTCCGCCGGAGCC ACCGCCACCGGGAAATGGGGCTCGC
AGGAGG (SEQ ID NO: 61)

[00368] A 50 ul PCR reaction was carried out with 25 pmol of HindIII-Sall-BpEI-Fc-F and BamHI-linker-Fc-R and template pSYN-Fc-001 using the following cycle: 95° C 2 minutes; 30 cycles of (95° C 30 seconds, 54° C 30 seconds, 72° C 1
15 minute). The expected sized band (~700 bp) was gel purified with a Gel Extraction kit (Qiagen, Valencia, Calif.) and cloned into the HindIII and BamHI restriction sites of pBUDCE4 (Invitrogen, Carlsbad, Calif.) to generate intermediate pSYN-FVII-007. Primers HindIII-Sall-BpEI-Fc-F and BamHI-linker-Fc-R amplify the Fc region starting at amino acid 221 (EU numbering) and add a HindIII and a Sail restriction
20 enzyme site immediately upstream of site Fc region, as well as a DNA fragment encoding a (GGGGS)_{4x} (SEQ ID NO: 6) linker followed by a BamHI site immediately downstream of the Fc coding region. Next, a 50 ul reaction was carried out with 25 pmol of BclI-Fc-F and scFc-EcoRI-R, and template pSYN-Fc-011 using the same cycles as above. The expected sized band (~700 bp) was gel purified as
25 above, cut with restriction enzymes BamHI and EcoRI, and cloned in the BclI/EcoRI restriction sites of pSYN-FVII-007 to generate the intermediate plasmid pSYN-FVII-008. The primer pair BclI-Fc-F and scFc-EcoRI-R amplifies the Fc region while adding a BclI and EcoRI restriction sites immediately upstream and downstream of the Fc coding region, respectively. To generate the last intermediate plasmid, a 50 ul
30 PCR reaction was carried out with 25 pmol of HindIII-Kozak-FVII-F and FVII-HC-BspEI-R and template pSYN-FVII-001 using the following cycle: 95° C 2 minutes; 30 cycles of (95° C 30 seconds, 55° C 30 seconds, 72° C 90 seconds). The primer pair amplifies the FVII coding region while adding a DNA fragment at the 3' end of the molecule encoding a (GGGGS)_{6x} (SEQ ID NO: 36) polypeptide linker followed by a
35 fragment of the Fc region ending at amino acid 221 (EU numbering). Primer HindIII-Kozak-FVII-F generates a HindIII restriction site at the 5' of the molecule followed by a Kozak sequence directly upstream of the FVII coding region. The FVII-HC-

5 BspEI-R primer introduces DNA encoding the polypeptide linker as well as the Fc portion. The expected sized band (~1500 bp) was gel purified as above and cloned into the HindIII/BspEI sites of pSYN-FVII-008 to generate pSYN-FVII-011.

[00369] Next, 2 DNA fragments were synthesized: Genescript-FVII-027-1 and
 Genscript-FVII-026-2. Genescript-FVII-027-1 consists of a DNA fragment encoding
 10 a portion of the Fc region (starting at nucleotide 1306, EU numbering) followed by
 the sequence RRRRS-(GGGGS)_{6x}-RKRRKR (SEQ ID NO: 50) followed by a portion
 of the MB9 scFv (residues 1-142). An EcoRI site was introduced in the coding
 sequence of MB9 using the degeneracy of the genetic code to preserve the proper
 amino acid sequence and overlaps the last 6 bases of Genescript-FVII-027-1. In
 15 addition, the first 6 bases at the 5' include a SapI site found within the Fc region.
 Genscript-FVII-026-2 consists of a DNA fragment encoding a portion of the MB9
 (residues 143-273) followed by a (GGGGS)_{6x} (SEQ ID NO: 36) polypeptide linker
 followed by the Fc region and an EcoRI site. An EcoRI site was introduced in the
 coding sequence of MB9 using the degeneracy of the genetic code to preserve the
 20 proper amino acid sequence and overlaps the first 6 bases of Genescript-FVII-026-2.

[00370] Genescript-FVII-027-1 was cloned into the SapI and EcoRI sites of
 pSYN-FVII-011 to generate pSYN-FVII-036. Next, Genscript-FVII-026-2 was
 cloned into the EcoRI site of pSYN-FVII-036 to generate pSYN-FVII-027. Correct
 orientation of the last cloning step was confirmed by restriction enzyme analysis and
 25 DNA sequencing. The final structure of translation of pSYN-FVII-027 is illustrated
 in Figure 1B.

Example 4. Transient transfections of cells

[00371] HEK-293-F cells were grown in suspension in Freestyle media
 (Invitrogen) supplemented with vitamin K3 (Sigma Aldrich, St. Louis, MO) to 2
 30 µg/liter (growth media) as suspension cells at 37° C/10% CO₂. Cells were subcultured
 every three to four days by seeding at cell density of 5x10⁵ cells/ml.

[00372] Twenty-four hours prior to transfection cells were seeded at a density
 of 7x10⁵ cells/ml in growth media supplemented with LONGTMR3IGF-1 (Sigma
 Aldrich, St. Louis, MO) to 20 µg/liter (transfection media). On the day of
 35 transfection, a transfection solution was made with a volume equal to 5% of the total
 volume of the cell culture to be transfected. In the transfection solution DNA was
 added (final concentration 20 mg/L) to a freshly made solution of PEI (60 mg/L) in

5 transfection media. The solution was swirled for 30 seconds and incubated for five minutes at room temperature before adding directly to the cell culture. Four hours later a volume equal to the cell culture volume of OptiCHO (Invitrogen) supplemented with vitamin K3 (when transfecting FIX or FVII constructs), LONGTMR3IGF-1 and 200 mM L-glutamine was added to the cells. The cell culture
10 was allowed to grow as shown above and daily media samples were taken to assess protein expression.

Example 5. Analysis of protein generated from transient transfections

[00373] For analysis of protein from transient transfections, conditioned media from transfections were subjected to protein A immunoprecipitation. Briefly, cell
15 culture supernatant was mixed with approximately 50 ul of protein A-Sepharose 50% slurry and incubated at 4°C with rocking for 1 hour, then centrifuged to pellet the protein A beads. Beads were washed twice by resuspending in 1 ml of PBS, spinning and aspirating. The beads were resuspended with sodium dodecylsulfate-
polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or nonreducing
20 conditions, heated for 5 minutes at 100°C, spun down and loaded on SDS-PAGE gels and run according to standard protocols. Gels were transferred to nitrocellulose membranes and Western blots were performed to detect the Fc region or the FVII light chain. For Fc detection, the antibody used was a goat anti-human IgG (Fc
specific)-horseradish peroxidase conjugate (Pierce ImmunoPure antibody, catalog
25 #31413). For FVII light chain detection an anti light chain monoclonal antibody was used (Green Mountain, clone 6MA-219). The antibodies were diluted 1:15,000 (for Fc detection) or 1:200 (for light chain detection) in PBST (PBS with 0.1% Tween-20) with 5% nonfat dry milk and incubated with the membrane for 1 hour at room
temperature. The membrane was then washed in PBST 3 times for 10 minutes and
30 signal was detected by a chemiluminescent method for Fc detection. For FVII light chain detection, the membrane was further incubated for one hour in a solution containing HRP-labeled goat anti-mouse antibody (Southern Biotech, # 1010-05) diluted 1:5000 in PBST. The membrane was also washed in PBST 3 times for 10 minutes and the signal was detected by a chemiluminescent method.
35 Chemiluminescent detection was performed using ECL Plus Western Blotting Detection System (Amersham Biosciences catalog #RPN2132) according to manufacturer's protocol. Signal was visualized in a Storm 840 Phosphorimager

- 5 (Molecular Devices). Alternatively, protein gels could be analyzed by Coomassie Blue staining

Example 6. The Generation of Constructs Comprising Alternate Processing Sites

- [00374] Cloning of pSYN-FIX-056 intermediate: Synthesis of DNA fragment
 10 Genscript-FVII-043 was outsourced (Genscript). This fragment consisted of a DNA molecule encoding a portion of the Fc region (residues 232 to 447, EU numbering) followed by a (GGGGS)₄x (SEQ ID NO: 6) polypeptide linker and another portion of the Fc region (residues 221 to 238, EU numbering). This DNA fragment was digested with BspEI and RsrII and subcloned into the BspEI/RsrII sites of pSYN-FrX-044 to
 15 generate pSYN-FIX-049. HindIII/EcoRI fragment from pSYN-FIX-049 comprising the FIX coding region followed by the single chain Fc coding region was subcloned in to the HindIII/EcoRI sites of pBUDCE4.1 (Invitrogen) to generate pSYN-FIX-056.

- [00375] Cloning of pSYN-FIX-050, -052 and -053: Synthesis of DNA fragments Genscript-FrX-050, -052, -053 was outsourced (Genscript). These
 20 fragments comprise a portion of the Fc region (from Sail site in pSYN-FFX-056), an RRRRS (SEQ ID NO: 38) sequence, a (GGGGS)₄x (SEQ ID NO: 6) polypeptide, a variable propeptide convertase cleavage site and a portion of the Fc region (up to the RsrII in the second Fc of pSYN-FFX-056). Each DNA fragment encodes for a different variable propeptide convertase cleavage site: genscript-FrX-050, RAGR;
 25 genscript-FIX-052 RSKR; and genscript-FIX-053, RKRRKR (SEQ ID NO: 39) (thus, they are identical to 044 with the exception of these sequences; 044 comprises the RRRR(SEQ ID NO: 40) site). Sall/RsrII fragments from Genscript-FIX-050, -052, and -053 were subcloned into Sall/RsrII sites of pSYN-FFX-056 to generate pSYN-FIX-050, -052, and -053, respectively.

30 **Example 7: FIXFc constructs with cscFc linker**

- [00376] Construct FFX-044 (Figure 3A) was made comprising a FFX molecule followed by an Fc region with a cscFc linker connecting both Fc moieties as described in Figure 3A. Figure 3B shows western blot data (Fc western) for FIX-044 following transfection and protein A pulldown. FIX-044 was cotransfected the Kex2, PC7,
 35 PACE or PC5.

Cloning of PC5

- [00377] The coding sequence for human PC5 was obtained by RT-PCR. The following primers were used (areas that anneal to the cDNA are indicated in bold):

Primers	SEQ ID NO	Sequences
PC5-KpnI-F:		5'-ATCTACACCATCTCCATCAGCAGC-3'
PC5-NotI-R:		5'-AAGGCGGCCGCTCAGCCTTGAAATGTACATGTTTTGC3'
PC5-UTR-F:		5'-AGCGAGGGAGCAGCGAGG-3'
PC5-HindIII-R:		5'-GGTAGTTGACATGGCGGTTGG-3'
PC5-M12-F:		5'-CAGCGACTTAAGCCACCATGGGCTGGGGGAGCCG-3'
PC5-KpnI-R:		5'-GTAGGTTGTGGCCAGCGTGG-3'

5

Coding sequence for human PC5 (GenBank accession no. NM-006200) was obtained in two pieces. The 3' -1750 bp were obtained using the primers PC5-KpnI-F and PC5-NotI-R with the Invitrogen SUPERSRIPT™ RT-PCR with PLATINUM™ Taq kit according to the manufacturer's standard protocol, from human liver mRNA.

- 10 The cycle used for the reverse transcription was 30 min at 50 °C followed by denaturing at 94 °C for 2 min and 35 cycles of 94 °C for 15 sec, 54 °C for 30 sec, 72 °C for 3 min, followed by 10 min extension at 72 °C and then storage at 4 °C. This produced a fragment from the internal KpnI site in the PC5 coding sequence through the stop codon, with a NotI site added at the 3' end. This fragment was then cloned
- 15 into pCR2.1 TOPO according to manufacturer's protocol to generate pSYN-PC5-001 (pCR2.1/PC5 (KpnI-NotI)). This fragment was then subcloned into pcDNA3.1/hygro using the KpnI and NotI restriction sites to generate pSYN-PC5-002 (pcDNA3.1/hygro/PC5 (KpnI-NotI)).

- 20 The 5' -1100 bp of PC5 was obtained in two steps. It was first amplified by RT-PCR using the primers PC5-UTR-F and PC5-HindIII-R to amplify a -1520 bp fragment from human liver mRNA, using similar conditions as above, with an annealing temperature of 57 °C. These primers have complete homology to the native PC5 sequence, in the untranslated 5' sequence and sequence 3' from the internal unique HindIII site, respectively. Note that this HindIII site is not present in the final
- 25 construct due to a silent nucleotide substitution. This DNA fragment was then gel purified and used as a template for a second PCR reaction with PC5-Afl2-F, which adds an AflII cloning site followed by a Kozak sequence to the N-terminal coding

5 sequence at the 5' end, and PC5-KpnI-R, which anneals 3' to the internal unique KpnI site, to generate an -1100 bp fragment. The reaction was carried out with the EXPAND™ High Fidelity System according to the manufacturer's standard protocol in a MJ Thermocycler using the following cycles: 94 °C, 2 min; 14 cycles of (94 °C, 30 sec, 57 °C, 30 sec, 72 °C, 2 min), followed by 72 °C, 10 min. This fragment was
10 then subcloned into pSYN-PC5-002 using the AflII and KpnI restriction sites to generate pSYN-PC5-003 (pcDNA3.1/hygro/PC5).

The nucleotide sequence encoding PC5 in pSYN-PC5-003 has the following sequence (SEQ ID NO:):

15	atgggctggg	ggagccgctg	ctgctgcccg	ggacgttttg	acctgctgtg
	cgtgctggcg	ctgctcgggg	gctgcctgct	ccccgtgtgt	cggacgcgcg
	tctacaccaa	ccactgggca	gtcaaaatcg	ccgggggctt	ccggagggcc
	aaccgtatcg	ccagcaagta	cggattcatc	aacataggac	agataggggc
	cctgaaggac	tactaccact	tctaccatag	caggacgatt	aaaaggctcag
	ttatctcgag	cagagggacc	cacagtttca	tttcaatgga	accaaaggtg
20	gaatggatcc	aacagcaagt	ggtaaaaaag	cggacaaaga	gggattatga
	cttcagtcgt	gcccagtcga	cctatttcaa	tgatcccaag	tgggccagta
	tgtggtatat	gcactgcagt	gacaatacac	atccctgcca	gtctgacatg
	aatatcgaag	gagcctggaa	gagaggctac	acgggaaaga	acatttgtgt
	cactatcctg	gatgacggaa	ttgagagaac	ccatccagat	ctgatgcaaa
25	actacgatgc	tctggcaagt	tgcgacgtga	atgggaatga	cttggaccca
	atgcctcgtt	atgatgcaag	caacgagaac	aagcatggga	ctcgtctgtc
	tggagaagtg	gcagccgctg	caaacaattc	gcactgcaca	gtcgggaattg
	ctttcaacgc	caagatcgga	ggagtgcgaa	tgctggacgg	agatgtcacg
	gacatggttg	aagcaaaatc	agttagcttc	aacccccagc	acgtgcacat
30	ttacagcgcc	agctggggcc	cggatgatga	tggaagact	gtggacggac
	cagccccct	cacccggcaa	gcctttgaaa	acggcggttag	aatggggcgg
	agaggcctcg	gctctgtgtt	tgtttgggca	tctggaaatg	gtggaaggag
	caaagaccac	tgctcctgtg	atggctacac	caacagcatc	tacaccatct
	ccatcagcag	cactgcagaa	agcggaaaga	aaccttggtg	cctggaagag
35	tgttcatcca	cgctggccac	aacctacagc	agcggggagt	cctacgataa
	gaaaatcatc	actacagatc	tgaggcagcg	ttgcacggac	aaccacactg
	ggacgtcagc	ctcagcccc	atggctgcag	gcatcattgc	gctggccctg
	gaagccaatc	cgtttctgac	ctggagagac	gtacagcatg	ttattgtcag
	gacttcccgt	gcgggacatt	tgaacgctaa	tgactggaaa	accaattgtg
40	ctggttttaa	ggtgagccat	ctttatggat	ttggactgat	ggacgcagaa
	gccatggtga	tggaggcaga	gaagtggacc	accgttcccc	ggcagcacgt
	gtgtgtggag	agcacagacc	gacaaatcaa	gacaatccgc	cctaacagtg
	cagtgcgctc	catctacaaa	gcctcaggct	gctcagataa	ccccaacccg
	catgtcaact	acctggagca	cgctggtgtg	cgcataacca	tcacccaccc
45	caggagagga	gacctggcca	tctacctgac	ctcgcctctc	ggaactaggt
	ctcagctttt	ggccaacagg	ctatttgatc	actccatgga	aggattcaaa
	aactgggagt	tcattgacat	tcattgctgg	ggagaaagag	ctgctggtga
	ctgggtcctt	gaagtattatg	atactccctc	tcagctaagg	aactttaaga
	ctccaggtaa	attgaaagaa	tggctcttgg	tcctctacgg	cacctccgtg
50	cagccatatt	caccaaccaa	tgaatttccg	aaagtggaa	ggttccgcta
	tagccgagtt	gaagacccca	cagacgacta	tggcacagag	gattatgcag
	gtccctgcga	ccctgagtgc	agtgaggttg	gctgtgacgg	gccaggacca
	gaccactgca	atgactgttt	gcactactac	tacaagctga	aaaacaatac
	caggatctgt	gtctccagct	gcccccttgg	ccactaccac	gccgacaaga
55	agcgctgcag	gaagtgtgcc	cccaactgtg	agtcctgctt	tgggagccat
	ggtgaccaat	gcatgtcctg	caaatatgga	tactttctga	atgaagaaac
	caacagctgt	gttactcact	gcctgatgg	gtcatatcag	gataccaaga
	aaaatctttg	ccggaaatgc	agtgaaaact	gcaagacatg	tactgaattc

```

5      cataactgta   cagaatgtag   ggatggggtta   agcctgcagg   gatcccgggtg
      ctctgtctcc   tgtgaagatg   gacggtatatt   caacggccag   gactgccagc
      cctgccaccg   cttctgcgcc   acttgtgctg   gggcaggagc   tgatgggtgc
      attaactgca   cagagggcta   cttcatggag   gatgggagat   gcgtgcagag
      ctgtagtatc   agctattact   ttgaccactc   ttcagagaaat   ggatacaaat
10     cctgcaaaaa   atgtgatatc   agttgtttga   cgtgcaatgg   cccaggattc
      aagaactgta   caagctgccc   tagtgggtat   ctcttagact   taggaatgtg
      tcaaattgga   gccatttgca   aggatgcaac   ggaagagtcc   tgggcggaag
      gaggccttctg   tatgcttggtg   aaaaagaaca   atctgtgcca   acggaagggt
15     cttcaacaac   tttgctgcaa   aacatgtaca   tttcaaggc

```

SEQ ID NO: contains substitutions from the GenBank sequence that do not affect the amino acid coding sequence. Specifically, the nucleotide at position 399 (corresponding to position 876 of GenBank accession no. NM-006200) is a T instead of a C, but preserves the amino acid Ser 133 (corresponding to amino acid numbering in GenBank accession no. NP-006191); nucleotide position 1473 (GenBank position 1950) is a C instead of a T, but preserves the amino acid Ala 491; and nucleotide position 1485 (GenBank position 1962) is an A instead of a G, but preserves the amino acid Ser 496. The nucleotide change at position 1473 eliminates a HindIII restriction site.

Cloning of PACE-SOL

The coding sequence for human PACE was obtained by RT-PCR. The following primers were used (areas that anneal to the cDNA are indicated in bold):

	SEQ ID NO	
PACE-F1:		5-GGTAAGCTT GGCATGGAGCTGAGGCCCTGGTTGC -3'
PACE-R1:		5- GTTTTCAATCTCTAGGACCCACTCGCC -3'
PACE-F2:		5- GCCAGGCCACATGACTACTCCGC -3'
PACE-R2:		5-GGTGAATT CTCACTCAGGCAGGTGTGAGGGCAGC -3'

The primer PACE-F1 adds a HindIII site to the 5' end of the PACE sequence beginning with 3 nucleotides before the start codon, while the primer PACE-R2 adds a stop codon after amino acid 715, which occurs at the end of the extracellular domain of PACE, as well as adding an EcoRI site to the 3' end of the stop codon. The PACE-R1 and PACE-F2 primers anneal on the 3' and 5' sides of an internal BamHI site, respectively. Two RT-PCR reactions were then set up using 25 pmol each of the primer pairs of PACE-F1/R1 or PACE-F2/R2 with 20 ng of adult human liver RNA (Clontech; Palo Alto, Calif.) in a 50 μ l RT-PCR reaction using the SUPERSCRIPT™ One-Step RT-PCR with PLATINUM® Taq system (Invitrogen, Carlsbad, CA.)

- 5 according to manufacturer's protocol. The reaction was carried out in a MJ Thermocycler using the following cycles: 50 °C 30 minutes; 94 °C 2 minutes; 30 cycles of (94 °C 30 seconds, 58 °C 30 seconds, 72 °C 2 minutes), followed by 72 °C 10 minutes. Each of these fragments was ligated into the vector pGEM T-Easy (Promega, Madison, WI) and sequenced fully. The F2-R2 fragment was then
 10 subcloned into pcDNA6 V5/His (Invitrogen, Carlsbad, CA) using the BamHI/EcoRI sites, and then the F1-R1 fragment was cloned into this construct using the HindIII/BamHI sites. The final plasmid, pcDNA6-PACE, produces a soluble form of PACE (amino acids 1-715), as the transmembrane region has been deleted. The sequence of PACE in pcDNA6-PACE is essentially as described in Harrison S *et al.*,
 15 (1998) *Semin Hematol* 35(2 Suppl 2):4-10.

Cloning of Kex2-SOL

The coding sequence for the yeast endoprotease, KEX2, was obtained by RT-PCR from *Saccharomyces cerevisiae* polyA-i- mRNA (BD Clontech, cat #6999-1) using the following primers (areas that anneal to the cDNA are indicated in bold):

Primers	SEQ ID NO	Sequences
KEX2-F:		5'-GCGCTAGCCGTACGGCCGCCACCATGAAAGTGAGGAAATATATTAC- TTTATGC-3'
KEX2-BglII-F:		5'-GCTATTGATCACAAAGATCTACATCCTCC-3'
KEX2-BglII-R:		5'-GGAGGATGTAGATCTTTGTGATCAATAGC-3'
KEX2-675-R:		5'-GCGAATTCCGGTCCGTCATTGCCTAGGGCTCGAGAGTTTTTTAGGA- GTGTTTGGATCAG-3'

- 20 These primers were used to obtain coding sequence for KEX2 (amino acids 1-675), the yeast homolog to PACE, in two pieces in a manner similar to that used for PACE-SOL, Example 3 above; similarly, the transmembrane region was removed to generate the soluble form of the protein.

Cloning of PC7-SOL

- 25 The coding sequence for PC7 was obtained by RT-PCR from human adult liver mRNA using the following primers (areas that anneal to the cDNA are indicated in bold):

	SEQ ID NO	
PC7-BamMut-F:		5'-GCATGGACTCCGATCCCAACG-3'
PC7-		5'-CGTTGGGATCGGAGTCCATGC-3'

BamMut-R:		
PC7-F:		5'-GGTAAGCTTGCCGCCACCATGCCGAAGGGGAGGCAGAAAG-3'
PC7-SOL-R:		5'-TTTGAATTCTC AGTTGGGGGTGATGGTGTAAACC -3'
PC7-Xma-F:		5'-GGCACCTGAATAACCGACGG -3'
PC7-Xma-R:		5'-CGTCACGTTGATGTCCCTGC -3'

5 [00378] These primers were used to obtain coding sequence for PC7 (amino acids 1-663) in three pieces in a manner similar to that used for PACE-SOL, above; similarly, the transmembrane region was removed to generate the soluble form of the protein.

[00379] The most efficient cleavage of the cscFc linker was observed when
 10 cotransfected with PC5 and PACE (reducing conditions, lanes 11 and 12). A series of constructs were generated (FIX-050, FIX-052 and FIX-053) where the second processing site was modified as shown in Figure 3A. These constructs we transiently transfected (with or without PC5 cotransfection) and the proteins analyzed by Fc western blot following a protein A pulldown as shown in Figure 3C. Optimal cleavage
 15 of the cscFc was observed for FFX-052 and -053 with PC5 cotransfection

Example 7: Intracellular processing of cscFc linker in FVIIaFc heterodimer that results in expression of active protease

[00380] Figure 2 reveals a FVIIFc construct (FVII-024) expressed as two-chain heterodimer where one chain consists of the FVII light chain followed by a
 20 (GGGGS)_{6x} linker followed by the first Fc moiety, while the other chain contains a FVII heavy chain followed by a (GGGGS)_{6x} linker followed by the second Fc moiety. The plasmid is designed to express the heterodimer as a single polypeptide where the C-terminus of the FVII heavy chain-linker-Fc chain is connected to the N-terminus of the heavy chain-linker-Fc chain by the following polypeptide sequence: RRRRS-
 25 (GGGGS)_{6x}-RKRRKR where the RRRRS and RKRRKR sequences are proprotein convertase cleavage sites. FVII-024 was cloned, transiently expressed and analyzed by western blot analysis as described herein. The plasmid expressing FVII-024 was cotransfected with PC5 to fully process the cscFc linker, described in the protein sequence, connecting the C-terminus of the first Fc moiety to the N-terminus of the
 30 heavy chain. The effect of PC5 on the processing of the proprotein convertase cleavage sites in the FVII-024 linker was tested as shown in Figure 2. Under nonreducing conditions the effect of PC5 on cleavage site processing cannot be

5 detected because the FVII light chain-Fc and FVII heavy chain-Fc subunits remain linked via 2 disulfide bonds in the Fc region (lanes 2 and 3). Under reducing conditions we observed partial processing of FVII-024 generated from cells not cotransfected with PC5 (lane 4), but full processing when the cells were cotransfected with PC5 (lane 5). Full cleavage of the processing sites results in generation of the active form of FVIIa, which requires a free N-terminus of the heavy chain to adopt an active conformation

Example 8: Processing of cscFc linker in FVIIIc

The FVIIIc construct illustrated in Figure 4 was cloned and purified. Briefly, the coding sequence of human recombinant B-domain deleted FVIII was obtained by reverse transcription-polymerase chain reaction (RT-PCR) from human liver poly A RNA (Clontech) using FVIII-specific primers. The FVIII sequence includes the native signal sequence for FVIII. The B-domain deletion starts after serine 743 (S743; 2287 bp) and ends before glutamine 1638 (Q1638; 4969 bp) for a total deletion of 2682 bp (SQ version).

20 The coding sequence for human recombinant Fc was obtained by RT-PCR from a human leukocyte cDNA library (Clontech) using Fc specific primers. Primers were designed such that the B-domain deleted FVIII sequence was fused directly to the N-terminus of the Fc sequence with no intervening linker. The FVIIIc DNA sequence was cloned into the mammalian dual expression vector pBUDCE4.1 (Invitrogen) under control of the CMV promoter.

A second identical Fc sequence including the mouse Igk signal sequence was obtained by RT-PCR and cloned downstream of the second promoter, EF1a, in the expression vector pBUDCE4.1. This final construct was designated pSYN-FVIII-013.

30 A second plasmid was created from similar constructs using PCR and standard molecular biology techniques, in order to express rFVIIIbDD-Fc-Fc in which the rFVIIIbDDFc coding sequence was fused to the second Fc sequence with a (GGGGS)₄ linker, allowing for production of only the rFVIIIbDD-Fc monomer-dimer hybrid in transient transfection. This construct was designated pSYN-FVIII-041. To make pSYN-FVIII-049, intermediate pSYN-FVIII-048 was generated by cloning NheI/XhoI fragment from pBUD-CE4.1 into pSYN-FVIII-013. The synthesis of a DNA fragment comprising the region from RsrII to XbaI sites of pSYN-FVIII-

5 049 was outsourced. This fragment was subcloned into the RsrII/XbaI sites of pSYN-FVIII-048 to generate pSYN-FVIII-049.

The protein was expressed by transient transfection as described herein. PC5 was cotransfected to fully process the cscFc linker, described in the protein sequence, connecting the C-terminus of the first Fc moiety to the N-terminus of the second Fc moiety. SDS PAGE analysis in Figure 4 reveals 3 distinct bands for purified FVIII-049 under reducing conditions: light chain-Fc (LC-Fc), heavy chain (HC) and Fc. This shows that the linker connecting both Fc moieties has been processed.

Example 9 Processing of cscFc linker in FVII-Fc

Cloning of pSYN-FVII-064

15 Synthesis of DNA sequence from HindIII to EcoRI of pSYN-FVII-064 was outsourced and cloned into HindIII/EcoRI sites of pBUDCE4.1 (Invitrogen)

Expression and purification of FVII-064

DNA was transiently transfected as described herein. The transfection DNA contained PC5 to fully process the cscFc linker, described in the protein sequence, connecting the C-terminus of the first Fc moiety to the N-terminus of the second Fc moiety

Characterization of cleavage of cscFc linker of FVII-064

FVII-064 was purified using Ion Exchange: Q sepharose 4FF from GE healthcare. Secondary capture was performed using shFcRn (soluble human FcRn) affinity (NHS- coupled shFcRn with sepharose 4FF beads). All steps were performed at 150cm/hrs linear Flow Rate. Figure 5 illustrates SDS PAGE analysis of FVII-064 following transient expression (cotransfected with PC5) and purification. Under reducing conditions we observe 2 distinct bands for FVII-Fc and Fc, demonstrating full cleavage of the cscFc linker

Example 10. Processing of cscFc of a FVIIIFc protein with a platelet targeting

construct

In this example FVII-027 (Figure 6) was transiently expressed with PC5 cotransfection to remove the cscFc linker described in the protein sequence. The construct was cloned, expressed and purified as described herein. SDS PAGE analysis of the purified protein in Figure 6 revealed two distinct bands for FVII-Fc and MB9-Fc under reducing conditions, showing that the cscFc linker was fully processed.

Example 11 Processing of cscFc of a Interferon-beta Fc fusion protein

In this example we generated a construct (IFN-beta-018) expressing interferon-beta followed by a linker and an Fc region where both Fc moieties are connected by a

- 5 cscFc linker. The construct was cloned as follows: synthesis of a DNA fragment from BsiWI/BspEI of pSYN-IFN-b-018 was outsourced and subcloned into BsiWI/BspEI sites of pSYN-FIX-053 to generate pSYN-IFN-b-018 . Figure 10 illustrates Western Blot analysis of IFN-beta-018 species following transient transfection of HEK 293 cells (with or without PC5 cotransfection) and protein A pulldown. Western blot data
10 revealed complete cleavage of the cscFc linkers when cotransfected with PC5.

Example 12. Additional Attempts at Expression of Activated Constructs

[00381] Several other constructs were made with the goal of expressing activated FVII, and are illustrated in Figure 7. However, these constructs did not successfully express activated molecules.

15 Cloning of pSYN-FVII-010

[00382] The FVII-010 construct is one in which the heavy chain of factor VII was expressed in the context of an scFc scaffold and the light chain was expressed separately.

- [00383] PCR-amplify with primer pairs FVII-HC-Hind3-IggKss-F/FVII-HC-BspEI-R, using pSYN-FVII-001. Clone in BspEI/HindIII sites of pSYN-FVII-008
20 (see supra), generating pSYN-FVII-009.

[00384] PCR amplify FVII light chain from pSYN-FVII-003 (refer to P0830) with primers FVII-LC-NotI-F/ FVII-LC-XhoI-R and clone in pSYN-FVII-009 to generate pSYN-FVII-010

25 Primers

FVI I -HC-BspE I-R
AGGAGTTCCGGAGCTGGGCACGGTGGGCATGTGTGAGTTTGTGCGGATCCCCGCCACCGGAACCTCCA
CCGCCTGATCCACCCACCTGATCCGCCGCCACCGGACCCACCTCCGCCGAGCCACCGCCACCGGGA
AATGGGGCTCGCAGGAGG

- 30 FVI I -HC-Hind3- IggKss-f
ACTGACAAGCTTGCCGCCACCATGGAGACAGACACTCCTGCTATGGGTACTGCTGCTCTGGGTTC
GGTCCACTGGTATTGTGGGGGCAAGGTGTGC

- 35 FVI I -LC-Not I - F
ACTGACGCGGCCGCGCCGCCACCATGGTCTCCAGG

FVI I -LC-XhoI - R
ACTGACCTCGAGTTATCGGCCTGGGGTTTGTCTGG

40 Cloning of pSYN-FVII-013

[00385] The FVII-013 construct is one in which the light chain was expressed in the context of an scFc scaffold and the heavy chain was expressed separately.

5 **[00386]** PCR-amplify with primer pair FVII-LC-linker-BamHI-R/ HindIII-Kozak-FVII-F from pSYN-FVII-001. The coding sequence of FVII was obtained by reverse transcription coupled to polymerase chain reaction from a human liver mRNA library (Ambion, Austin, Texas) using the following primers:

FVII-F1

10 GGAATGTCAACAGGCAGGG

FVII-R1

CTTGGCTTTCTCTCCACAGGC

A 50 µl reaction was carried out with 10 pmol of each primer using the Superscript One-step RT-PCR with Platinum Taq system (Invitrogen, Carlsbad, Calif.) according to the manufacturer's standard protocol in a MJ thermocycler. The cycle used was 50° C for 30 minutes for the reverse transcription followed by denaturing at 94° C for 2 minutes and 30 cycles of (94° C 30 seconds, 53° C 30 seconds, 72° C 90 seconds) followed by 10 minutes at 72° C. The expected sized band (~1400 bp) was gel-purified with a Gel Extraction kit (Qiagen, Valencia, Calif.) and cloned in pCR2.1 TOPO using the TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif.) to produce the intermediate plasmid pSYN-FVII-001 and cloned in BamHI/HindIII sites of pSYN-FVII-011, generating pSYN-FVII-012. PCR-amplify FVII-HC from pSYN-FVII-009 using primer pair FVII-HC-NotI-F/FVII-HC-XhoI-R and subclone in pSYN-FVII-012 to generate pSYN-FVII-013

25

Primers

FVII-LC-6xlinker-BamHI-

RACTGACGGATCCCCGCCACCGGAACCTCCACCGCCTGATCCACCCACCTGATCCGCCGCCACCGG

ACCCACCTCCGCCGGAGCCACCGCACCTCGGCCTTGGGGTTTGCTGGC

30

HindIII-Kozak-FVII-F

CGACAAGCTTGCCGCCACCATGGTCTCCAGGCCCTCAGG

FVII-HC-NotI-F

ACTGACGCGCCGCGCCGCCACCATGGAGACAGAC

FVII-HC-XhoI-R

35

ACTGACCTCGAGTTAGGGAAATGGGGCTCGCAGGAG

Cloning of pSYN-FVII-018

[00387] For the FVII-018 construct, the heavy chain of FVII was expressed as an Fc fusion protein and the light chain of FVII was separately expressed as a separate Fc fusion protein.

40

[00388] Primers FVII-HC-Hind3-IggKss-F/scFc-EcoRI-R were used to PCR amplify HCFVII-linker-Fc, using pSYN-FVII-010 as template. Subclone in HindIII/EcoRI sites of pBUDCE4. This makes pSYN-FVII-017. Next, PCR-amplify

5 from pSYN-FVII-013 with primers FVII-LC-NotI-F/FC-XhoI-R and subclone in XhoI/NotI sites of FVII-017. This makes PSYN-FVII-018

Primers

scFc-EcoRI-R

ACTGACGAATTCTCATTTACCCGGAGACAGGGAG

10

Fc-XhoI-R

AGCTCTCGAGTCATTTACCCGGAGACAGGG

Figure 8 illustrates Western Blot analysis of FVIIFc species following transient
15 transfection of HEK 293 cells and protein A pulldown of the molecules illustrated in Figure 7. Western blot data show that the FVII heavy chain cannot be expressed with a free N terminus using a common method of fusing a heterologous signal peptide to the N-terminus of the heavy chain.

Example 13. Alternative attempts to express activated FVII-Fc constructs

20 Failure to express the FVII heavy chain with a free N terminus led us to generate the constructs described in Figure 11. Here FVIIFc is expressed as a heterodimer where one subunit comprises the FVII light chain and an Fc moiety, and the other subunit comprises the heavy chain preceded by an RKRRKR processing site (FVII-019) or by a light chain C terminal fragment and an RKRRKR processing site (FVII-020). We
25 hypothesized that this may facilitate expression the heavy chain -Fc moiety subunit since the heavy chain would not adopt the active conformation until cleavage of the processing site in the Golgi. These constructs were analyzed by Fc western blot following a protein A pulldown of transiently transfected material. FVII-011 (FVIIFc with a single chain Fc) was used as a control. Only light chain-Fc was observed,
30 suggesting that heavy chain Fc can not be expressed from FVII-019 or FVII-020

Example 14. Expression of activated FVIIFc proteins in the monomer and heterodimer structures

Cloning of pSYN-FVII-025.

Synthesis of a DNA fragment comprising the FVII coding region from XbaI to BsiWI
35 with an RKRRKR amino acid insertion between R152 and 1153 (mature sequence numbering) was outsourced. The DNA fragment was subcloned in the XbaI/BsiWI sites of pSYN-FVII-011 (the sequence of which is included herein) to generate pSYN-FVII-025

Figure 9 illustrates a heterodimeric cscFc construct (FVII-024) and a monomeric Fc
40 construct (FVII-025). These constructs were made and Western blots were performed

5 following protein A pulldown and transient expression with or without PC5
contransfection. The data show intracellular activation in the context of either the
heterodimer or the monomer enabled expression of the separate heavy chain, but that
the intracellular activation is more efficient in the context of the heterodimer (FVII-
024) than the monomer (FVII-025).

10

Example 15: Protein purification

Protein Purification of FVII-064

FVII-064 molecules were purified from conditioned media 1) Anion exchange
chromatography with pseudo-affinity elution (e.g. Q sepharose 4FF (GE Healthcare)
15 followed by elution with varying levels of CaCl₂ to selectively elute the most active
species), followed by 2) shFcRn (soluble human FcRn) affinity (NHS- coupled
shFcRn with sepharose 4FF beads) chromatography, binding Fc-containing proteins at
low pH (e.g. pH 6.2) and eluting at neutral pH (e.g. pH 8.0). These purification steps
utilized standard methods known to those in the art to generate purified proteins of
20 >95% purity by SEC analysis and SDS-PAGE.

Purification of FVIII-049

FVIII-049 was purified from clarified and chemically defined harvest media using a
two column purification process, including a FVIII-specific affinity purification step
25 (McCue 2009 Journal of Chromatography A. 1216:7824) followed by anion
exchange with standard NaCl elution. These purification steps utilized standard
methods known to those in the art to generate purified proteins of >95% purity by
SEC analysis and SDS-PAGE.

5

Informal Sequence Listing

	pSYN-FVII-024 nucleotide sequence					
10	atggtctctccc	aggccctcag	gctcctctgc	cttctgcttg	ggcttcaggg	ctgcctggct
	gcagtccttcg	taaccacagga	ggaagccac	ggcgctcctgc	accggcgccg	gcgcgccaac
	gcgttcctgg	aggagctgcg	gccgggctcc	ctggagaggg	agtgcaggga	ggagcagtg
	tccttcgagg	aggcccgga	gatcttcaag	gacgcggaga	ggacgaagct	gttctggatt
	tcttacagtg	atggggacca	gtgtgcctca	agtccatgcc	agaatggggg	ctcctgcaag
15	gaccagctcc	agtccctatat	ctgcttctgc	ctccctgcct	tcgagggccg	gaactgtgag
	acgcacaagg	atgaccagct	gatctgtgtg	aacgagaacg	gcggctgtga	gcagtactgc
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60	catgaggctc	tgcacaacca	ctacacgcag	aagagcctct	ccctgtctcc	gggtaaatga
	FVII-024 amino acid sequence. Signal sequence is shown in dotted underline, propeptide is double underlined, linker region connecting FVII light chain or heavy chain to Fc region is underlined, Fc region is shown in italics and linker with proprotein convertase processing sites is shown in bold.					
65	^ .SQALRLLC .L.LLGLQGCLA <u>AVFVTOEEAH</u> <u>GVLHRRRRAN</u> AFLEELRPGS LERECKEEQC					
	SFEAREIFK	DAERTKLFW I	<u>SYSDGDQCAS</u>	<u>SPCQNGGSCK</u>	DQLQSYICFC	LPAFEGRNCE
	THKDDQLICV	NENGGCEQYC	SDHTGTRKSC	RCHEGYSLLA	DGVSTPTVE	YPCGKIPILE
	KRNASKPQGR	GGGSGGGGS	GGGSGGGGS	GGGSGGGGS	<u>DKTHTCPPCP</u>	<u>APELLGGPSV</u>
70	<u>FLFPKPKKDT</u>	<u>LMISRTPEVT</u>	<u>CWVDVSHED</u>	<u>PEVKFNWYVD</u>	<u>GVEVHNAKTK</u>	<u>PREEQYNSTY</u>
	<u>RVVSVLTVLH</u>	<u>QDWLNGKEYK</u>	<u>CKVSNKALPA</u>	<u>PIEKTISKAK</u>	<u>GQPREPQVYT</u>	<u>LPPSRDELTK</u>
	<u>NOVSLTCLVK</u>	<u>GFYPSDIAVE</u>	<u>WESNGOPENN</u>	<u>YKTTTPVLDS</u>	<u>DGSFFLYSKL</u>	<u>TVDKSRWQQG</u>

5	<i>NVFSCSVME</i>	<i>ALHNHYTQKS</i>	<i>LSLSPGKRRR</i>	<i>RSGGGGSGGG</i>	<i>GSGGGGSGGG</i>	<i>GSGGGGSGGG</i>
	GSRKKRRKRV	GGKVC PKGEC	PWQVLLLVNG	AQLCGGTLIN	TIWWSAAHC	FDKIKNWRNL
	IAVLGEHDL	EHGDDEQSR	VAQVIIPSTY	VPGTTNH DIA	LLRLHQPVVL	TDHWPLCLP
	ERTFSERTLA	FVRFSLVSGW	GQLDRGATA	LELMVLNVPR	LMTQDCLQOS	RKVGDSPNIT
	EYMF CAGYSD	GSKDSCKGDS	GGPHATHYRG	TWYLTGIVSW	GQGCATVGHF	GVYTRVSQYI
10	EWLQKLMRSE	PRPGVLLRAP	FPGGGGSGGG	GSGGGGSGGG	GSGGGGSGGG	<i>GSDKTHTCPP</i>
	<i>CPAPELLGGP</i>	<i>SVFLFPPKPK</i>	<i>DTLMISRTPE</i>	<i>VTCTVWDVSH</i>	<i>EDPEVKFNWY</i>	<i>VDGVEVHNAK</i>
	<i>TKPREEQYNS</i>	<i>TYRWSVLTV</i>	<i>LHQDWLNGKE</i>	<i>YKCKVSNKAL</i>	<i>PAPIEKTISK</i>	<i>AKGQPREPQV</i>
	<i>YTLPPSRDEL</i>	<i>TKNQVSLTCL</i>	<i>VKGFYPSDIA</i>	<i>VEWESNGQPE</i>	<i>NNYKTTPPVL</i>	<i>DSDGSFFLYS</i>
	<i>KLTVDKSRWQ</i>	<i>QGNVFSCSV</i>	<i>HEALHNHYTQ</i>	<i>KSLSLSPGK*</i>		

15

pSYN-FVII- 027 DNA seq- u.ence

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5	tacaacagca ggcaaggagt atctcctaaag gatgagctga gacatcgccg cccggtgttg aggtggcagc tacacgcaga	cgtaccgtgt acaagtgcaa ccaaagggca ccaagaacca tgagtgagg actccgacgg aggggaacgt agagcctctc	ggtcagcgctc ggctctccaac gccccgagaa ggtcagcctg gagcaatggg ctcctctcttc cttctcatgc cctgtctccg	ctcaccgtcc aaagccctcc ccacaggtgt acctgcctgg cagccggaga ctctacagca tccgtgatgc ggtaaatga	tgcaccagga cagcccccat acaccctgcc tcaaaggctt acaactacaa agctcaccgt atgaggctct gcacaaccac	ctgggtgaat cgagaaaacc cccatcccg ctatcccagc gaccacgcct ggacaagagc gcacaaccac
10						
15	FVII-027 amino acid sequence . Signal sequence is shown in dotted underline, propeptide is double underlined, linker region connecting FVII or MB9 to Fc region is underlined, and linker with proprotein convertase processing sites is shown in bold					
20	^ . SQALRLLC . L . LLGLQGCLA SFEEAREIFK THKDDQLICV KRNASKPQGR NLI AVLGEHD LPERTFSERT ITEYMLFCAGY YIEWLQKLMR PPCPAPPELLG AKTKPREEQY QVYTLPPSRD YSKLTVDKSR	. L . LLGLQGCLA DAERTKLFWI NENGGCEQYC IVGGKVC PKG LSEHDGDEQS LAFVRFSLVS SDGSKDSCKG SEPRPGVLLR GSPVFLFPFK NSTYRWSVL ELTKNQVSLT WQQGNVFSKS	AVFVTOEEAH SYSDGQDCAS SDHTGTRKSC ECPWQVLLLV RRVAQVIIPS DSGGPHATHY APFPGGGGSG PKDTLMISRT TVLHQDWLNG CLVKGFYPSD VMHEALHNHY	GVLHRRRRRAN SPCQNGGSK RCHEGYSLLA NGAQLCGGTL TYVPGTTNHD RGTWYLTGIV GGGSGGGGSG PEVTCVWDV KEYCKKVSNN IAVEWESNGQ TQKSLSLSPG	AFLEELRPGS DQLQSYICFC DGVSTPTVE INTIWWSA IALRLHQPV PRLMTQDCLQ SWGQGCATVG GGGSGGGGSG SHEDPEVKFN ALPAPIEKTI PENNYKTPP KRRRRSGGGG	LERECKEEQC LPAFEGRNCE YPCGKIPILE HCFDKIKNWR VLTDHWPLC QSRKVGDSFN HFGVYTRVSQ GGGSDKTHTC WYVDGVEVHN SKAKGQPREP VLDSGGSFFL SGGGSGGGG
25						
30	SGGGSGGGG RQAPGQGLEW RGRALYNRND VAPGQTARIT LTISRVEAGD GGGSGGGGGS TPEVTCWVD GKEYCKKVSNN DIAVEWESNG YTQKSLSLSP	SGGGSGSRKRR MGWINPNSSG RSPNWFDPWG CGNNIGSKS EADYYCQVWD GGGSGGGGGS VSHEDPEVKF KALPAPIEKT QPENNYKTPP GK*	KRAEVQLVQS TNYAQKFQGW QGTTLVTSSG VQWYQKPGQ SSSDHWFGG GGGSGDKTHT NWYVDGVEVH ISKAKGQPRE PVLDSGGSFF	VTMTRDTS IS SASAPTLKLE APVLWYDDS GTKLTVLGQP CPPCPAPPELL NAKTKPREEQ PQVYTLPPSR LYSKLTVDKS	TAYMELSRRLR EGEFSEARVQ DRPSGIPERF KAAPSVTLFP GGPSVFLFPF YNSTYRVVSV DELTKNQVSL RWQQGNVFSC	TFTGYMHVW SDDTAVYCA AVLTQPPSVS SGSNSGNMAT PSAAAGGGGS KPKDTLMISR LTVLHQDWLN TCLVKGFYPS SVMHEALHNH
35						
40						
45	pSYN-FIX-0 44 DNA sequence atgcagcgcg ggatatctac gcttgccctt gatttgacag aacatcacag aaaaacaaag ctaaagaatt tgaatcgccc agagagaatg ctgaaagaac catgtttaa ttggatttga gcgagcagtt atcgacttgc tttctgtttc atgtaaattc ttaatgactt aggttgtttt ggattgtaac aacataatat ctcaccacaa tggacgaacc acacgaacat acaaagggag catgtcttcg aaggaggtag ggaccagttt atggaatata ctgacaaaac tcttctctct	tgaacatgat tcagtgtctga tagatataga caatattgaa atcttggtc actttcttaa attcttttac aaagaggtat tatggaagaa aactgaattt tgccggcagt aggaaagaac ttgtaaaaat agaaaaccag acaaacttct tactgaagct cactcgggtt gaatggtaaa tgctgccac tgaggagaca ctacaatgca cttagtgcta cttctcaaa atcagcttta atctacaaag agattcatgt cttaactgga taccgaagta tcacacatgc cccccaaaa	catggcagaa atgtacaggt aatatctgat gagcttaaca catgccctaa gagatgtaaa atttcagttt aattcaggt aagttagtt tggaagcag tgcaaggatg tgtgaattag agtgtgata aagtcctgtg aagctcacc gaaaccattt gttggtggag gttgatgeat tgtgttgaaa gaacatacag gctattaata aacagctacg tttgatctg gttcttcagt ttcaccatct caaggagata attattagct teceggtag ccaccgtgcc cccaaggaca	tcaccaggcc ttgtttcctt gctgtcttct gccagcacgc agagaaattg atcttcatga ttcttgatca aattggaaga ttgaagaagc atggtgatgg acattaattc atgtaacatg acaagggtgt aaccagcag gtgetgagae tggataacat aagatgccaa tctgtggagg ctgggtgttaa ageaaaageg agtacaacca ttacacctat gctatgtaag accttagagt ataacaacat gtgggggacc ggggtgaaga tcaactggat cagctccgga cctcatgat	tcatcaccat ttttaaata tcactaaatt aggttggtaa gctttcagat tgttttcttt tgaaaacgee gtttgttcaa acgagaagtt agatcagtt ctatgaatgt taacattaag ttgtcctgt gccatttcca tgtttttcct cactcaaagc accaggtcaa etctategtt aattacagtt aaatgtgatt tgacattgee ttgeattget tggctgggga tccacttgtt gttctgtgct ccatgttact gtgtgcaatg taaggaaaaa actcctggga ctccggacc	ctgectttta cattgagtat ttgattacat gtactgtggg tatttgatt tttgctaaaa aacaaaattc gggaatctag tttgaaaaa agatccaatc tgggtgtccct aatggcagat actgagggat tgtggaagag gatgtggact acccaatcat ttcccttggc aatgaaaaat gtcgaggtg cgaattattc cttctggaac gacaaggaat agagcttcc gaccgagcca ggcttccatg gaagtggaa aaaggcaaat acaaagctca ggaccgtcag cctgaggtca
50						
55						
60						
65						
70						

5	catgcgtggt acggcggtga accgtgtggt agtgcgaagt aaggcgagcc	ggtggacgtg ggtgcataat cagcgtcctc ctccaacaaa ccgagaacca	agccacgaag gccaagacaa accgtcctgc gccctcccag caggtgtaca	accctgaggt agccgcggga accaggactg cccccatcga ccctgcccc	caagttcaac ggagcagtag gctgaatggc gaaaaccatc atccccggat	tggtacgtgg aacagcacgt aaggagtaca tccaaagcca gagctgacca
10	agaaccaggt agtgggagag ccgacggctc ggaacgtctt gcctctccct	cagcctgacc caatgggcag cttcttcctc ctcatgctcc gtctccgggt	tgcttggtca ccggagaaca tacagcaagc gtgatgcatg aaacggcgcc	aaggcttcta actacaagac tcaccgtgga aggctctgca gccggagcgg	tcccagcgac cacgcctccc caagagcagg caaccactac tgccggcgga	atcgccgtgg gtgttggtg tggtgagcag acgcagaaga tcaggtgggg
15	gtggatcagg acacatgccc ccccaaaacc tggaactgag tgcataatgc	cgggtggaggt accgtgccc caaggacacc ccacgaagac caagacaaa	tccggtggcg gcaccgggaa ctcatgatct cctgaggtca ccgcgggagg	ggggatccc tcctggggcg cccggacccc agttcaactg agcagtagaa	ccggcgggcg accgtcagtc tgaggtcaca gtacgtggac cagcacgtac	gacaaaactc ttcctcttc tgctgtgtgg ggcgtggagg cgtgtgtgta
20	cgcctctcac ccaacaaagc gagaaccaca gcctgacctg atgggcagcg	accgtgccc ctccccagcc ggtgtacacc cctggtcaaa ggagaacaac	caggactggc cccatcgaga ctgcccccat ggcttctatc tacaagacca	tgaatggcaa aaaccatctc cccgggatga ccagcgacat cgcttcccgt	ggagtacaag caaagccaaa gctgaccaag cgccgtggag gttggactcc	tgcaaggtct gggcagcccc aaccaggtca tggtgagagca gacggctcct
25	tcttctctca catgctccgt ctccgggtaa	gagcaagctc gatgcatgag atga	accgtggaca gctctgcaca	agagcaggtg accactacac	gcagcagggg gcagaagagc	aacgtctctc ctctccctgt
30	FIX-044 amino acid sequence. Signal sequence is shown in dotted underline, propeptide is double underlined, and linker with proprotein convertase processing sites is shown in bold.					
35	MORVNMIMAE	SPGHIXICIL	GYLISAFCTV	FLDHENANKI	LNRPKRYNSG	KLEEFVQGNL
40	EREEMEEKCS	FEEAREVFEN	TERTFEFWKQ	YVDGDQCESN	PCLNGGSKCD	DINSYECWCP
45	FGFEGKNCEL	DVTCNIKNGR	CEQFCKNSAD	NKWCSCTEG	YRLAENQKSC	EPAVPFPCGR
50	VSVSQTSLKT	RAE TVFPDVD	YVNSTEAEIT	LDNITQSTQS	FNDFTRVVGG	EDAKPGQFPW
55	QVVLNGKVDA	FCGGS IVNEK	WIVTAAHCEV	TGVKITWAG	EHNIETEHT	EQKRNVI RI
60	PHHNYNAAIN	KYNHDIALLE	LDEPLVLNSY	VTPICIIDKE	YTNIFLKFSG	GYVSGWGRVF
65	HKGRSALVLQ	YLRVP LVDRA	TCLRSTKFTI	YNNMFCAGFH	EGGRDSCQGD	SGGPHVTEVE
70	GTSFLTGI IS	WGEECAMKKG	YGI YTKVSRY	VNWIKETKTL	TDKTHTCPPC	PAPELLGGPS
75	VFLFPPKPKD	TLMISRTPEV	TCVWDVSHE	DPEVKFNWYV	DGVEVHNAKT	KPREEQYNST
80	YRWSVLTVL	HQDWLNGKEY	KCKVSNKALP	APIEKTISKA	KGQPREPQVY	TLPPSRDEL
85	KNQVSLTCLV	KGFYPDSIAV	EWESNGQPEN	NYKTTTPVLD	SDGSFFLYSK	LTVDKSRWQQ
90	GNVFSCVMH	EALHNHYTQK	SLSLSPGKRR	RRSGGGGSGG	GGSGGGGSGG	GGRRRRDKT
95	HTCPPCPAPE	LLGGPSVFLF	PPKPKDTLMI	SRTPEVTCW	VDVSHEDPEV	KFNWYVDGVE
100	VHNAKTKPRE	EQYNSTYRVV	SVLTVLHQDW	LNGKEYKCKV	SNKALPAPIE	KTISKAKGQP
105	REPQVYTLPP	SRDELTKNQV	SLTCLVKGFY	PSDIAVEWES	NGQPENNYKT	TPPVLDSDGS
110	FFLYSKLTVD	KSRWQQGNVF	SCSVMHEALH	NHYTQKSLSL	SPGK*	
115	Genscr ipt-FIX-044 DNA sequence					
120	tccggaactc	ctgggaggac	cgtagctctt	cctcttcccc	ccaaaaccca	aggacaccct
125	catgatctcc	cggacccttg	aggtcacatg	cgtggtggtg	gacgtgagcc	acgaagaccc
130	tgaggtcaag	ttcaactggt	acgtggacgg	cgtggaggtg	cataatgcca	agacaaagcc
135	gcgggaggag	cagtacaaca	gcacgtaccg	tgtggtcagc	gtcctcaccg	tcctgcacca
140	ggactggctg	aatggcaagg	agtacaagtg	caaggtctcc	aacaaagccc	tcccagcccc
145	catcgagaaa	accatctcca	aagccaaagg	gcagccccga	gaaccacagg	tgtacaccct
150	cccccatccc	cgggatgagc	tgaccaagaa	ccaggtcagc	ctgacctgcc	tggtcaaagg
155	cttctatccc	agcgacatcg	ccgtggagtg	ggagagcaat	gggcagccgg	agaacaacta
160	caagaccacg	cctcccggtg	tggactccga	cggctccttc	ttcctctaca	gcaagctcac
165	cgtggacaag	agcaggtggc	agcaggggaa	cgtcttctca	tgctccgtga	tgcatgaggc
170	tctgcacaac	cactacacgc	agaagagcct	ctccctgtct	ccgggtaaac	ggcgccggcg
175	gagcggtgcc	ggcggatcag	gtgggggtgg	atcaggcggt	ggaggttccg	gtggcggggg
180	atcccccgcc	cggcgcgaca	aaactcacac	atgccaccgc	tgcccagcac	cggacaccct
185	gggcggaccg					
190	Genscr ipt-FVII-027-1 DNA sequence					
195	gaagagcctc	tcctgtctc	cgggtaa acg	gcgcgcggcg	agcgggtggc	gcggatcagg
200	tggtgggtgga	tcaggcggtg	gaggttc egg	tggtggggga	tcggcggtg	gaggttcggg
205	tggtgggtgga	tcaagggaaga	ggaggaa gag	ggcggaagtg	cagctggtgc	agtctggagc
210	tgaggtgaat	aagcctgggg	cctcagt gaa	ggtctcctgc	aaggcttctg	gatacacctt
215	caccggctac	tatatgcact	gggtgcg aca	ggccccctgga	caagggttg	agtggatggg
220	atgatacaac	cctaacagtg	gtggcac aaa	etatgeacag	aagtttcagg	gctgggtcac
225	catgaccagg	gacacgtcca	tcagcac cgc	ctacatggag	ctgagcaggc	tgagatctga
230	cgacacggcc	gtgtattact	gtgcgag agg	ccgtgctttg	tataacegga	acgaccggtc

5 ccccaactgg ttgcagccct ggggccaggg aaccctggtc accgtctcct cagggagtgc
 atccgcccc acccttaagc ttgaagaagg tgaattc

Genscript-FVII-0 26-2 DNA sequence

10 gaattctcag aagcacgcgt acaggctgtg ctgactcagc cgccctcggt gtcagtggcc
 ccaggacaga cggccaggat tacctgtggg ggaacaaca ttggaagtaa aagtgtgcag
 tggtaccagc agaagccagg ccaggcccct gtgctggtcg tctatgatga tagcagccgg
 ccctcaggg tccttgagcg attctctggc tccaactctg ggaacatggc caccctgacc
 atcagcaggg tcgaagccgg ggatgaggcc gactattact gtcagggtg ggatagtagt
 agtgatcatg tggatattcg cggagggacc aagctgaccg tcctaggtca gccaagggt
 gccccctcgg tcaactctgtt cccgcgcgtc gcggccgctg gtggcgggtg ctccggcgga
 ggtgggtccg gtggcgccgg atcaggtggg ggtggatcag gcggtggagg ttccgggtgg
 gggggatcag aaaaaactca cacatgccc ccgtgcccag cacctgaact cctgggagga
 ccgtcagctc tcctcttccc cccaaaaccc aaggacaccc tcatgatctc ccggaccctc
 gaggtcacat gcgtgggtgt ggacgtgagc cacgaagacc ctgagggtcaa gttcaactgg
 tacgtggacg gcgtggaggt gcataatgcc aagacaaagc cgcgggagga gcagtaaac
 agcaggtacc ggtgggtcag cgctctcacc gtcctgcacc aggactggct gaatggcaag
 gagtacaagt gcaaggtctc caacaaagcc ctcccagccc ccatcgagaa aaccatctcc
 aaagccaaag ggagcccccg agaaccacag gtgtacaccc tgccccatc ccgcgatgag
 ctgaccaaga accaggtcag cctgacctgc ctggtcaaag gcttctatcc cagcgacatc
 gcccgtggagt ggagagcaa tgggcagccg gagaacaact acaagaccac gcctcccgtg
 ttggactccg acggctcctt cttcctctac agcaagctca ccgtggacaa gagcaggtgg
 cagcagggga acgtcttctc atgctccgtg atgcatgagg ctctgcacaa ccactacacg
 cagaagagcc tctccctgtc tccgggtaaa tgagaattc

25 **B domain deleted FVIII amino acid sequence:** Signal peptide
 underlined; 14 amino acid linker (containing the remaining B domain)
 between the HC and LC sequence is double underlined, with the
 S743/Q1638 fusion site indicated in bold.

30 1 **MQIELSTCFF** **LCLLRFCFSA** TRRYYLGAWE LSWDYMQSDL GELPVDARFP
 35 51 PRVPKSFPPN TSWYKKTFLF VEFTDHLFNI AKPRPPWMGL LGPTIQAEVY
 101 DTWITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR EKEDDKVFP
 151 GSHTYVWQVL KENGPASDP LCLTYSYLSH VDLVKDLNSG LIGALLVCRE
 201 GSLAKEKTQT LHKF ILLFAV FDEGKSWHSE TKNSLMQDRD AASARAWPKM
 251 HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL EGHTFLVRNH
 40 301 RQASLEISPI TFLTAQTLLM DLGQFLFCH ISSHQHDGME AYVKVDSCPE
 351 EPQLRMKNNE EAEDYDDDLT DSEMDWRFD DDNSPSFIQI RSVAKKHKPT
 401 WVHYIAEEEE DWDYAP LVLA PDDRSYKSQY LNNGPQRIGR KYKKVRFMAY
 451 TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR PYN IYPHGIT
 501 DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP TKSDPRCLTR
 45 551 YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR NVILFSVFDE
 601 NRSWYL TENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV FDSLQLSVCL
 651 HEVAYWYILS IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF PFSGETVFMS
 701 MENPGLWILG CHNSDFRNRG MTALLKVSSC DKNTGDYIED SYEDISAYLL
 751 SKNNAIEPRIL **FSQNPPVLKR** **HOEITRTTL** QSDQEEIDYD DTISVEMKKE
 50 801 DFDI YDEDEN QSPRSFQKKT RHYFIAAVER LWGYGMS SSP HVLRNRAQSG
 851 SVPQFKKWF QEFTDGSFTQ PLYRGELNEH LGLLGPYIRA EVEDNIMVTF
 901 RNQASRPYSF YSSLISYEED QRQGAEPKRN FVKPNETKTY FVKVQHMAP
 951 TKDEFDCKAW AYFSDVDLEK DVHSLIGPL LVCHTNLTNP AHGRQVTQVE
 1001 FALFFTIFDE TKSIFYTENM ERNCRAPCNI QMEDPTFKEN YRFHAINGYI
 55 1051 MDLPLGLVMA QDQIRIWYLL SMGSNENIHS IHFSGHVF TV RKKEEYKMAL
 1101 YNLYPGVFET VEMPLSKAGI WRVECLIGEH LHAGMSTLFL VYSNKCQTPL
 u s 1 GMASGHIRDF QITASGQYQG WAPKLARLHY SGSINAWSTK EPFSWIKVDL
 1201 LAPMI IHGIK TQGARQKFSS LYISQFI IMY SLDGKKWQTY RGNSTGTLMV
 1251 FFGNVDSSGI KHNIFNPPII ARYIRLHPTH YSIRSTLRME LMGCDLNSCS
 60 1301 MPLGMESKAI SDAQITASSY FTNMFATWSP SKARLHLQGR SNAWRPQVNN
 1351 PKEWLQVDFQ KTMKVTGVTT QGVKSLLTSM YVKEFLISSS QDGHQWTLFF
 1401 QNGKVKVFQ NQDSFTPVVN SLDPPLLTTRY LRIHPQSWVH QIALRMEVLG
 1451 CEAQDLY

65 Full length FVIII amino acid sequence: Signal peptide underlined

70 1 **MQIELSTCFF** **LCLLRFCFSA** TRRYYLGAWE LSWDYMQSDL GELPVDARFP
 51 PRVPKSFPPN TSWYKKTFLF VEFTDHLFNI AKPRPPWMGL LGPTIQAEVY
 101 DTWITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR EKEDDKVFP
 151 GSHTYVWQVL KENGPASDP LCLTYSYLSH VDLVKDLNSG LIGALLVCRE
 201 GSLAKEKTQT LHKF ILLFAV FDEGKSWHSE TKNSLMQDRD AASARAWPKM
 251 HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL EGHTFLVRNH

5	301	RQASLEISPI	TFLTAQTLLM	DLGQFLLFCH	ISSHQHDGME	AYVKVDSCPE
	351	EPQLRMKNNE	EAEDYDDDLT	DSEMDWRFD	DDNSPSFIQI	RSVAKKHPKT
	401	WVHYIAAEEE	DWDYAP LVLA	PDDRSYKSQY	LNNGPQRIGR	KYKKVRFMAY
	451	TDETFTKTREA	IQHESGILGP	LLYGEVGDITL	LIIFKNQASR	PYNIYPHGIT
	501	DVRPLYSRRL	PKGVKHLKDF	PILPGEIFKY	KWTVTVEDGP	TKSDPRCLTR
10	551	YYSSFVNMER	DLASGLIGPL	LICYKESVDQ	RGNQIMSDKR	NVILFSVFDE
	601	NRSWYLTENI	QRFLPNPAGV	QLEDPEFQAS	NIMHSINGYV	FDSLQLSVCL
	651	HEVAYWYILS	IGAQTDFLSV	FFSGYTFKHK	MVYEDTLTLF	PFSGETVFMS
	701	MENPGLWLIG	CHNSDFRNRG	MTALLKVSSC	DKNTGDYED	SYEDISAYLL
	751	SKNNAIEPRS	FSQNSRHPST	RQKQFNATTI	PENDIEKTD	WFAHRTMPK
15	801	IQNVSSDDL	MLLRQSPTPH	GLSLSDLQEA	KYETFSDDP	PGAIDSNNSL
	851	SEMTHFRPQL	HHSGLMVFTF	ESGLQLRLNE	KLGTATAATL	KKLDFKVSST
	901	SNNLISTIPS	DNLAAGTDNT	SSLGPPSMPV	HYDSQLDITL	FGKKSSPLTE
	951	SGGPLSLSEE	NNDSKLLSEG	LMNSQESSWG	KNVSSTESGR	LFGKGRAHGP
	1001	ALLTKDNALF	KVSISLLKTN	KTSNNSATNR	KTHIDGPSLL	IENSPSVWQN
20	1051	ILESDETFKK	VTPLIHDRML	MDKNATALRL	NHMSNKTSS	KNMENVQKK
	1101	EGPIPPDAQN	PDMSFFKMLF	LPESARWIQR	THGKNSLNSG	QGPSPKQLVS
	1151	LGPEKSVEGQ	NFLSEKNKW	VGKGEFTKDV	GLKEMVFPSS	RNLFLTNLND
	1201	LHENNTHNQE	KKIQEE IEKK	ETLIQENWL	PQIHVTGTGK	NFMKNLFLLS
	1251	TRQNVESYD	GAYAPVLQDF	RSNDSTNRT	KKHTAHFSKK	GEEENLEGLG
25	1301	NQTKQIVEKY	ACTTRISPNT	SQONFVTQRS	KRALKQFRLP	LEETLEKRI
	1351	IVDDTSTQWS	KNMKHLTPST	LTQIDYNEKE	KGAITQSPLS	DCLTRSHSIP
	1401	QANRSPLPIA	KVSSFPSIRP	IYLRVLFQD	NSSHLPAASY	RKKDSGVQES
	1451	SHFLQGAKKN	NLSLAILTLE	MTGDQREVGS	LGTSATNSVT	YKKVENTVLP
30	1501	KPDLPKTSKG	VELLPKVIHY	QKDLFPTETS	NGSPGHLDLV	NGSLDQGTG
	1551	AIKWNEANRP	GKVPFLRVAT	ESSAKTPSKL	LDPLAWDNHY	GTQIPKEEWK
	1601	SQEKSPKTA	FKKKDTILSL	NACESNHAIA	AINEGQNKPE	IEVTWAKQGR
	1651	TERLCSQNPP	VLKRHQRE IT	RTTLQSDQEE	IDYDDTISVE	MKKEDFDI YD
	1701	EDENQSPRSF	QKKTRHYF IA	AVERLWDYGM	SSSPHVLNR	AQSGSVPOFK
	1751	KVVFQEFDTG	SFTQPLYRGE	LNEHLGGLGP	YIRAEVEDNI	MVTFRNQASR
35	1801	PYSFYSSLIS	YEEDQRQGA	PRKNFVKPNE	TKTYFWKVQH	HMAPTKDEFD
	1851	CKAWAYFSDV	DLEKDVHSG	IGPLLVCNTN	TLNPAHGRQV	TVQEFALFFT
	1901	IFDETKSWYF	TENMERNCR	PCNIQMEDPT	FKENYRFHAI	NGYIMDTLPG
	1951	LVMAQDQIR	WYLLSMGSNE	NIHSIHFSGH	VFTVRKKEEY	KMALYNLYPG
	2001	VFETVEMLPS	KAGIWRVECL	IGEHLHAGMS	TLFLVYSNKC	QTPLGMASGH
40	2051	IRDFQITASG	QYGQWAPKLA	RLHYSGS INA	WSTKEPFSWI	KVDLLAPMI I
	2101	HGIKTQGARQ	KFSSLYISQF	IIMYSLDGKK	WQTYRGNSTG	TLMVFFGNVD
	2151	SSGIKHNI	PIIARYIRL	HPHYSIRST	LRMELMGCDL	NSCSMPLGME
	2201	SKAISDAQIT	ASSYFTNMFA	TWSPSKARLH	LQGRSNAWRP	QVNNPKEWLQ
	2251	VDFQKTMKVT	GVTTQGVKSL	LTSMYVKEFL	ISSSQDGHQW	TLFFQNGKVK
45	2301	VFQGNQDSFT	PVVNSLDPPL	LTRYLRIHPQ	SWVHQIALRM	EVLGCEAQDL
	2351	Y				

50	FIX amino acid sequence. Signal sequence is shown in dotted underline, propeptide is double underlined					
	<u>MORVNMIMAE</u>	<u>SPGLITICLL</u>	<u>GILLSAEC TV</u>	<u>FLDHENANKI</u>	<u>LNRPKRYNSG</u>	<u>KLEEFVQGNL</u>
	ERECMEKCS	FEAREVFEN	TERTTEFWKQ	YVDGDQCESN	PCLNGGSCKD	DINSYECWCP
	FGFEGKNCEL	DVCNINKNR	CEQFCNSAD	NKWCSTEG	YRLAENQKSC	EPAPVPFCGR
	VSVSQTSLT	RAE TVFPD	YVNSTEAETI	LDNITQSTQS	FNDFTRVVGG	EDAKPGQFPW
55	QVVLNGKVDA	FCGS IVNEK	WIVTAAHCVE	TGVKITWAG	EHNIETEHT	EQKRNVI RI
	PHHNYNAIN	KYNHDIALLE	LDEPLVLSNY	VTPICIADKE	YTNIFLKFGS	GYVSGWGRVF
	HKGRSALVLQ	YLRVP LVDRA	TCLRSTKFTI	YNNMFCAGFH	EGGRDSCQGD	SGGPHVTEVE
	GTSFLTGI IS	WGEECAMKGK	YGI YTKVSRY	VNWIKEKTKL	T	

60	FIX DNA sequence					
	atgcagcgcg	tgaacatgat	catggcagaa	tcaccaggcc	tcataccat	ctgccttttta
	ggatatctac	tcaagtctga	atgtacagtt	tttcttgatc	atgaaaacgc	acaaaatt
65	ctgaatcgcc	caaagaggta	taattcaggt	aaattggaag	agtttggtca	aggggaatcta
	gagagagaat	gtatggaaga	aaagtgtagt	tttgaagaag	cacgagaagt	ttttgaaaac
	actgaaagaa	caactgaatt	ttggaagcag	tatgttgatg	gagatcagtg	tgagtccaat
	ccatgtttta	atggcggcag	ttgcaaggat	gacattaatt	cctatgaatg	ttggtgtccc
	tttggatttg	aaggaaagaa	ctgtgaatta	gatgtaacat	gtaacattaa	gaatggcaga
70	tgcgagcagt	tttgtaaaaa	tagtgctgat	aacaagggtg	tttgctcctg	tactgagga
	tatcgacttg	cagaaaaacca	gaagtcctgt	gaaccagcag	tgccatttcc	atgtgggaaga
	gtttctgttt	cacaaacttc	taagctcacc	cgtgctgaga	ctgtttttcc	tgatgtggac
	tatgtaaatt	ctactgaagc	tgaaccatt	ttggataaca	tcactcaaag	cacccaatca

5 ttttaatgact tcaactcggt tgttggtgga gaagatgcca aaccagggtca attcccttgg
 cagggtgttt tgaatggtaa agttgatgca ttctgtggag gctctatcgt taatgaaaaa
 tggattgttaa ctggtgtgaa ctggtgtgaa actggtgtta aaattacagt tgtcgaggt
 gaacataata ttgaggagac agaacataca gagcaaaagc gaaatgtgat tgaattatt
 cctcaccaca actacaatgc agctattaat aagtacaacc atgacattgc ccttctggaa
 10 ctggacgaac ccttagtgct aaacagctac gttacaccta tttgcattgc tgacaaggaa
 tacacgaaca tcttcctcaa atttgatct ggctatgtaa gtggctggg aagagtcttc
 cacaaaggga gatcagcttt agttcttcag taccttagag ttccacttgt tgaccgagcc
 acatgtcttc gatctacaaa gttcaccatc tataacaaca tgttctgtgc tggcttccat
 gaaggaggta gagattcatg tcaaggagat agtgggggac cccatgttac tgaagtggaa
 15 gggaccagtt tcttaactgg aattattagc tggggtgaag agtgtgcaat gaaaggcaaa
 tatggaatat ataccaaggt atcccgtat gtcaactgga ttaaggaaaa aacaaagctc
 acttga

20 FX amino acid sequence. Signal sequence is shown in dotted underline,
 propeptide is double underlined

20 MGRPLHLVLL SASLAGLILL GESLF IRREO ANNILARVTR ANSFLEEMKK GHLERECMEE
 TCSYEAREV FEDSDKTNEF WNKYKDGQDC ETSPCQNQCK CKDGLGEYTC TCLEGFEGKN
 CELFTRKLC LDNGDCDQFC HEEQNSVVC CARGYTLADN GKACIPTGPY PCGKQTLERR
 25 KRSVAQATSS SGEAPDSITW KPYDAADLDP TENPFDDLDF NQTQPERGDN NLTRIVGGQE
 CKDGECPWQA LLINEENEGF CGGTILSEFY ILTAAHCLYQ AKRFKVRVGD RNTEQEEGGE
 AVHEVEWIK HNRFTKETD FDIIVLRLKT PITFRMNVAP ACLPERDWAE STLMTQKTGI
 VSGFGRTHEK GRQSTRKML EVPYVDRNSC KLSSSFITQ NMF CAGYDTK QEDACQDSG
 GPHVTRFKDT YFVTGIVSWG EGCARKGKYG IYTKVTAFLK WIDRSMKTRG LPKAKSHAPE
 VITSSPLK

30 FX DNA sequence
 atggggcgcc cactgcacct cgtctgctc agtgcctccc tggctggcct cctgctgctc
 ggggaaagtc tgttcatccg cagggagcag gccacaaca tcttggcgag ggtcacgagg
 gccaatctct ttcttgaaga gatgaagaaa ggacacctcg aaagagagtg catggaagag
 35 acctgctcat acgaagagtc ccgcgaggtc tttgaggaca gcgacaagac gaatgaattc
 tggaataaat acaaagatgg cgaccagtgt gagaccagtc cttgccagaa ccagggcaaa
 tgtaaaagacg gcctcgggga atacacctgc acctgtttag aaggattcga aggcacaaac
 tctgaattat tcaacacgaa gctctgcagc ctggacaacg gggactgtga ccagttctgc
 acagaggaaac agaactctgt ggtgtgctcc tgcgcccgcg ggtacacctc ggtgacaaac
 40 ggcaaggcct gcattccac agggccctac cctgtgagg aacagacctc ggaacgcagg
 aagaggtcag tggcccagc caccagcagc agcggggagg cccctgacag catcacatgg
 aagccatatg atgcagccga cctggacccc accgagaacc ccttcgacct gcttgacttc
 aaccagacgc agcctgagag gggcgacaac aacctcacca ggatcgtggg aggcaggaa
 tgcaaggacg gggagtgtcc ctggcaggcc ctgctcatca atgaggaaaa cgagggttct
 45 tgtggtggaa ccattctgag cgagtctac atcctaaccg cagcccactg tctctaccaa
 gccaaagagat tcaaggtgag ggtaggggac cggaacacgg agcaggagga gggcggtgag
 gcgggtgcacg aggtggaggt ggtcatcaag cacaaccggt tcacaaagga gacatatgac
 ttcgacatcg cgtgctccg gctcaagacc cccatcacct tccgcatgaa cgtggcgctc
 gcctgcctcc ccgagcgtga ctgggcccag tccacgctga tgacgcagaa gacggggatt
 50 gtgagcggct tcggggcgac ccacgagaag ggccggcagt ccaccaggct caagatgctg
 gaggtgccct acgtggaccg caacagctgc aagctgtcca gcagcttcat catcacccag
 aacatgttct gtgcccgtc cgacaccaag caggaggatg cctgccaggg ggacagcggg
 ggcccgcacg tcaccgctt caaggacacc tacttcgtga caggcatcgt cagctgggga
 gagggctgtg cccgtaaggg gaagtacggg atctacacca aggtcaccgc cttcctcaag
 55 tggatcgaca ggtccatgaa aaccaggggc ttgcccagg ccaagagcca tgccccggag
 gtcataacgt cctctccatt aaagtga

60 DNA sequence for pSYN-FIX-053
 1 ATGCAGCGCG TGAACATGAT CATGGCAGAA TCACCAGGCC TCATCACCAT CTGCCTTTTA
 61 GGATATCTAC TCAGTGCTGA ATGTACAGGT TTGTTTCCTT TTTTAAAAATA CATTGAGTAT
 121 GCTTGCCTTT TAGATATAGA AATATCTGAT GCTGTCTTCT TCACTAAATT TTGATTACAT
 181 GATTTGACAG CAATATTGAA GAGTCTAACA GCCAGCACGC AGGTTGGTAA GTACTGTGGG
 241 AACATCACAG ATTTTGGCTC CATGCCCTAA AGAGAAATTG GCTTTCAGAT TATTTGGATT
 65 301 AAAAACAAG ACTTCTTAA GAGATGTAAA ATTTTCATGA TGTTCCTTTT TTTGCTAAAA
 361 CTAAGAATT ATTCTTTTAC ATTTCAAGTT TTCTTGATCA TGAAAACGCC AACAAAATTC
 421 TGAATCGGCC AAAGAGGTAT AATTCAGGTA AATTGGAAGA GTTTGTTCAT GGAATCTAG
 481 AGAGAGAATG TATGGAAGAA AAGTGTAGTT TTGAAGAAGC ACGAGAAGTT TTTGAAAACA
 541 CTGAAAGAAC AACTGAATTT TGGAAAGCAGT ATGTTGATGG AGATCAGTGT GAGTCCAATC
 70 601 CATGTTTAAA TGGCGGCAGT TGCAAGGATG ACATTAATTC CTATGAATGT TGGTGTCCCT
 661 TTGGATTTGA AGGAAAGAAC TGTGAATTAG ATGTAACATG TAACATTAAG AATGGCAGAT
 721 GCGAGCAGTT TTGTAAAAAT AGTGCTGATA ACAAGGTGGT TTGCTCCTGT ACTGAGGGAT
 781 ATCGACTTGC AGAAAACCAG AAGTCTCTGT AACCAGCAGT GCCATTTCCA TGTGGAAGAG
 841 TTTCTGTTTC ACAAACTTCT AAGCTCACCC GTGCTGAGAC TGTTTTCCTT GATGTGGACT

5	901	ATGTAAATTC	TACTGAAGCT	GAAACCATT	TGGATAACAT	CACTCAAAGC	ACCCAATCAT
	961	TTAATGACTT	CACTCGGTT	GTTGGTGGAG	AAGATGCCAA	ACCAGGTCAA	TTCCCTTGGC
	1021	AGGTTGTTTT	GAATGGTAAA	GTTGATGCAT	TCTGTGGAGG	CTCTATCGTT	AATGAAAAAT
	1081	GGATTGTAA	TGCTGCCAC	TGTGTTGAAA	CTGGTGTAA	AATACAGTT	GTCGCAGGTG
	1141	AACATAATAT	TGAGGAGACA	GAACATACAG	AGCAAAAGCG	AAATGTGATT	CGAATTATTC
10	1201	CTCACCACAA	CTACAATGCA	GCTATTAATA	AGTACAACCA	TGACATTGCC	CTTCTGGAAC
	1261	TGGACGAACC	CTTAGTGCTA	AACAGCTACG	TTACACCTAT	TTGCATTGCT	GACAAGGAAT
	1321	ACACGAACAT	CTTCTCAAAA	TTTGGATCTG	GCTATGTAAG	TGGCTGGGGA	AGAGTCTTCC
	1381	ACAAAGGGAG	ATCAGCTTTA	GTTCTTCAGT	ACCTTAGAGT	TCCACTTGTT	GACCGAGCCA
	1441	CATGTCTTCG	ATCTACAAAG	TTCACCATCT	ATAACAACAT	GTTCTGTGCT	GGCTTCCATG
15	1501	AAGGAGGTAG	AGATTCTATG	CAAGGAGATA	GTGGGGGACC	CCATGTTACT	GAAGTGGAAG
	1561	GGACCAGTTT	CTTAAGTGA	ATTATTAGCT	GGGTGAAGA	GTGTGCAATG	AAAGGCAAT
	1621	ATGGAATATA	TACCAAGGTG	TCCGGTATG	TCAACTGGAT	TAAGGAAAAA	ACAAAGCTCA
	1681	CTGACAAAAC	TCACACATGC	CCACCGTGCC	CAGCTCCGGA	ACTCTGGGA	GGACCGTCAG
	1741	TCTTCTCTT	CCCCCAAAA	CCCAAGGACA	CCCTCATGAT	CTCCCGGACC	CCTGAGGTCA
20	1801	CATGCGTGGT	GGTGGACGTG	AGCCACGAAG	ACCCTGAGGT	CAAGTTCAAC	TGGTACGTGG
	1861	ACGGCGTGGA	GGTGCATAAT	GCCAAGACAA	AGCCGCGGGA	GGAGCAGTAC	AACAGCAGCT
	1921	ACCGTGTGGT	CAGCGTCCCT	ACCGTCTGCT	ACCAGGACTG	GCTGAATGGC	AAGGAGTACA
	1981	AGTGCAAGGT	CTCCAACAAA	GCCCTCCAG	CCCCATCGA	GAAAACCATC	TCCAAGGCCA
	2041	AAGGGCAGCC	CCGAGAACCA	CAGGTGTACA	CCCTGCCCCC	ATCCCGGGAT	GAGCTGACCA
25	2101	AGAACCAGGT	CAGCCTGACC	TGCTGGTCA	AAGGCTTCTA	TCCCGAGGAC	ATCGCGGTGG
	2161	AGTGGGAGAG	CAATGGGAC	CCGAGAACA	ACTACAAGAC	CACGCTCC	GTGTTGGACT
	2221	CCGACGGCTC	CTTCTTCCCT	TACAGCAAGC	TCACCGTCGA	CAAGAGCAGG	TGGCAGCAGG
	2281	GGAACGTCTT	CTCATGTCTC	GTGATGCATG	AGGCTCTGCA	CAACCACTAC	ACGCAGAAGA
30	2341	GCCTCTCCCT	GTCTCCGGGT	AAACGGCGCC	GCCGAGCGG	TGGCGGCGGA	TCAGGTGGGG
	2401	CGGTGATCAGG	CGGTGGAGGT	TCCGTGGCG	GGGGATCCAG	GAAAGAGGAG	AAGAGGGACA
	2461	AAACTCACAC	ATGCCACCG	TGCCCAGCAC	CGGAACCTCT	GGGCGGACCG	TCAGTCTTCC
	2521	TCTTCCCCC	AAAACCCAAG	GACACCTCA	TGATCTCCCG	GACCCCTGAG	GTCACATCGG
	2581	TGGTGGTGGA	CGTGAGCCAC	GAAGACCTTG	AGGTCAAGTT	CAACTGGTAC	GTGGACGGCG
35	2641	TGGAGGTGCA	TAATGCCAAG	ACAAAGCCGC	GGGAGGAGCA	GTACAACAGC	ACGTACCGTG
	2701	TGGTCAGCGT	CCTCACCGTC	CTGCACAGG	ACTGGCTGAA	TGGCAAGGAG	TACAAGTGCA
	2761	AGGTCTCCAA	CAAAGCCCTC	CCAGCCCCCA	TCGAGAAAAC	CATCTCCAAA	GCCAAAGGGC
	2821	AGGCCCGAGA	ACCACAGGTG	TACACCTGTC	CCCCATCCCG	GGATGAGCTG	ACCAAGAACC
	2881	AGGTGAGCCT	GACCTGCTCT	GTCAAAGGCT	TCTATCCAG	CGACATCGCC	GTGGAGTGGG
40	2941	AGAGCAATGG	GCAGCCGGAG	AACAACCTACA	AGACCACGCC	TCCCGTGTG	GACTCCGACG
	3001	GCTCTCTCTT	CTCTACAGC	AAGCTCACCG	TGGACAAGAG	CAGGTGGCAG	CAGGGGAACG
	3061	TCTTCTCATG	CTCCGTGATG	CATGAGGCTC	TGCACAACCA	CTACACGCAG	AAGAGCCTCT
	3121	CCCTGTCTCC	GGGTAATGA				

45 FIX-053 amino acid sequence. Signal sequence is shown in dotted underline, propeptide is double underlined, and linker with proprotein convertase processing sites is shown in bold

50	i	<u>MQRVNMIMAE</u>	<u>SPGLITICLL</u>	<u>GYLLSAECTV</u>	<u>FLDHENANKI</u>	<u>LNRPKRYNSG</u>	KLEEFVQGNL
	61	ERECMEEKCS	FEEAREVFEN	TERTTEFWKQ	YVDGDQCESN	PCLNGGSKCD	DINSYECWCP
	121	FGFEGKNCCL	DVTCNIKNGR	CEQFCNKSAD	NKVVCSCTEG	YRLAENQKSC	EPAPVFPCCR
	181	VSVSQTSLKT	RAETVFPDVD	YVNSTEAETI	LDNITQSTQS	FNDFTRWGG	EDAKPGQFPW
	241	QWLNGKVDA	FCGGSIVNEK	WIVTAAHCVE	TGVKITWAG	EHNIETEHT	EQKRVIRI
55	301	PHHNYNAAIN	KYNHDIALLE	LDEPLVLNSY	VTPICIADE	YTNIFLKFSG	GYVSGWGRVF
	361	HKGRSALVLQ	YLRVPLVDRA	TCLRSTKFTI	YNNMFCAGFH	EGGRDSCQGD	SGGPHVTEVE
	421	GTSFLTGLIS	WGEECAMKKG	YGI YTKVSRY	VNWIKEKTKL	TDKTHTCPPC	PAPELLGGPS
	481	VFLFPPKPKD	TLMISRTPEV	TCVWDVSHE	DPEVKFNWVY	DGVEVHNAKT	KPREEQYNST
	541	YRWSVLTVL	HQDWLNGKEY	KCKVSNKALP	APIEKTIEKA	KGQPREPQVY	TLPPSRDEL
60	601	KNQVSLTCLV	KGFYPSDIAV	EWESNGQPEN	NYKTTTPVLD	SDGSFFLYSK	LTVDKSRWQQ
	661	GNVFSVSMH	EALHNHYTQK	SLSLSPGKRR	RRSGGGGSGG	GGSGGGGSGG	GGSRKRKR:
	721	KTHTCPPCPA	PELLGGPSVF	LFPPKPKDTL	MISRTPEVTC	VWDVSHEDP	EVKFNWYVDG
	781	VEVHNAKTKP	REEQYNSTYR	VVSVLTVLHQ	DWLNGKEYKC	KVSNKALPAP	IEKTISKAKG
	841	QPREPQVYTL	PPSRDELTKN	QVSLTCLVKG	FYPDSIAVEW	ESNGQPENNY	KITPPVLDSD
65	901	GSFFLYSKLT	VDKSRWQQGN	VFSCSVMHEA	LHNHYTQKSL	SLSPGK*	

DNA sequence for pSYN-FVII-064

70	1	ATGGTCTCCC	AGGCCCTCAG	GCTCTCTGCT	CTTCTGCTTG	GGCTTCAGGG	CTGCCCTGGCT
	61	GCAGTCTTCG	TAACCCAGGA	GGAAGCCAC	GGCGTCTGCT	ACCGGCGCCG	GCAGCCCAAC
	121	GGTTCCTCGG	AGGAGCTGCG	GCCGGGCTCC	CTGGAGAGGG	AGTGCAAGGA	GGAGCAGTGC
	181	TCCTTCGAGG	AGGCCCGGGA	GATCTTCAAG	GACGCGGAGA	GGACGAAGCT	GTTCTGGATT
	241	CTTTACAGTG	ATGGGGACCA	GTGTGCCTCA	AGTCCATGCC	AGAAATGGGGG	CTCTGCAAG
	301	GACCAGCTCC	AGTCTTATAT	CTGCTTCTGC	CTCCCTGCCT	TCGAGGGCCG	GAAGTGTGAG
75	361	ACGCACAAGG	ATGACCAGCT	GATCTGTGTG	AACGAGAACG	GCGGCTGTGA	GCAGTACTGC
	421	AGTGACCACA	CGGGCACCAA	GCGTCTCTGT	CGGTGCCACG	AGGGGTACTC	TCTGCTGGCA

5	481	GACGGGGTGT	CCTGCACACC	CACAGTTGAA	TATCCATGTG	GAAAAATACC	TATTCTAGAA
	541	AAAAGAAATG	CCAGCAAAAC	CCAAGGCCGA	ATTGTGGGGG	GCAAGGTGTG	CCCCAAAGGG
	601	GAGTGTCCAT	GGCAGGTCCT	GTTGTTGGTG	AATGGAGCTC	AGTTGTGTGG	GGGGACCCCTG
	661	ATCAACACCA	TCTGGGTGGT	CTCCGCGGCC	CACTGTTTCG	ACAAAAT CAA	GAAGTGGAGG
10	721	AACCTGATCG	CGGTGCTGGG	CGAGCACGAC	CTCAGCGAGC	ACGACGGGGA	TGAGCAGAGC
	781	CGGCGGGTGG	CGCAGGTCAT	CATCCCCAGC	ACGTACGTCC	CGGGCACCAC	CAACCACGAC
	841	ATCGCGCTGC	TCCGCCTGCA	CCAGCCCGTG	GTCTCACTG	ACCATGTGGT	GCCCTCTCTGC
	901	CTGCCCGAAC	GGACGTTCTC	TGAGAGGACG	CTGGCCTTCG	TGCGCTTCTC	ATTGGTCAGC
	961	GGCTGGGGCC	AGCTGCTGGA	CCGTGGCGCC	ACGGCCCTGG	AGCTCATGGT	CCTCAACGTG
15	1021	CCCCGGCTGA	TGACCCAGGA	CTGCCTGCAG	CAGTCACGGA	AGGTGGGAGA	CTCCCCAAT
	1081	ATCACGGAGT	ACATGTTCTG	TGCCGGCTAC	TCGGATGGCA	GCAAGGACTC	CTGCAAGGGG
	1141	GACAGTGGAG	GCCACATGC	CACCCACTAC	CGGGGCACGT	GGTACCTGAC	GGGCATCGTC
	1201	AGCTGGGGCC	AGGGCTGCGC	AACCGTGGGC	CACCTTGGGG	TGTACACCAG	GGTCTCCAG
	1261	TACATCGAGT	GGCTGCAAAA	GCTCATGCGC	TCAGAGCCAC	GCCCAGGAGT	CCTCTCTCGA
20	1321	GCCCCATTTT	CCGGTGGCGG	TGGCTCCGGC	GGAGGTGGGT	CCGGTGGCGG	CGGATCAGGT
	1381	GGGGGTGGAT	CAGGCGGTGG	AGGTTCCGGT	GGCGGGGGAT	CCGACAAAAC	TCACACATGC
	1441	CCACCGTGCC	CAGTCTCCGA	ACTCTGGGA	GGACCGTCAG	TCTTCTCTTT	CCCCCAAAA
	1501	CCCAAGGACA	CCCTCTACAT	CACCCGGGAG	CCTGAGGTCA	CATGCGTGGT	GGTGGACGTG
	1561	AGCCACGAAG	ACCCTGAGGT	CAAGTTCAAC	TGGTACGTGG	ACGGCGTGA	GGTGCATGGA
25	1621	GCCAAGACAA	AGCCGCGGGA	GGAGCAGTAC	AACAGCACGT	ACCGTGTGGT	CAGCGTCTCT
	1681	ACCGTCTGTC	ACCAGGACTG	GCTGAATGGC	AAGGAGTACA	AGTGCAAGGT	CTCCAACAAA
	1741	GCCCTCCAG	CCCCATCGA	GAAAACCATC	TCCAAAGCCA	AAGGCGAGCC	CCGAGAACCA
	1801	CAGGTGTACA	CCCTGCCCCC	ATCCCGGGAT	GAGCTGACCA	AGAACCAGGT	CAGCCTGACC
	1861	TGCTTGCTCA	AAGCTTCTA	TCCCAGCGAC	ATCGCCGTGG	AGTGGGAGAG	CAATGGGCAG
30	1921	CCGGAGAACA	ACTACAAGAC	CACGCTCTCC	GTGTTGGACT	CCGACGGCTC	CTTCTTCTCT
	1981	TACAGCAAGC	TCACCGTCGA	CAAGAGCAGG	TGGCAGCAGG	GGAACGTCTT	CTCATCTCTC
	2041	GTGATGCATG	AGGCTCTGCA	CAACCACTAC	ACGCAGAAGA	GCCTCTCCCT	GTCTCCGGGT
	2101	AAACGGCGCC	GCCGGAGCGG	TGGCGGCGGA	TCAGGTGGGG	GTGGATCAGG	CGGTGGAGGT
	2161	TCCGGTGGCG	GGGGATCCAG	GAAGAGGAGG	AAGAGGGACA	AAACTCACAC	ATGCCACCCG
35	2221	TGCCCAGCAC	CGGAACCTCT	GGGCGGACCG	TCAGTCTTCC	TCTTCCCCCC	AAAACCCAAG
	2281	GACACCTCT	ACATCACCCG	GGAGCTGAG	GTACATGCG	TGGTGGTGA	CGTGAGCCAC
	2341	GAAGACCCTG	AGGTCAAGTT	CAACTGGTAC	GTGGACGGCG	TGGAGGTGCA	TAATGCCAAG
	2401	AACAAAGCCG	GGGAGGAGCA	GTACAACAGC	ACGTACCGTG	TGGTCAGCGT	CCTCACCGTC
	2461	CTGCACCAGG	ACTGGCTGAA	TGGCAAGGAG	TACAAGTGCA	AGGTCTCCAA	CAAAGCCCTC
40	2521	CCAGCCCCCA	TCGAGAAAAA	CATCTCCAAA	GCCAAAGGGC	AGCCCCGAGA	ACCACAGGTG
	2581	TACACCTGTC	CCCCATCCCG	GGATGAGCTG	ACCAAGAACC	AGGTACGCGT	GACCTGCCTG
	2641	GTCAAAGGCT	TCTATCCAG	CGACATCGCC	GTGGAGTGGG	AGAGCAATGG	GCAGCCGGAG
	2701	AACAACTACA	AGACCACGCC	TCCCGTGTTC	GACTCCGACG	GCTCCTTCTT	CCTCTACAGC
	2761	AAGCTCACCG	TGGACAAGAG	CAGGTGGCAG	CAGGGGAACG	TCTTCTCATG	CTCCGTGATG
45	2821	CATGAGGCTC	TGCACAACCA	CTACACGCAG	AAGAGCCTCT	CCCTGTCTCC	GGGTAAATGA

FVII-064 amino acid sequence. Signal sequence is shown in dotted underline, propeptide is double underlined, linker connecting FVII to Fc is underlined, and linker with proprotein convertase processing sites is shown in bold

	i	MVSQALRLLC.	..LLGLQGCLA	<u>AVFVTOEEAH</u>	<u>GVLHRRRRAN</u>	AFLEELRPGS	LERECKEEQC
50	61	SFEARE IFK	DAERTKLFwi	SYSDDQDCAS	SPCQNGGSK	DQLQSYICFC	LPFAFEGRNCE
	121	THKDDQLICV	NENGGCEQYC	SDHTGTRKSC	RHEGYSLLA	DGVSTPTPVE	YPCGKIPILE
55	181	KRNASKPQGR	IVGGKVC PKG	BCPWQVLLLV	NGAQLCGGTL	INTIWSAA	HCDFDKIKNR
	241	NLIAVLGEHD	LSEHDGDEQS	RRVAQVIIPS	TYVPGTTNHD	IALLRLHQPV	VLDTHVPLC
	301	LPERTFSERT	LAFVRFSLVS	GWGQLDRGA	TALELMVLNV	PRLMTQDCLQ	QSRKVGDSNP
	361	ITEYMFACGY	SDGSKDSCCK	DSGGPHATHY	RGTWYLTGIV	SWGQGCATVG	HFGVYTRVSP
	421	Y IEWLQKLMR	SEPRPGVLLR	APFPQGGGSG	<u>GGGSGGGGSG</u>	<u>GGGSGGGGSG</u>	<u>GGGSPKTHTC</u>
60	481	PPCPAPPELLG	GPSVFLFPPK	PKDTLYI TRE	PEVTCVWDV	SHEDPEVKFN	WYVDGVEVHN
	541	AKTKPREEQY	NSTYRVSVL	TVLHQDWLNG	KEYKCKVSNK	ALPAPIEKTI	SKAKGQPREP
	601	QVYTLPPSRD	ELTKNQVSLT	CLVKGFYPSD	IAVEWESNGQ	PENNYKTTTP	VLDSDGSFFL
	661	YSKLTVDKSR	WQQGNVFS	VMHEALHNHY	TQKSLSLSPG	KRRRRSGGGG	SGGGSGGGGG
	721	SGGGGSRKRR	KRDKTHTCPP	CPAPELLGGP	SVFLFPPKPK	DTLYITREPE	VTCWVDVSH
65	781	EDPEVKFNWY	VDGVEVHNAK	TKPREEQYNS	TYRWSVLT	LHQDWLNGKE	YKCKVSNKAL
	841	PAPIEKTIEK	AKGQPREPQV	YTLPPSRDEL	TKNQVSLTCL	VKGF YPSDIA	VEWESNGQPE
	901	NNYKTTTPVL	DSGGSFFLYS	KLTVDKSRWQ	QGNVFS	HEALHNHYTQ	KSLSLSPGK*

DNA sequence for pSYN-FVIII-049

70	1	ATGCAAAATAG	AGCTCTCCAC	CTGCTTCTTT	CTGTGCCTTT	TGCGATTCTG	CTTTAGTGCC
	61	ACCAGAAGAT	ACTACCTGGG	TGCAGTGGAA	CTGTCATGGG	ACTATATGCA	AAGTGATCTC
	121	GGTGAGCTGC	CTGTGGACGC	AAGATTTCCT	CCTAGAGTGC	CAAAATCTTT	TCCATTCAAC

5	181	ACCTCAGTCG	TGTACAAAAA	GACTCTGTTT	GTAGAATTCA	CGGATCACCT	TTTCAACATC
	241	GCTAAGCCAA	GGCCACCCTG	GATGGGTCTG	CTAGGTCCCTA	CCATCCAGGC	TGAGGTTTAT
	301	GATACAGTGG	TCATTACACT	TAAGAACATG	GCTTCCCATC	CTGTCAGTCT	TCATGCTGTT
	361	GGTGATCCT	ACTGGAAAGC	TTCTGAGGGA	GCTGAATATG	ATGATCAGAC	CAGTCAAAAGG
10	421	GAGAAAGAAG	ATGATAAAGT	CTTCCTGTT	GGAAGCCATA	CATATGTCTG	GCAGGTCCTG
	481	AAAGAGAATG	GTCCAATGGC	CTCTGACCCA	CTGTGCCTTA	CCTACTCATA	TCTTTTCAT
	541	GTGGACCTGG	TAAAAGACTT	GAATTCAGGC	CTCATTGGAG	CCCTACTAGT	ATGTAGAGAA
	601	GGGAGTCTGG	CCAAGGAAAA	GACACAGACC	TTGCACAAAT	TTATACTACT	TTTTGCTGTA
	661	TTTGATGAAG	GGAAAAAGTTG	GCACTCAGAA	ACAAAGAAGT	CCTTGATGCA	GGATAGGGAT
	721	GCTGCATCTG	CTCGGGCCTG	GCCTAAAATG	CACACAGTCA	ATGGTTATGT	AAACAGGTCT
15	781	CTGCCAGGTC	TGATTGGATG	CCACAGGAAA	TCAGTCTATT	GGCATGTGAT	TGGAATGGGC
	841	ACCACTCCTG	AAGTGCACCT	AATATTCCTC	GAAGGTCACA	CATTTCCTGT	GAGGAACCAT
	901	CGCCAGGCGT	CCTTGGAAT	CTCGCCAATA	ACTTTCCTTA	CTGCTCAAAC	ACTCTTGATG
	961	GACCTTGGAC	AGTTTCTACT	GTTTTGTCT	ATCTCTTCCC	ACCAACATGA	TGGCATGGAA
20	1021	GCTTATGTCA	AAGTAGACAG	CTGTCCAGAG	GAACCCCAAC	TACGAATGAA	AAATAATGAA
	1081	GAAGCGGAAG	ACTATGATGA	TGATCTTACT	GATTCTGAAA	TGGATGTGGT	CAGGTTTGAT
	1141	GATGACAACT	CTCCTTCCTT	TATCCAAATT	CGCTCAGTTG	CCAAGAAGCA	TCCTAAAACCT
	1201	TGGGTACATT	ACATTGCTGC	TGAAGAGGAG	GACTGGGACT	ATGCTCCCTT	AGTCTCGCC
	1261	CCCGATGACA	GAAGTTATAA	AAGTCAATAT	TTGAACAATG	GCCCTCAGCG	GATTGGTAGG
25	1321	AAGTACAAAA	AAGTCCGATT	TATGGCATA	ACAGATGAAA	CCTTTAAGAC	TCGTGAAGCT
	1381	ATTCAGCATG	AATCAGGAAT	CTTGGGACCT	TTACTTTATG	GGGAAGTTGG	AGACACACTG
	1441	TTGATTATAT	TTAAGAATCA	AGCAAGCAGA	CCATATAACA	TCTACCCTCA	CGGAATCACT
	1501	GATGTCCGTC	CTTTGTATTC	AAGGAGATTA	CCAAAAGGTG	TAAAACATTT	GAAGGATTTT
	1561	CCAATTCTGC	CAGGAGAAAT	ATTCAAATAT	AAATGGACAG	TGACTGTAGA	AGATGGGCCA
30	1621	ACTAAATCAG	ATCCTCGGTG	CCTGACCCGC	TATTACTCTA	GTTTCGTTAA	TATGGAGAGA
	1681	GATCTAGCTT	CAGGACTCAT	TGGCCCTCTC	CTCATCTGCT	ACAAAGAATC	TGTAGATCAA
	1741	AGAGGAAACC	AGATAATGTC	AGACAAGAGG	AATGTCATCC	TGTTTTCTGT	ATTTGATGAG
	1801	AACCGAAGCT	GGTACCTCAC	AGAGAATATA	CAACGCTTTC	TCCCAATCC	AGCTGGAGTG
	1861	CAGCTTGAGG	ATCCAGAGTT	CCAAGCTCC	AACATCATGC	ACAGCATCAA	TGGCTATGTT
35	1921	TTTGATAGTT	TGCAGTTGTC	AGTTTTGTTT	CATGAGGTGG	CATACTGGTA	CATTCTAAGC
	1981	ATTGGAGCAC	AGACTGACTT	CCTTCTCTGT	TTCTTCTCTG	GATATACCTT	CAAACACAAA
	2041	ATGGTCTATG	AAGACACACT	CACCCTATT	CCATTCTCAG	GAGAACTGT	CTTCATGTCC
	2101	ATGGAAAACC	CAGGTCTATG	GATTCTGGGG	TGCCACAAC	CAGACTTTCG	GAACAGAGGC
	2161	ATGACCGCCT	TACTGAAGGT	TTCTAGTTGT	GACAAGAACA	CTGGTGATTA	TTACGAGGAG
40	2221	AGTTATGAAG	ATATTTACAG	ATACTTGCTG	AGTAAAAACA	ATGCCATTGA	ACCAAGAAGC
	2281	TTCTCTCAAA	ACCCACCAGT	CTTGAAACGC	CATCAACGGG	AAATAACTCG	TACTACTCTT
	2341	CAGTCAGATC	AAGAGGAAAT	TGACTATGAT	GATACCATAT	CAGTTGAAAT	GAAGAAGGAA
	2401	GATTTTGACA	TTTATGATGA	GGATGAAAAT	CAGAGCCCCC	GCAGCTTTCA	AAAGAAAACA
	2461	CGACACTATT	TTATTGCTGC	AGTGGAGAGG	CTCTGGGATT	ATGGGATGAG	TAGTCCCCCA
45	2521	CATGTTCTAA	GAAACAGGGC	TCAGAGTGGC	AGTGTCCCTC	AGTTCAAGAA	AGTTGTTTTT
	2581	CAGGAATTTA	CTGATGGCTC	CTTTACTCAG	CCCTTATACC	GTGGAGAACT	AAATGAACAT
	2641	TTGGGACTCC	TGGGGCCATA	TATAAGAGCA	GAAGTTGAAG	ATAATATCAT	GGTAACCTTC
	2701	AGAAATCAGG	CCTCTCGTCC	CTATTCTTCT	TATTCTAGCC	TTATTCTTAA	TGAGGAAGAT
	2761	CAGAGGCAAG	GAGCAGAACC	TAGAAAAAAC	TTTGTCAGGC	CTAATGAAAC	CAAAACTTAC
50	2821	TTTTGGAAAG	TGCAACATCA	TATGGCACCC	ACTAAAGATG	AGTTTGACTG	CAAAAGCTGG
	2881	GCTTATTCTT	CTGATGTTGA	CCTGGAAAAA	GATGTGCACT	CAGGCCGTAG	TGGACCCCTT
	2941	CTGGTCTGCC	ACACTAACAC	ACTGAACCCT	GCTCATGGGA	GACAAGTGAC	AGTACAGGAA
	3001	TTTGCTCTGT	TTTTACCAT	CTTTGATGAG	ACCAAAAAGCT	GGTACTTCAC	TGAAAAATATG
	3061	GAAGAAACT	GCAGGGCTCC	CTGCAATATC	CAGATGGAAG	ATCCCACTTT	TAAAGAGAAT
55	3121	TATCGCTTCC	ATGCAATCAA	TGGCTACATA	ATGGATACAC	TACCTGGCTT	AGTAATGGCT
	3181	CAGGATCAAAA	GGATTGATG	GTATCTGCTC	AGCATGGGCA	GCAATGAAAA	CATCCATTCT
	3241	ATTCATTTCA	GTGGACATGT	GTTCACTGTA	CGAAAAAAG	AGGAGTATAA	AATGGCACTG
	3301	TACAATCTCT	ATCCAGGTGT	TTTTGAGACA	GTGGAAATGT	TACCATCCAA	AGCTGGAATT
	3361	TGGCGGGTGG	AATGCCTTAT	TGGCGAGCAT	CTACATGCTG	GGATGAGCAC	ACTTTTTCTG
	3421	GTGTACAGCA	ATAAGTGTCA	GACTCCCTCG	GGAATGGCTT	CTGGACACAT	TAGAGATTTT
60	3481	CAGATTACAG	CTTCAGGACA	ATATGGACAG	TGGGCCCCAA	AGCTGGCCAG	AGTTCTATTAT
	3541	TCCGGATCAA	TCAATGCCTG	GAGCACCAAG	GAGCCCTTTT	CTTGGATCAA	GGTGGATCTG
	3601	TTGGCACCAA	TGATTATTCA	CGGCATCAAG	ACCCAGGGTG	CCCGTCAGAA	GTCTCCAGC
	3661	CTCTACATCT	CTCAGTTTAT	CATCATGTAT	AGTCTTGATG	GGAAGAAGTG	GCAGACTTAT
65	3721	CGAGGAAATT	CCTCTGGAAC	CTTAATGGTC	TTCTTTGGCA	ATGTGGATTG	ATCTGGGATA
	3781	AAACACAATA	TTTTTAACCC	TCCAATTATT	GCTCGATACA	TCCGTTTGCA	CCCAACTCAT
	3841	TATAGCATTC	GCAGCACTCT	TCGCATGGAG	TTGATGGGCT	GTGATTTAAA	TAGTTGCAGC
	3901	ATGCCATTGG	GAATGGAGAG	TAAAGCAATA	TCAGATGCAC	AGATTACTGC	TTTCACTTAC
	3961	TTTACCAATA	TGTTTGCCAC	CTGGTCTCCT	TCAAAAAGCTC	GACTTCACCT	CCAAGGGAGG
70	4021	AGTAATGCCT	GGAGACCTCA	GGTGAATAAT	CCAAAAGAGT	GGCTGCAAGT	GGACTTCCAG
	4081	AAGACAATGA	AAGTCACAGG	AGTAACTACT	CAGGGAGTAA	AATCTCTGCT	TACCAGCATG
	4141	TATGTGAAGG	AGTTCTCTAT	CTCCAGCAGT	CAAGATGGCC	ATCAGTGGAC	TCTCTTTTTT
	4201	CAGAATGGCA	AAGTAAAGGT	TTTTCAGGGA	AATCAAGACT	CCTTCACACC	TGTGGTGAAC
	4261	TCTCTAGACC	CACCGTTACT	GACTCGCTAC	CTTCGAATTC	ACCCCCAGAG	TTGGGTGCAC
75	4321	CAGATTGCC	TGAGGATGGA	GGTCTGGGC	TGCGAGGCAC	AGGACCTCTA	CGACAAAAC
	4381	CACACATGCC	CACCGTGCCC	AGCACCTGAA	CTCTGGGAG	GACCGTCAGT	CTTCTCTTTC
	4441	CCCCCAAAAC	CCAAGGACAC	CCTCATGATC	TCCCGGACCC	CTGAGGTCAC	ATGCGTGGTG
	4501	GTGGACGTGA	GCCACGAAGA	CCCTGAGGTC	AAGTTCAACT	GGTACGTGGA	CGCGGTGGAG
	4561	GTGCATAATG	CCAAGACAAA	GCCGCGGGAG	GAGCAGTACA	ACAGCACGTA	CCGTGTGGTC
80	4621	AGCGTCCTCA	CCGTCTGCA	CCAGGACTGG	CTGAATGGCA	AGGAGTACAA	GTGCAAGGTC
	4681	TCCAACAAAG	CCCTCCAGC	CCCCATCGAG	AAAACCATCT	CCAAAGCCAA	AGGGACGCC
	4741	CGAGAACCAC	AGGTGTACAC	CTGCCCCCA	TCCCGCGATG	AGTGACCAA	GAACCAAGTC

5 4801 AGCCTGACCT GCCTGGTCAA AGGCTTCTAT CCCAGCGACA TCGCCGTGGA GTGGGAGAGC
 4861 AATGGGCAGC CGGAGAACAA CTACAAGACC ACGCCTCCCG TGTTGGACTC CGACGGCTCC
 4921 TTCTTCCTCT ACAGCAAGCT CACCGTCGAC AAGAGCAGGT GGCAGCAGGG GAACGTCTTC
 4981 TCATGCTCCG TGATGCATGA GGCTCTGCAC AACCACTACA CGCAGAAAGAG CCTCTCCCTG
 5041 TCCTCCGGTA AACGCGCCG CCGGAGCGGT GCGGCGGAT CAGGTGGGGG TGGATCAGGC
 10 5101 GGTGGAGGTT CCGGTGGCGG GGGATCCGGC GGTGGAGGTT CCGGTGGGGG TGGATCAAGG
 5161 AAGAGGAGGA AGAGGGACAA AACTCACACA TGCCCAACCT GCCCAGCTCC AGAACTCCTG
 5221 GGCGGACCGT CAGTCTTCCT CTTCCCCCA AAACCAAGG ACACCCTCAT GATCTCCCGG
 5231 ACCCTGAGG TCACATGCGT GGTGGTGGAC GTGAGCCACG AAGACCCTGA GGTCAAGTTC
 15 5341 AACTGGTACG TGGACGGCGT GGAGGTGCAT AATGCCAAGA CAAAGCCGCG GGAGGAGCAG
 5401 TACAACAGCA CGTACCGTGT GGTACGCGTC CTCACCGTCC TGCACCAGGA CTGGCTGAAT
 5461 GGCAAGGAGT ACAAGTGCAA GGTCTCCAAC AAAGCCCTCC CAGCCCCCAT CGAGAAAACC
 5521 ATCTCCAAAG CCAAAGGGCA GCCCGAGAA CCACAGGTGT ACACCCTGCC CCCATCCCGG
 5581 GATGAGCTGA CCAAGAACCA GGTCAGCCTG ACCTGCCTGG TCAAAGGCTT CTATCCCGCT
 20 5641 GACATCGCCG TGGAGTGGGA GAGCAATGGG CAGCCGGAGA ACAACTACAA GACCAACGCT
 5701 CCCGTGTTGG ACTCCGACGG CTCCTTCTTC CTCTACAGCA AGCTCACCCT GGACAAGAGC
 5761 AGGTGGCAGC AGGGGAACGT CTTCTCATGC TCGTGATGC ATGAGGCTCT GCACAACCAC
 5821 TACACGCAGA AGAGCCTCTC CCTGTCTCCG GGTAAATGA

25 FVIII-049 amino acid sequence. Signal sequence is shown in dotted underline, and linker with proprotein convertase processing sites is shown in bold

30 i MQIELSTCFF...LCLLRFCFSA TRRYYLGAWE LSWDYMQSDL GELPVDARFP PRVPKSFPPN
 61 TSWYKTLF VFTDHLFNI AKPRPPWMGL LGPTIQAEVY DTWITLKNM ASHPVSLHAV
 121 GVSYYWKASEG AEYDDQTSQR EKEDDKVFPD GSHTYVWQVL KENGPMSADP LCLTYS YLSH
 181 VDLVKDLNSG LIGALLVCRE GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
 241 AASARAWPKM HTVNGYVNRSLPGLIGCHRK SVYWHVIGMG TTPEVHSIFL EGHFTFLVRNH
 301 RQASLEISPI TFLTAQTLLM DLGQFLFLFCH ISSHQHDGME AYVKVDSCPE EPQLRMKNNE
 361 EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI RSVAKKHPKT WWHYIAAEEE DWDYAPLVLA
 421 PDDRSYKSQY LNNGPQRIGR KYKKVRFMAY TDETFKTREA IQHESGILGP LLYGEVDTL
 481 LIIFKNQASR PYNIYPHGIT DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
 541 TKSDPRCLTR YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR NVILFSVFDE
 601 NRSWYLTEINI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV FDSLQLSVCL HEVAYWYILS
 661 IGATQDFLSV FFSGYTFKKH MYEDTLTLF PFSGETVFMS MENPGLWILG CHNSDFRNRG
 721 MTALLKVSSC DKNTGDYED SYEDISAYLL SKNNAIEPRS FSQNPVPLKR HOREITRTTL
 781 QSDQEEIDYD DTISVEMKKE DFDI YDEDEN QSPRSFQKKT RHYF IAAVER LDWDYGMSSSP
 841 HVLNRNRAQSG SVPQFKKWF QEFTDGSFTQ PLYRGELNEH LGLLGPYIRA EVEDNIMVTF
 901 RNQASRPYSF YSSLISYEED QRQGAERPNK FVKPNETKTY FWKVQHMAP TKDEFDCRAW
 961 AYFSDVDLEK DVHSGI IGPL LVCHTNTLNP AHGRQVTVQE FALFFTIFDE TKSIFYFTENM
 1021 ERNCRAPCNI QMEDPTFKEN YRFHAINGYI MDTLPLGLVMA QDQIRWYLL SMGSNENIHS
 1081 IHFSGHVFTV RKKEEYKMAL YNLYPGVFET VEMLPKAGI WRVECLIGE HLAGMSTLFL
 1141 VYSNKCQTPL GMASGHIRDF QITASQYQG WAPKLARLHY SGSINAWSTK EFPWSIKVDL
 1201 LAPMIIHGIK TQGARQKFSS LYISQFI IMY SLDGKKWQTY RGNSTGTLMV FFGNVDSSGI
 1261 KHNIFNPPII ARYIRLHPTH YSIRSTLRME LMGCIDLNSCS MPLGMESKAI SDAQITASSY
 1321 FTMNFATWSP SKARLHLQGR SNAWRPQVNN PKEWLQVDFQ KTMKVTVGTT QGVKSLTSM
 1381 YVKEFLISSS QDGHQWTLFF QNGKVVFQNG NQDSFTPNW SLDPPLLTRY LRIHPQSWVH
 1441 QIALRMEVLG CEAQDL YDKT HTCPPCPAPE LLGGPSVFLF PPKPKDTLMI SRTPEVTCW
 1501 VDVSHEDPEV KFNWYVDGVE VHNKTKPRE EQYNSTYRVV SVLTVLHQDW LMGKEYKCKV
 1561 SNKALPAIE KTISKAKGQP REPQVYTLPP SRDELTKNQV SLTCLVKGFY PSDIAVEWES
 1621 NQQPENNYKT TPPVLDSDGS FFLYSKLTVD KSRWQQGNVF SCSVMHEALH NYHTQKSLSL
 1681 SPGKRRRRSG **GGGSGGGGSG** **GGGSGGGGSG** **GGGSGGGGSG** KRRKRDKTHT CPPCPAPELL
 1741 GGPSVFLFPP KPKDTLMISR TPEVTCVWD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ
 1801 YNSTYRWSV LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAKGQPRE PQVYTLPPSR
 1861 DELTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTP PVLDSDGSFF LYSKLTVDKS
 60 1921 RWQQGNVFCF SVMHEALHNH YTQKSLSLSP GK*

DNA sequence for IFN-b-018

65 1 ATGACCAACA AGTGTCTCCT CCAAATTGCT CTCCTGTTGT GCTTCTCCAC TACAGCTCTT
 61 TCCATGAGCT ACAACTTGCT TGGATTCTTA CAAAGAAGCA GCAATTTTCA GTGTCAAGAG
 121 CTCTGTGGC AATTGAATGG GAGGCTTGAA TATTGCCTCA AGGACAGGAT GAACCTTGAC
 181 ATCCCTGAGG AGATTAAGCA GCTGCAGCAG TTCCAGAAGG AGGACGCCGC ATTGACCATC
 241 TATGAGATGC TCCAGAACAT CTTTGCTATT TTCAGACAAG ATTCATCTAG CACTGGCTGG
 301 AATGAGACTA TTGTTGAGAA CCTCTGGCT AATGTCTATC ATCAGATAAA CCATCTGAAG
 361 ACAGTCTGG AAGAAAAACT GAGAGAAAGAA GATTTCACCA GGGGAAAACT CATGAGAGT
 70 421 CTGCACCTGA AAAGATATTA TGGGAGGATT CTGCATTACC TGAAGGCCAA GGAGTACAGT
 481 CACTGTGCCT GGACCATAGT CAGAGTGGAA ATCCTAAGGA ACTTTTACTT CATTAACAGA
 541 CTTACAGGTT ACCTCCGAAA CGGTGGCGGC GGCTCCGGTG GAGGCGGGTC CGCGCGTGA
 601 GGGAGCGACA AAACCTCACAC ATGCCCACCG TGCCAGCTC CGGAACCTCT GGGAGGACCG
 661 TCAGTCTTCC TCTTCCCCC AAAACCCAAG GACACCCTA TGATCTCCCG GACCCCTGAG
 75 721 CTCACATCG TGGTGGTGA CGTGAGCCAC GAAGACCCTG AGGTCAAGTT CACTGGTAC
 781 GTGGACGGCG TGGAGGTGCA TAATGCCAAG ACAAGCCGCG GGGAGGAGCA GTACAACAGC

5
 841 ACGTACCGTG TGGTCAGCGT CCTCACCGTC CTGCACCAGG ACTGGCTGAA TGGCAAGGAG
 901 TACAAGTGCA AGGTCTCCAA CAAAGCCCTC CCAGCCCCCA TCGAGAAAAC CATCTCCAAA
 961 GCCAAAGGGC AGCCCCGAGA ACCACAGGTG TACACCCTGC CCCCATCCCG GGATGAGCTG
 10 1021 ACCAAGAACC AGGTCAGCCT GACCTGCCTG GTCAAAGGCT TCTATCCAG CGACATCGCC
 1081 GTGGAGTGGG AGAGCAATGG GCAGCCGGAG AACAACTACA AGACCACGCC TCCCGTGTG
 1141 GACTCCGACG GCTCCTTCTT CCTCTACAGC AAGCTCACCG TCGACAAGAG CAGGTGGCAG
 1201 CAGGGGAACG TCTTCTCATG CTCCGTGATG CATGAGGCTC TGCACAACCA CTACACGCAG
 1261 AAGAGCCTCT CCCTGTCTCC GGGTAAACGG CGCCGCCGGA GCGGTGGCGG CCGATCAGGT
 1321 GGGGGTGGAT CAGGCGGTGG AGGTTCCGGT GGCGGGGGAT CCAGGAAGAG GAGGAAGAGG
 1381 GACAAAACCT ACACATGCCC ACCGTGCCCA GCACCGGAAC TCCTGGGCGG ACCGTCAGTC
 1441 TTCTCTTCC CCCCAAAACC CAAGGACACC CTCATGATCT CCGGACCCC TGAGGTCACA
 15 1501 TCGTGGTGG TGGACGTGAG CCACGAAGAC CCTGAGGTCA AGTTCAACTG GTACGTGGAC
 1561 GCGTGGAGG TGCATAATGC CAAGACAAAG CCGCGGGAGG AGCAGTACAA CAGCACGTAC
 1621 CGTGTGGTCA GCGTCCTCAC CGTCTGCAC CAGGACTGGC TGAATGGCAA GGAGTACAAG
 1681 TGCAAGGTCT CCAACAAAGC CCTCCCAGCC CCCATCGAGA AAACCATCTC CAAAGC CAAA
 20 1741 GGGCAGCCCC GAGAACCACA GGTGTACACC CTGCCCCCAT CCGGGATGA GTGACCAAG
 1801 AACCAGGTCA GCCTGACCTG CCTGTCAAAA GGCTTCTATC CCAGCGACAT CGCCGTGGAG
 1861 TGGGAGAGCA ATGGGCAGCC GGAGAACAAAC TACAAGACCA CGCTCCCCGT GTTGGACTCC
 1921 GACGGCTCCT TCTTCTCTA CAGCAAGCTC ACCGTGGACA AGAGCAGGTG GCAGCAGGGG
 25 1981 AACGTCTTCT CATGCTCCGT GATGCATGAG GCTCTGCACA ACCACTACAC GCAGAAGAGC
 2041 CTCTCCCTGT CTCCGGGTAA ATGA

IFN-b-018 amino acid sequence. Signal sequence is shown
 in dotted underline, linker region connecting IFN-b to Fc
 30 region is underlined, and linker with proprotein
 convertase processing sites is shown in bold

1 MTNKCLOIA. .LLLCFSTAL..SMSYNLLGFL QRSSNFQCQK LLWQLNGRLE YCLKDRMNF
 35 61 IPEE IKQLQQ FQKEDAALTI YEMLQNI FAI FRQDSSSTGW NETIVENLLA NVYHQINHLK
 121 TVLEEKLEKE DFTRGKLMSS LHLKRYGRI LHYLKAKEYS HCAWTIVRVE ILRNFYFINR
 181 LTGYLRN GGG GSGGGGSGGG GSDKTHTCPP CPAPELLGGP SVFLFPPKPK DTLMI SRTPE
 241 VTCVWDVSH EDPEVKFNWY VDGVEVHNAK TKPREEQYNS TYRVVSVLTV LHQDWLNGKE
 301 YKCKVSNKAL PAPIEKTIEK AKGQPREPQV YTLPPSRDEL TKNQVSLTCL VKGFYPSDIA
 361 VEWESNGQPE NNYKTTPPV L DSDGSFFLYS KLTVDKSRWQ QGNVFSCSVM HEALHNHYTQ
 40 421 KSLSLSPGKR RRRSGGGGSG GGGSGGGGSG GGGSRKRKR DKTHTCPPCP APELLGGPSV
 481 FLFPPKPKDT LMISRTPEVT CVWDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY
 541 RWSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTIEKAK GQPREPQVYT LPPSRDELTK
 601 NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTTPVLDS DGSFFLYSKL TVDKSRWQQG
 45 661 NVFSCSVMHE ALHNHYTQKS LSLSPGK*

This invention is further illustrated by the following examples which should
 not be construed as limiting. The contents of all references, patents and published
 50 patent applications cited throughout this application are incorporated herein by
 reference.

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What is claimed is:

1. A polypeptide, comprising (i) at least one biologically active moiety, (ii) an Fc region comprising at least two Fc moieties and (iii) a cleavable scFc (cscFc) linker interposed between the two Fc moieties, wherein the cscFc linker is linked to at least one enzymatic cleavage site which results in cleavage of the cscFc linker.
2. The polypeptide of claim 1, wherein at least one biologically active moiety comprises a clotting factor.
3. The polypeptide of claim 2, wherein the clotting factor is selected from the group consisting of FVII, FVIIa, FVIII, FFX, FFXa, FX, and FXa.
4. The polypeptide of claim 1 comprising the moieties A-F1-P1-L-P2-B-F2 wherein A is optionally present and, if present, is a biologically active moiety, F1 is an Fc moiety or domain, P1 is optionally present and, if present, is an enzymatic cleavage site, L is a polypeptide linker, P2 is optionally present and, if present, is an enzymatic cleavage site, B is optionally present and, if present, is a biologically active moiety, and F2 is an Fc moiety.
5. The polypeptide of claim 4, where A is present and is selected from the group consisting of: an antigen binding portion of an antibody; a non immunoglobulin binding molecule, a binding portion of a ligand, and a binding portion of a receptor, and a clotting factor.
6. The polypeptide of claim 4, wherein A comprises the light chain of a clotting factor and B comprises the heavy chain of a clotting factor which when associated form an active molecule.
7. The polypeptide of claim 4, where B is present and is selected from the group consisting of: an antigen binding portion of an antibody; a non immunoglobulin binding molecule, a binding portion of a ligand, a binding portion of a receptor, and a clotting factor.
8. The polypeptide of claim 4, wherein F1 and F2 each comprise a CH2 and a CH3 domain.

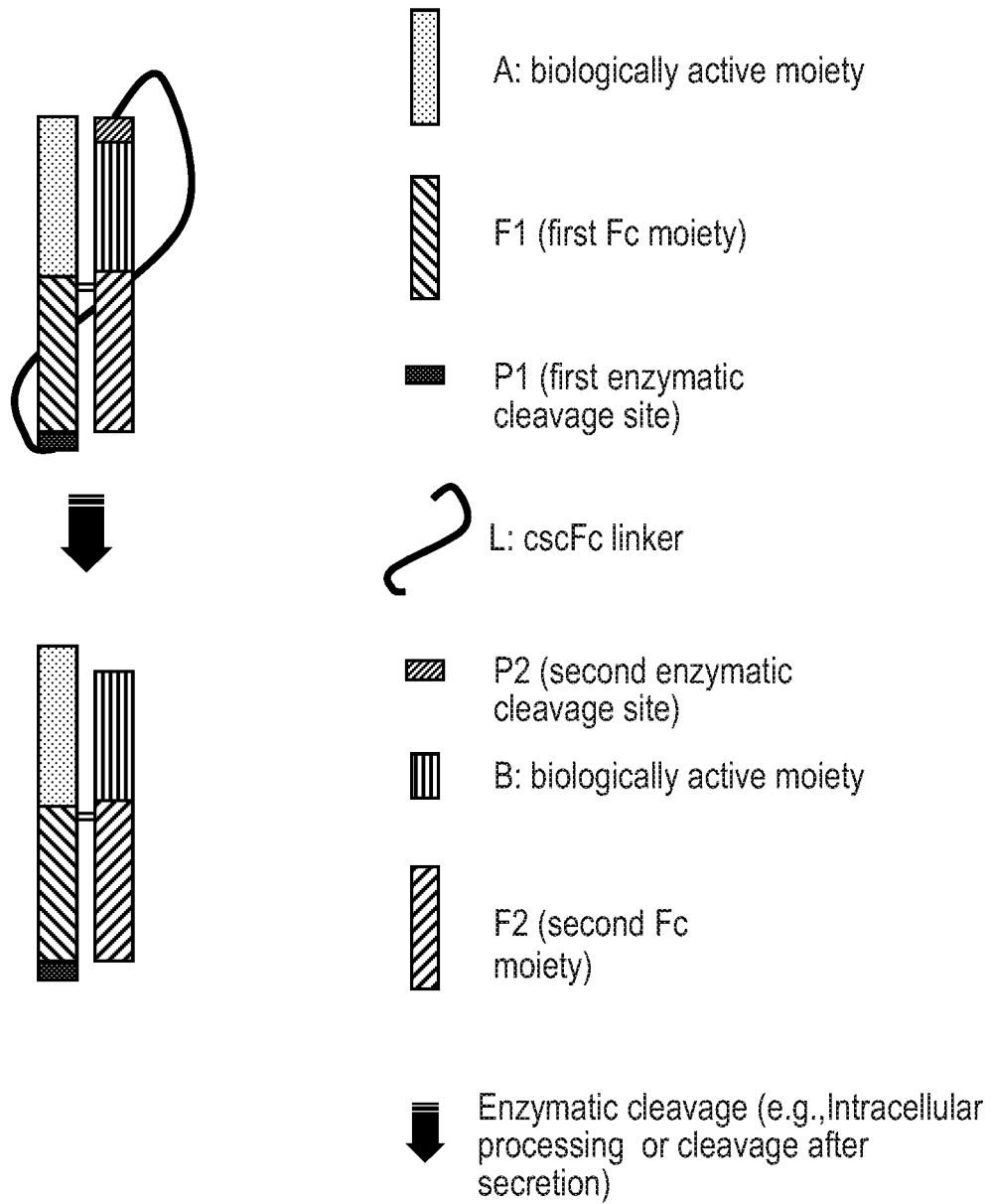
- 5 9. The polypeptide of claim 4, which comprises the moieties A-FI-B-P1-L-P2-F2 or A-F1-P1-L-P2 B-F2 in linear sequence from amino to carboxy terminus.
10. The polypeptide of claim 4 which has the formula selected from the group consisting of: A-FI-P1- L-P2-F2; FI-P1- L-P2-B-F2; A-F1-P1-
10 L- F2; FI-P1- L- B-F2; A-FI- L-P2-F2; FI- L-P2-B-F2, and A-F1-P1-L-P2-B-F2 in linear sequence from amino to carboxy terminus..
11. The polypeptide of claim 1, which comprises one biologically active moiety.
12. The polypeptide of claim 3, wherein A and B are both present and are
15 different biologically active moieties.
13. The polypeptide of claim 4 , wherein PI and P2 are both present and are recognized by the same or by different enzymes.
14. The polypeptide of claim 13, wherein at least one of PI or P2 comprises comprises the amino acid sequence Arg-Arg-Arg-Arg.
- 20 15. The polypeptide of claim 13, wherein at least one of PI or P2 comprises the amino acid sequence Arg-Lys-Arg-Arg-Lys-Arg.
16. The polypeptide of claim 13, wherein at least one of PI or P2 comprises the amino acid sequence Arg-Arg-Arg-Arg-Ser.
17. The polypeptide of claim 4, wherein PI and P2 are both present and
25 PI comprise the sequence Arg-Arg-Arg-Arg and P2 comprises the sequence Arg-Lys-Arg-Arg-Lys-Arg.
18. The polypeptide of claim 4, wherein PI or P2 comprises an amino acid sequence selected from the group consisting of: TQSFNDFTR and SVSQTSKLTR, DFLAEGGGVR, TTKIKPR, LVPRG, and ALRPR.
- 30 19. The polypeptide of claim 1, wherein the cscFc linker has a length of about 1 to about 50 amino acids.
20. The polypeptide of claim 19, wherein the cscFc linker has a length of about 20 to about 30 amino acids.
21. The polypeptide of claim 1, wherein the cscFc linker comprises a
35 gly/ser peptide.
22. The polypeptide of claim 21, wherein the gly/ser peptide is of the formula (Gly₄Ser)_n or S(Gly₄Ser)_n, wherein n is a positive integer selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10.

23. The polypeptide of claim 22, wherein the S(Gly₄ Ser)_n linker is S(Gly₄ Ser)₆ or S(Gly₄ Ser)₄.
24. A polypeptide comprising two polypeptide chains, wherein the first polypeptide chain comprises a light chain of a clotting factor linked to a first Fc moiety and a second polypeptide chain comprises a heavy chain of a clotting factor linked to a second Fc moiety, wherein the light chain and the heavy chain associate to form an enzymatically active clotting factor.
25. The polypeptide of claim 24, wherein the light chain of the clotting factor is linked to the first Fc moiety and the heavy chain of the clotting factor is linked to the second Fc moiety and wherein the clotting factor is enzymatically active upon secretion by a cell.
26. The polypeptide of claim 24, wherein the clotting factor is selected from the group consisting of FVII, FVIIa, FFX, FFXa, FX, and FXa.
27. The polypeptide of claim 1 or 24 which comprises a targeting moiety.
28. The polypeptide of claim 27, wherein the targeting moiety binds to resting platelets.
29. The polypeptide of claim 27 wherein the targeting moiety selectively binds to activated platelets.
30. The polypeptide of claim 28, wherein the targeting moiety selectively binds to a target selected from the group consisting of: GPIIb, GPVI, and the nonactive form of GPIIb/IIIa.
31. The polypeptide of claim 29, wherein the targeting moiety selectively binds to a target selected from the group consisting of: the active form of GPIIb/IIIa, P selectin, GMP-33, LAMP-1, LAMP-2, CD40L, and LOX-1.
32. The polypeptide of claim 27, wherein the targeting moiety binds to the GPIIb complex
33. The polypeptide of claim 27, which comprises a targeting moiety, wherein the targeting moiety is a peptide selected from the group consisting of: PS4, OS1, and OS2.

- 5 34. The polypeptide of claim 27, wherein the targeting moiety comprises an antibody variable region from an antibody selected from the group consisting of: SCE5, MB9, and AP3.
35. The polypeptide of claim 24, where A is the light chain of FVII and B is the heavy chain of FVII.
- 10 36. A composition comprising the polypeptide of claim 1 or 24.
37. The composition of claim 36, wherein the composition comprises a cell culture supernatant.
38. A nucleic acid molecule encoding the polypeptide of claim 1 or 24.
39. The nucleic acid molecule of claim 38, wherein the nucleic acid
15 molecule is present in a vector.
40. The vector of claim 39, wherein the vector further comprises a nucleotide sequence encoding an enzyme which cleaves at least one of the intracellular processing sites.
41. A processed polypeptide comprising at least two amino acid chains
20 which polypeptide is encoded by the nucleic acid molecule of claim 38.
42. A host cell comprising the vector of claim 39, wherein the host cell expresses an enzyme which cleaves the polypeptide linker.
43. The host cell of claim 42, wherein the enzyme is endogenous to the cell.
- 25 44. The host cell of claim 42, wherein the enzyme is exogenous to the cell.
45. A method for producing a polypeptide comprising culturing the host cell of claim 43 or 44 in culture such that a mature polypeptide comprising two amino acid chains is produced.
- 30 46. A processed, heterodimeric polypeptide comprising two polypeptide chains, wherein said processed, heterodimeric polypeptide is made by expressing the vector of claim 45 in a cell cultured in cell culture medium and isolating the mature, heterodimeric polypeptide from the culture medium.
- 35 47. A composition comprising the processed polypeptide of claim 46 and a pharmaceutically acceptable carrier.
48. A composition comprising the nucleic acid molecule of claim 38 and a pharmaceutically acceptable carrier.

- 5 49. A method for treating or preventing a disease or disorder in a
 subject, comprising administering the composition of claim 47 or 48.
50. The method of claim 49, wherein the disease or disorder is selected
 from the group consisting of a clotting disorder, a neurological disorder,
 an inflammatory disorder, an autoimmune disorder, and a neoplastic
10 disorder.
51. The method of claim 49, wherein the disease or disorder is a
 disorder affecting hemostasis.
52. The method of claim 51, wherein the composition promotes clot
 formation.
- 15

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**Fig. 1A**

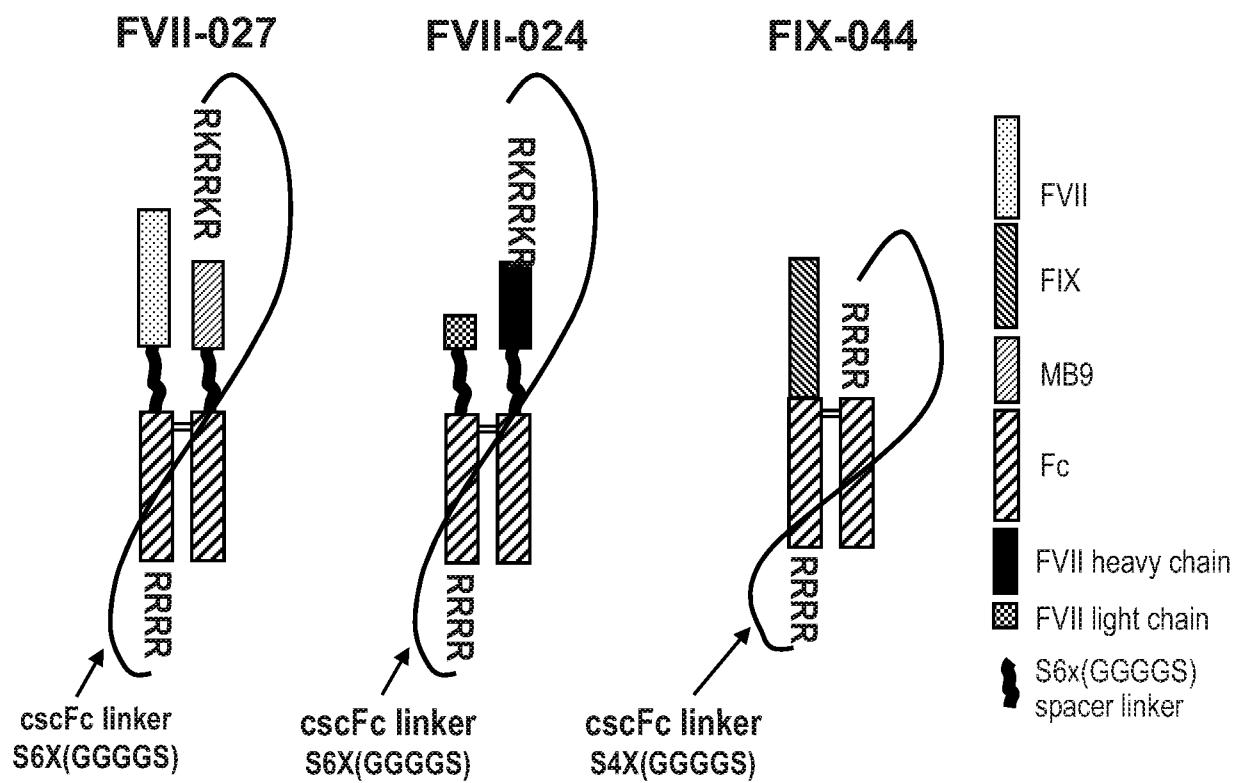
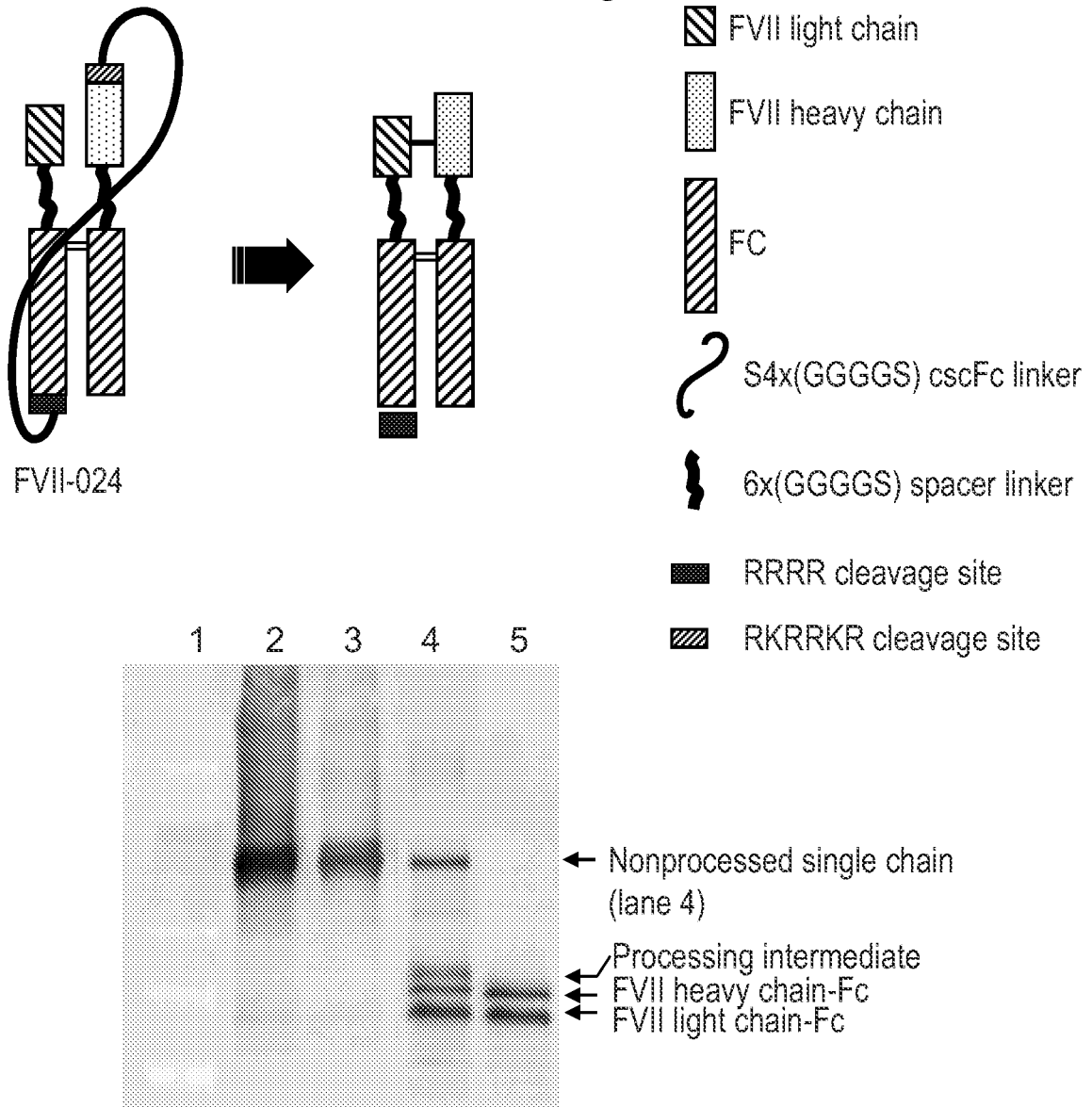
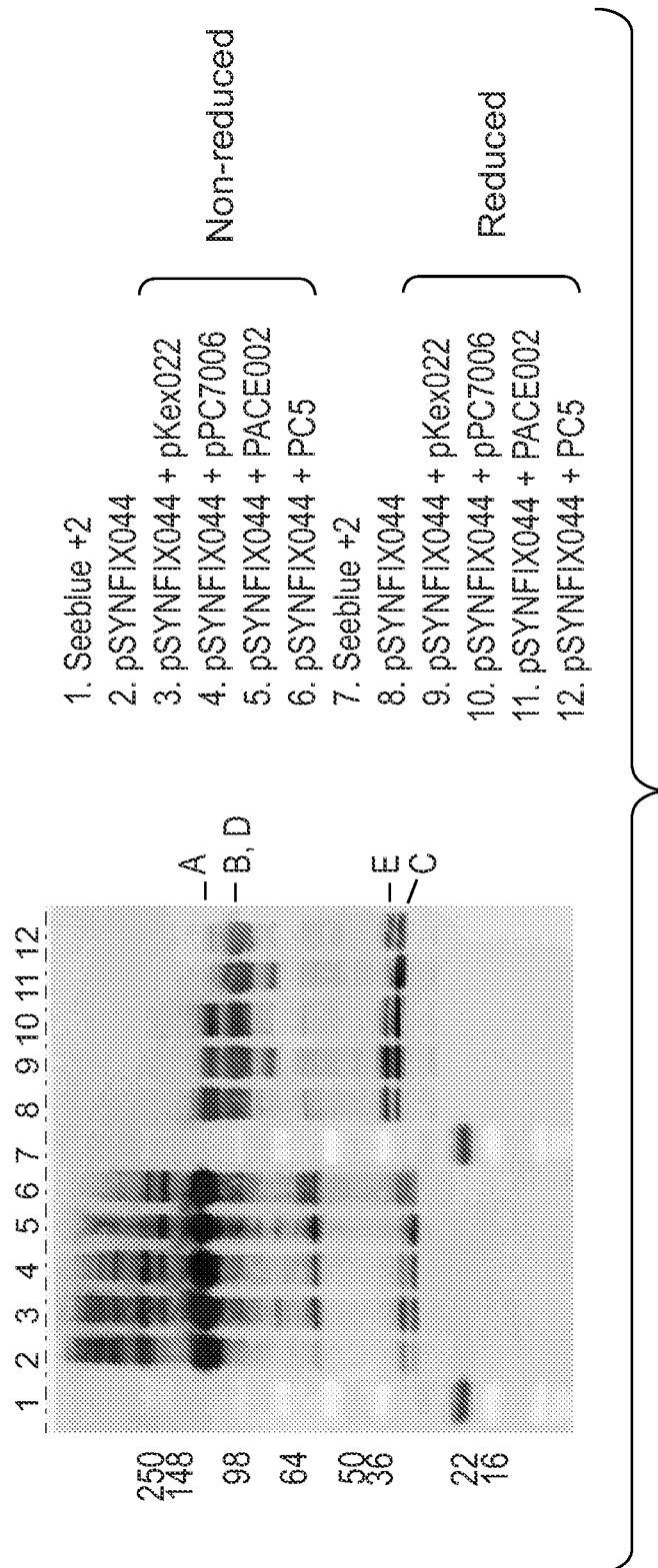


Fig. 1B

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Western blot of protein A immunoprecipitation following transient transfection of pSYN-FVII-024 with or without pSYN-PC5-003. Lane 1, SeeBlue Plus 2 molecular weight marker; Lane 2, pSYN-FVII-024, non reducing; lane 3, pSYN-FVII-024+pSYN-PC5-003, non reducing; lane 4, pSYN-FVII-024, reducing; lane 5, pSYN-FVII-024+pSYN-PC5-003, reducing.

**Fig. 2**



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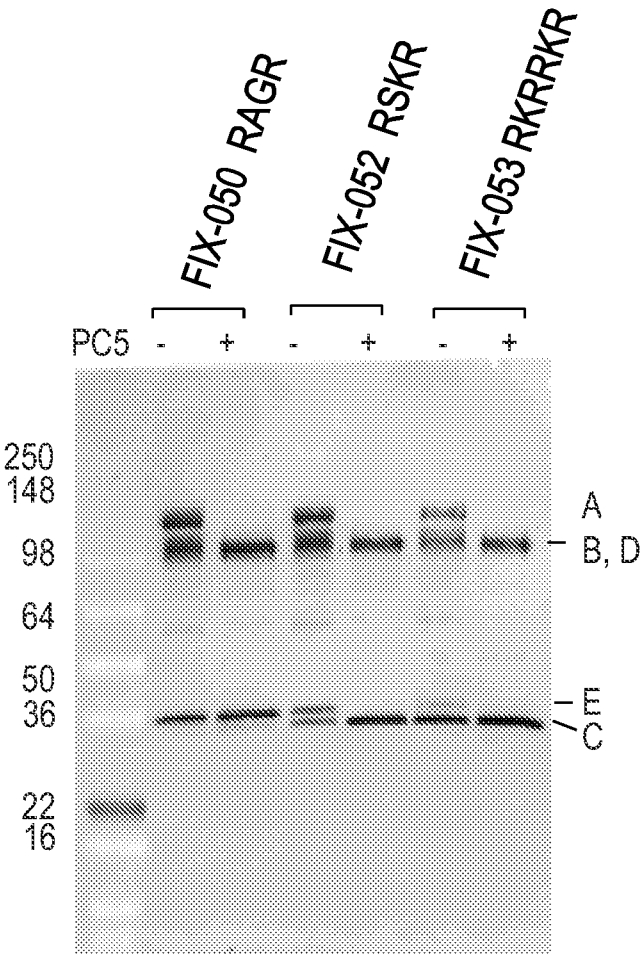
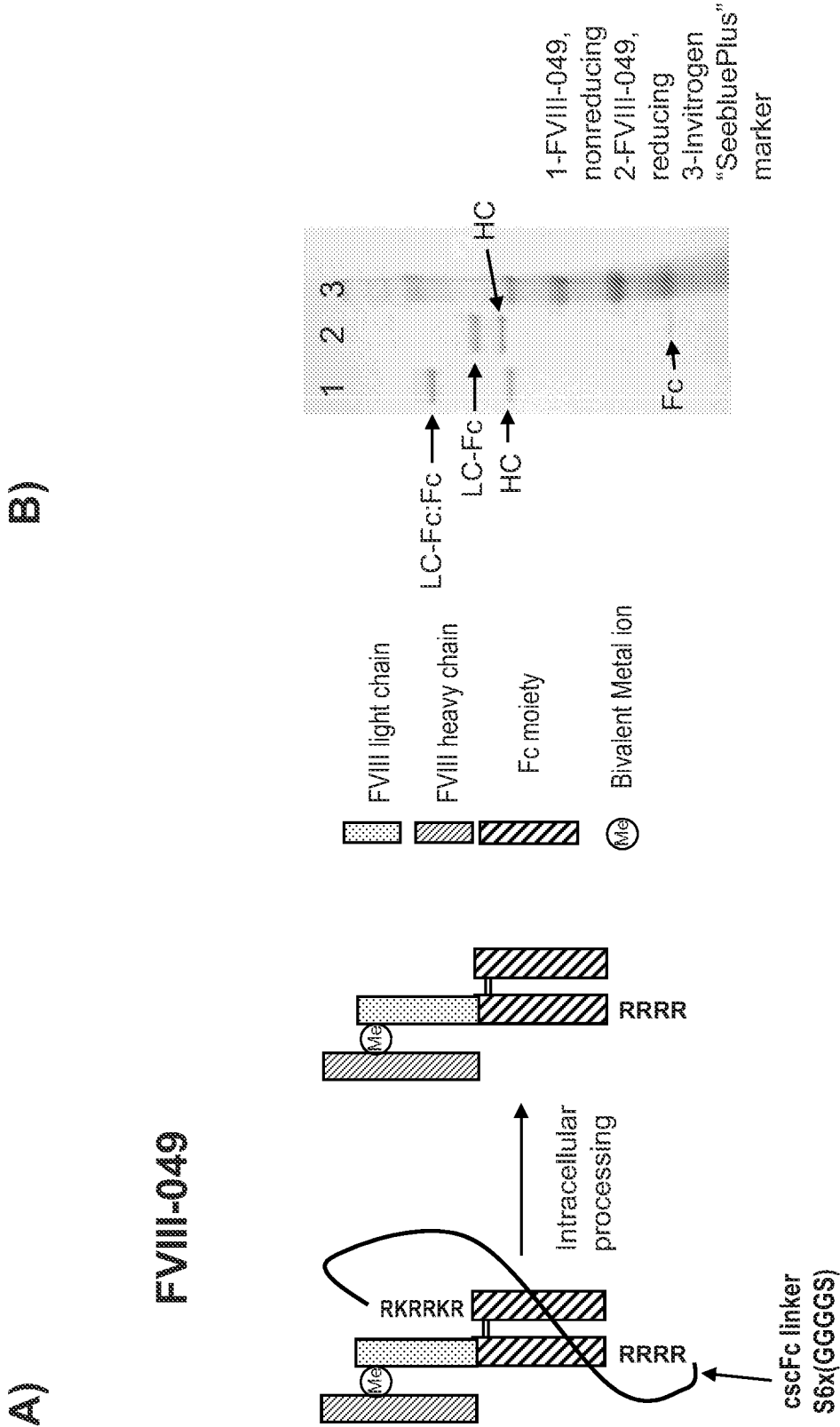
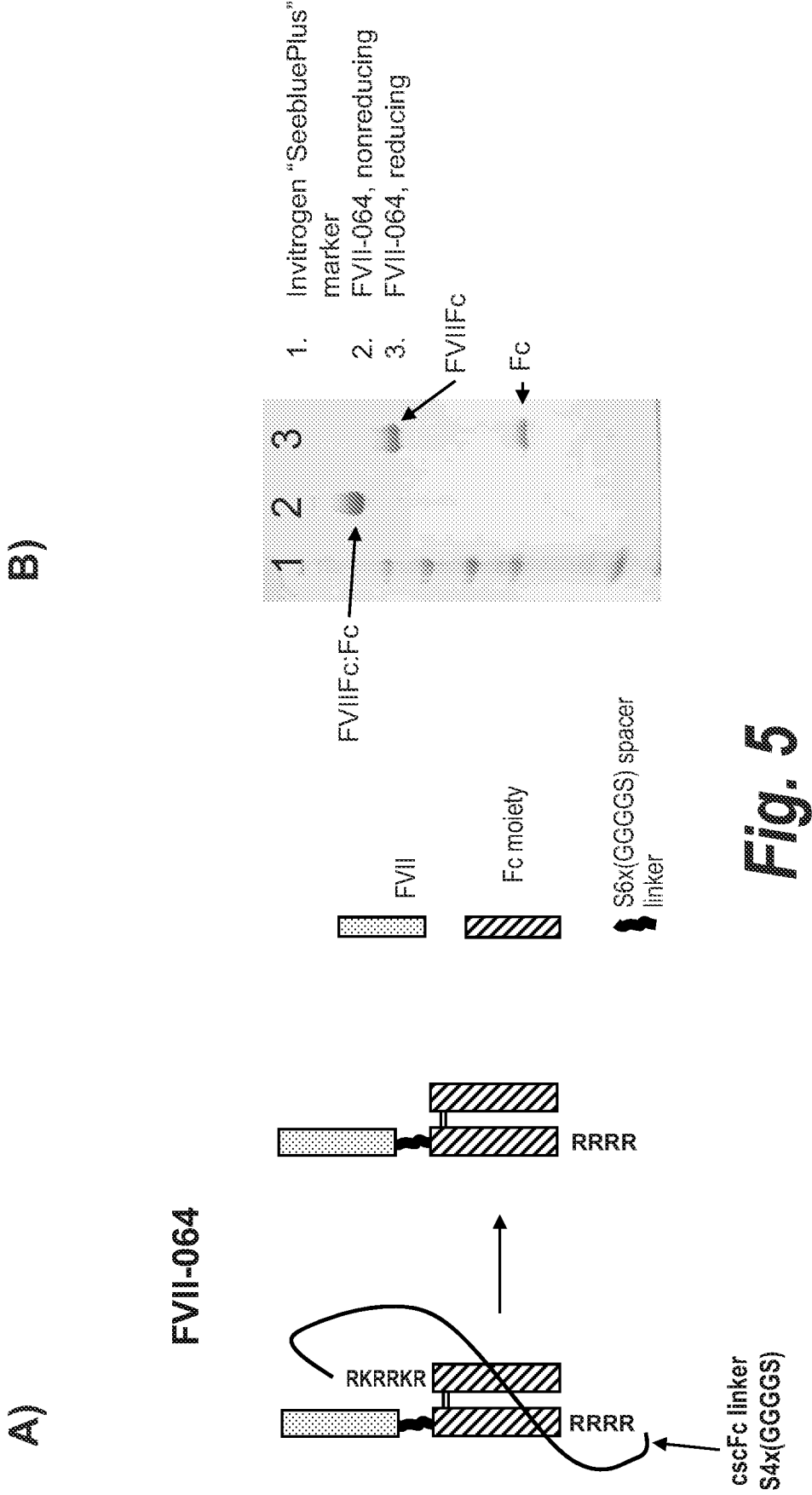


Fig. 3C

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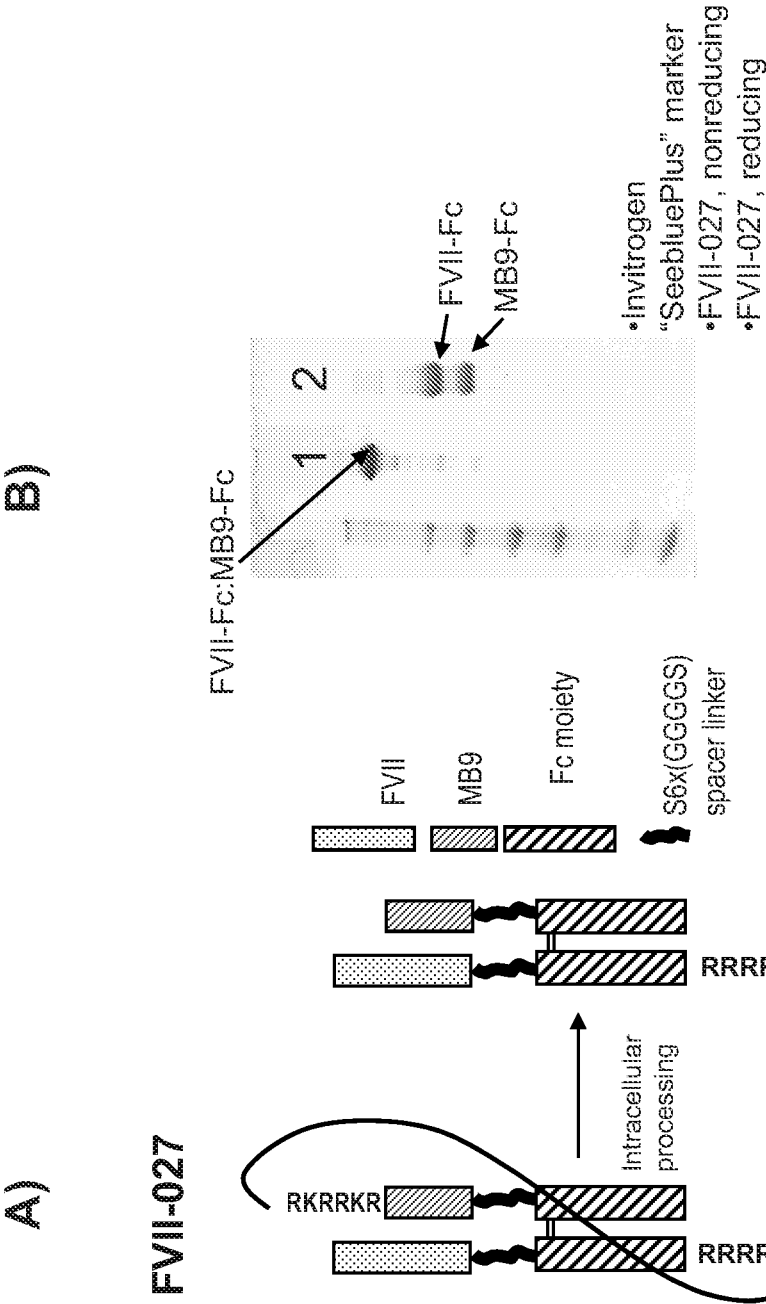
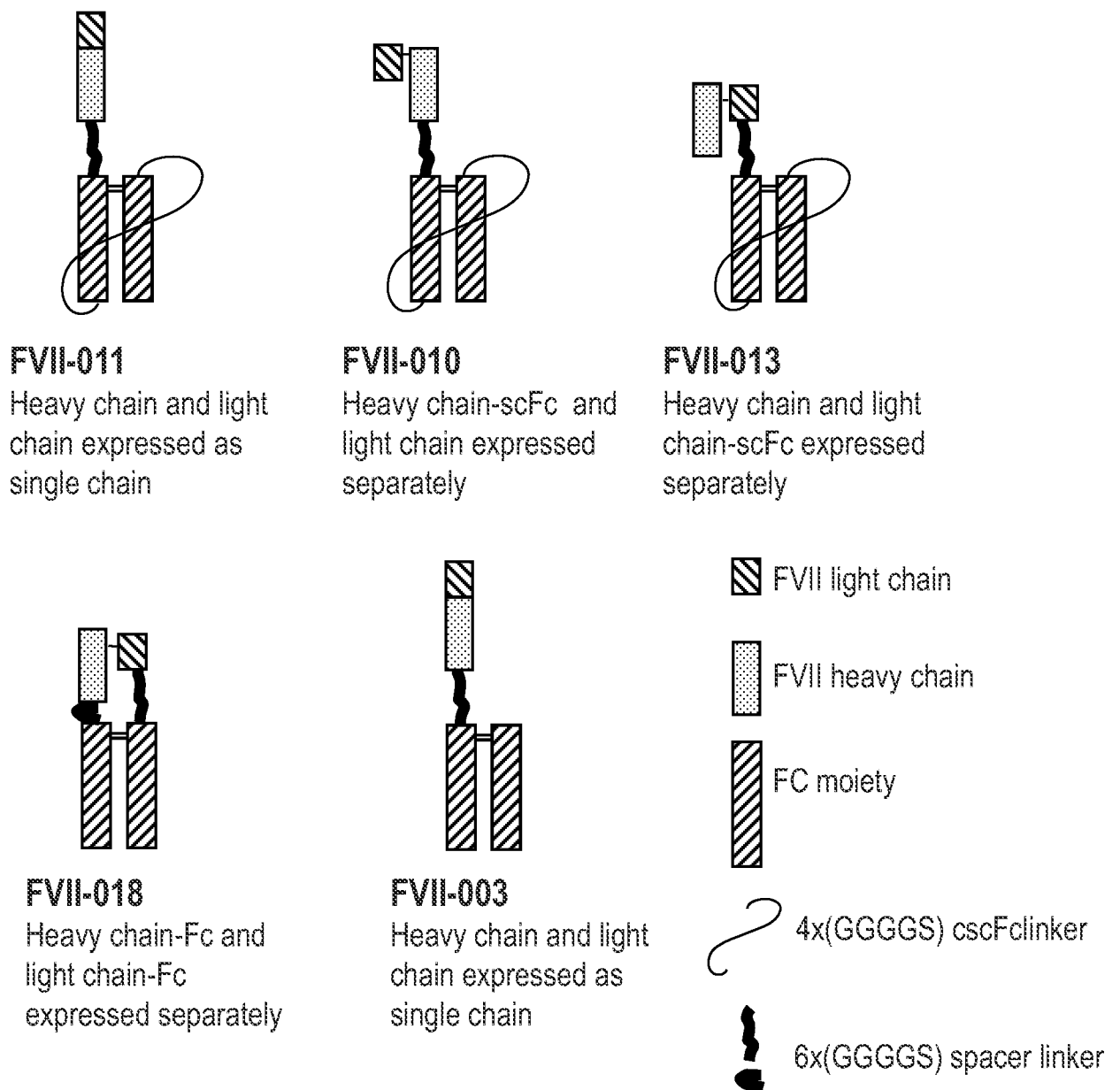


Fig. 6

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**Fig. 7**

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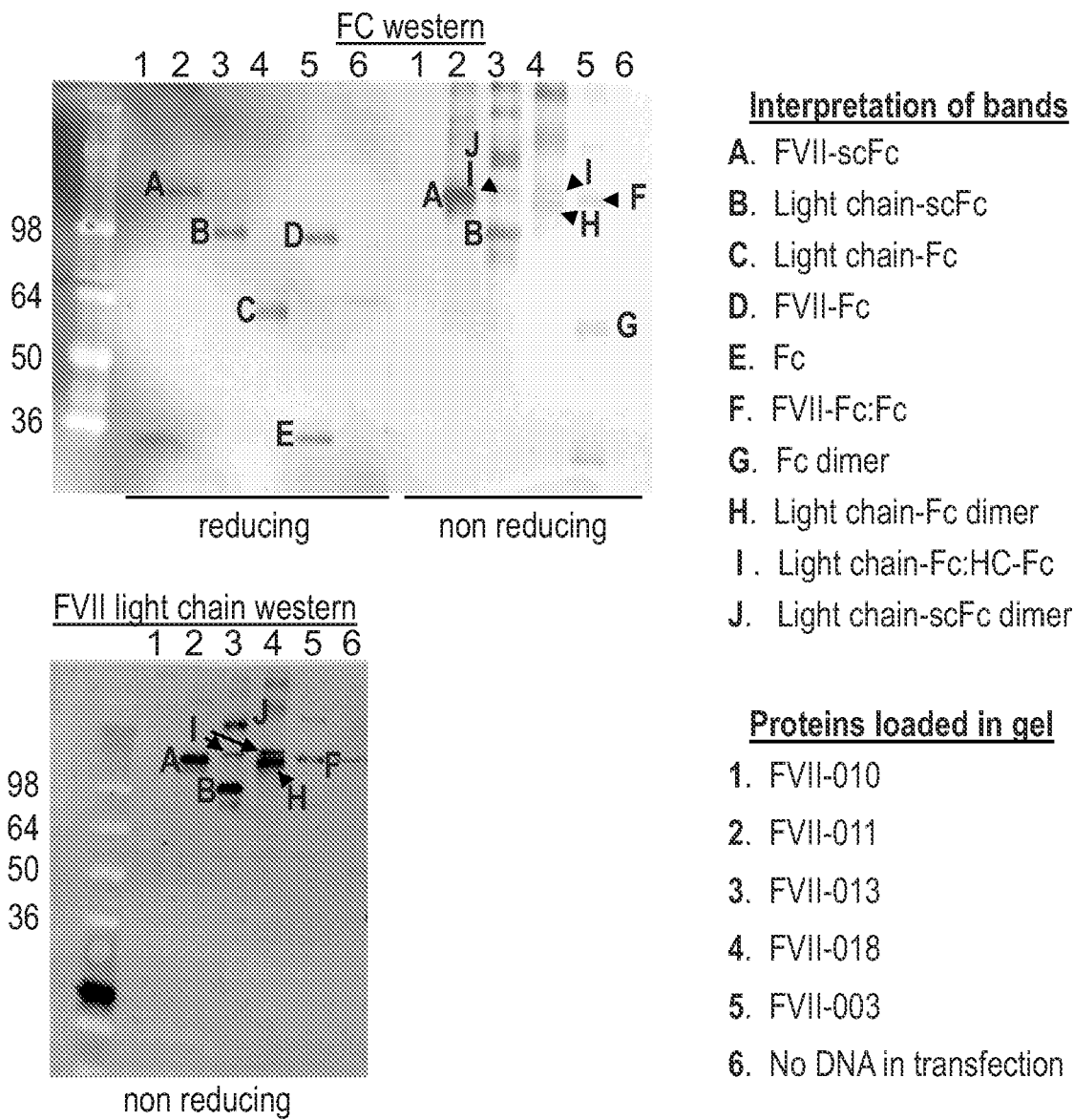


Fig. 8

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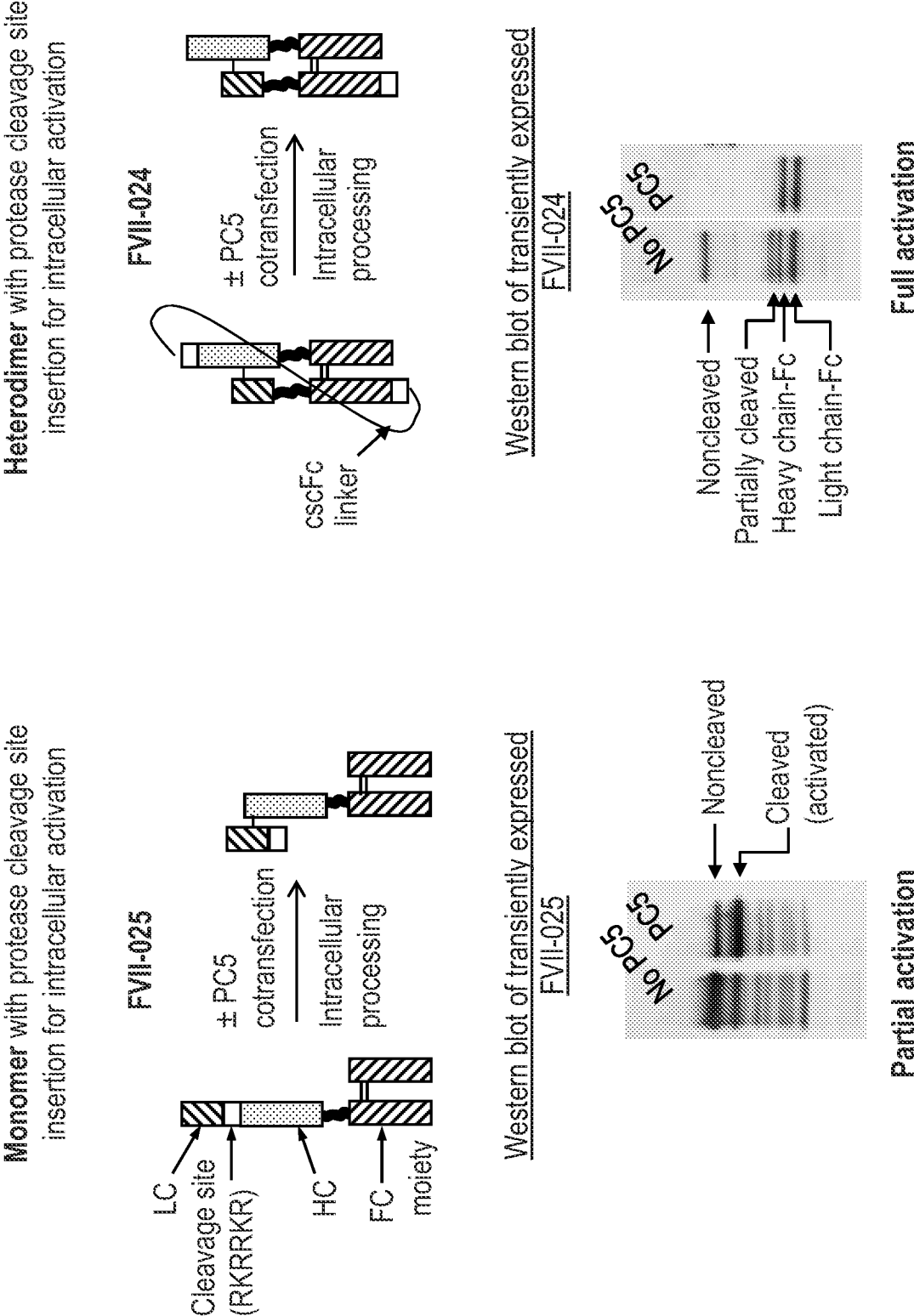


Fig. 9

Protein A pulldown of transiently expressed
IFNb-018 followed by Western blot analysis
(anti-Fc antibody)

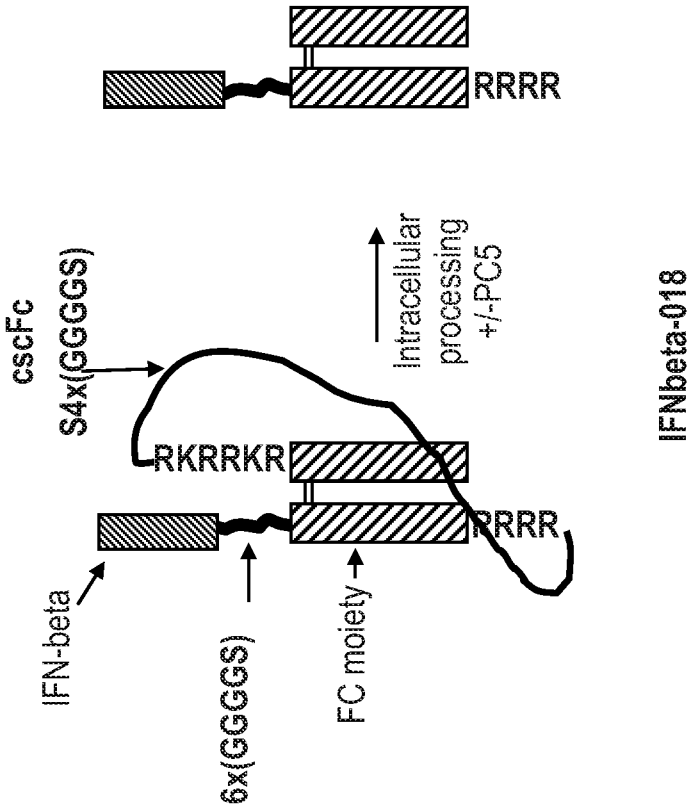
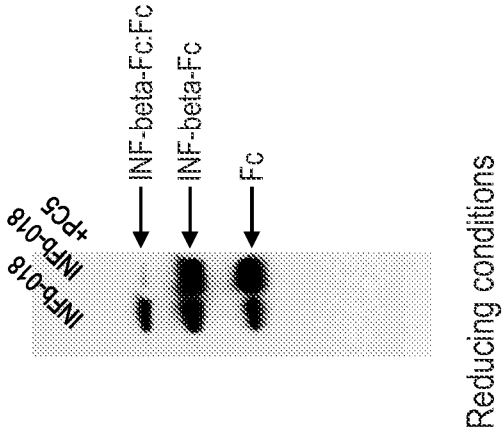


Fig. 10

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Western blot analysis (Fc western) of FVIIFc species following transient transfection of HEK 293 cells and protein A pulldown

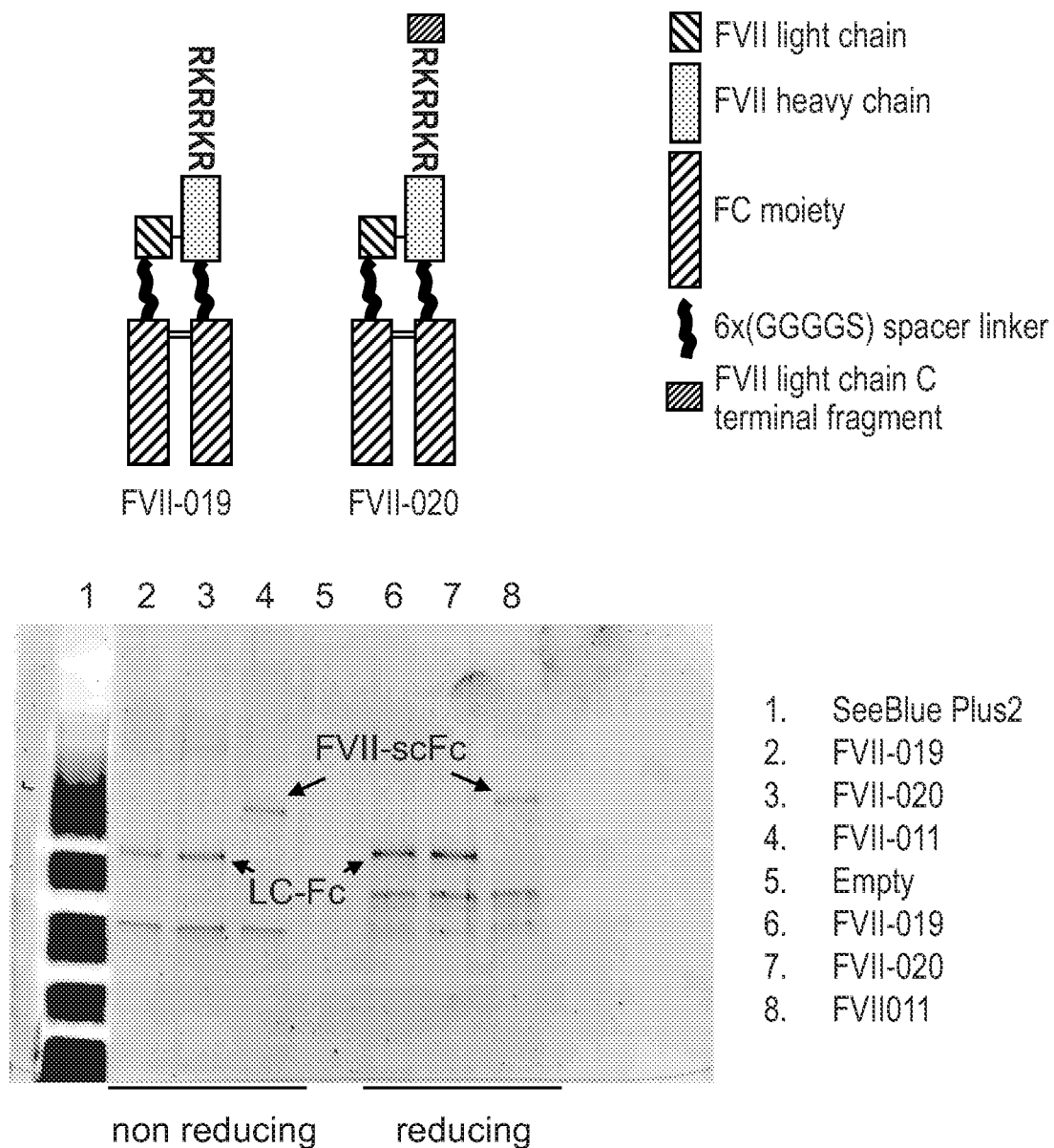


Fig. 11

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2011/043599

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/46 C07K19/00 A61K47/42
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal , BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	wo 2006/113665 A2 (MACROGENICS INC [US] ; JOHNSON LESLIE S [US] ; HUANG LING [US]) 26 October 2006 (2006-10-26)	1-26, 35-52
Y	abstract paragraphs [0018] - [0024] , [0032] , [0033] , [0038] , [0073] , [0091] , [0094] , [0098] - [0100] , [0190] , [0196] - [0198] figures 2, 9, 14, 17 ----- -/- .	27-34



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

23 November 2011

Date of mailing of the international search report

01/12/2011

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Chavanne, Franz

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2011/043599

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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Y	abstract page 3, line 12 - line 30 page 4, line 34 - line 38 page 6, line 14 - line 16 page 7, line 37 - page 11, line 32 examples 1,3 -----	27-34
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Y	abstract paragraphs [0012] , [0013] , [0056] , [0060] , [0061] , [0066] , [0071] , [0083] , [0085] , [0108] examples 3,9 figure 2 -----	27-34
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	abstract page 1, line 4 - line 11 page 2, line 22 - line 29 page 3, line 18 - line 29 page 4, line 17 - line 19 page 5, line 18 - line 26 page 6, line 22 - line 31 example 1 figure 1 ----- -/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2011/043599

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WO 2009/140598 A1 (BAYER HEALTHCARE LLC [US]; FELDMAN RICHARD [US]; KIM JI-YUN [US]; JIAN) 19 November 2009 (2009-11-19) abstract paragraphs [0002], [0007], [0014] - [0023], [0026] - [0045] examples 1-5,8 -----</p>	27-34

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2011/043599

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. (means)

☐

on paper

☒

in electronic form

b. (time)

☐

in the international application as filed

☐

together with the international application in electronic form

☒

subsequently to this Authority for the purpose of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2011/043599

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Information on patent family members

International application No

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