

CLAIMS

1. A binding protein comprising:

at least one pseudoFab portion comprising (1) a first VL domain (VL_a) paired with a first VH domain (VH_a) to form a first functional antigen binding site that binds target antigen A; (2) a first stabilized knockout VH domain (VHX) paired with a first stabilized knockout VL domain (VLX) to form a first stabilized knockout domain; and

wherein the stabilized knockout domain comprises (3) one or more inactivating mutations which abolish its binding to a target antigen; and (4) one or more engineered interchain disulfide bonds.
2. The binding protein of claim 1, wherein the binding protein is a multispecific binding protein further comprising at least a second VL domain (VL_b) paired with a second VH domain (VH_b) to form a second functional antigen binding site that binds target antigen B.
3. A multispecific binding protein comprising:
 - a) a first pseudoFab portion comprising (1) a first VL domain (VL_a) paired with a first VH domain (VH_a) to form a first functional antigen binding site that binds target antigen A; (2) a first stabilized knockout VH domain (VHX) paired with a first stabilized knockout VL domain (VLX) to form a first stabilized knockout domain; and
 - b) a first Fab portion comprising (3) a second VL domain (VL_b) paired with second VH domain (VH_b) to form a second functional antigen binding site that binds target antigen B; (4) a first CH1 domain paired with a first CL domain; and

wherein the stabilized knockout domain comprises (5) one or more inactivating mutations which abolish its binding to a target antigen; and (6) one or more engineered interchain disulfide bonds.
4. A multispecific binding protein comprising:
 - a) a first pseudoFab portion comprising (1) a first VL domain (VL_a) paired with a first VH domain (VH_a) to form a first functional antigen binding site that binds target antigen A; (2) a first stabilized knockout VH domain (VHX) paired with a first stabilized knockout VL domain (VLX) to form a first stabilized knockout domain; and
 - b) a first Fab portion comprising (3) a second VL domain (VL_b) paired with second VH domain (VH_b) to form a second functional antigen binding site that binds target antigen B; (4) a first CH1 domain paired with a first CL domain; and
 - c) a linker portion which operably links the first Fab portion and the first pseudoFab portion,

wherein the stabilized knockout domain comprises (5) one or more inactivating mutations which abolish its binding to a target antigen; and (6) one or more engineered interchain disulfide bonds.
5. The binding protein according to any one of claims 1-2 or the multispecific binding protein according to any one of claims 3-4, wherein the linker portion is a peptide linker.
6. The binding protein or the multispecific binding protein according to claim 5, wherein the peptide linker is a Gly-Ser linker of the formulation (Gly₄Ser)_n, wherein n is 1-10.

7. The binding protein or the multispecific binding protein according to any one of claims 1-6, wherein the linker portion is a heterodimerization domain.
8. The binding protein or the multispecific binding protein according to any one of claims 1-7, comprising independently one or two first pseudoFab portion(s) and one or two first Fab portion(s).
9. The binding protein or the multispecific binding protein according to any one of claims 1-8, comprising separate proteins chains selected from one of the following groups:

(a) VHa-CH1-L1-VHb-L2-VHX and VLa-CL and VLb-L3-VLX;

(b) VHa-L2-VHX-L1-VHb-CH1 and VLa-L3-VLX and VLb-CL;

(c) VHa-CH1-L1-VHa-CH1 and VHb-L2-VHX-L3-VHb-L4-VHX and two chains VLb-L5-VLX and two chains VLa-CL;

wherein the chains of (a) and (b) can be present once or twice, and wherein L1, L2, L3, L4 and L5 are linkers, which may independently be the same or different .

10. The binding protein or the multispecific binding protein according to any one of claims 1-9, wherein the first pseudoFab portion comprises a first polypeptide chain having a structure represented by the formula:

(Ia) N-VHa-L1-VHX-C

and a second polypeptide chain having a structure represented by the formula:

(IIa) N-VLa-L2-VLX-C

wherein L1 and L2 are linkers, which may independently be present or absent, and wherein N and C represent the N- and C-terminal ends, respectively.

11. The binding protein or the multispecific binding protein according to any one of claims 1-10, wherein the first pseudoFab portion comprises of a first polypeptide chain having a structure represented by the formula:

(Ib) N-VHX-L1-VHa-C

and a second polypeptide chain having a structure represented by the formula:

(IIb) N-VLX-L2-VLa-C

wherein L1 and L2 are linkers, which may independently be present or absent, and wherein N and C represent the N- and C-terminal ends, respectively.

12. The binding protein or the multispecific binding protein according to any one of claims 1-11, wherein the first pseudoFab portion comprises of a first polypeptide chain having a structure represented by the formula:

(Ic) N-VLa-L1-VHX-C

and a second polypeptide chain having a structure represented by the formula:

(IIc) N-VHa-L2-VLX-C

wherein L1 and L2 are linkers, which may independently be present or absent, and wherein N and C represent the N- and C-terminal ends, respectively.

13. The binding protein or the multispecific binding protein according to any one of claims 1-12, wherein the first pseudoFab portion comprises of a first polypeptide chain having a structure represented by the formula:

(Id) N-VHX-L1-VLa-C

and a second polypeptide chain having a structure represented by the formula:

(IIId) N-VLX-L2-VHa-C

wherein L1 and L2 are linkers, which may independently be present or absent, and wherein N and C represent the N- and C-terminal ends, respectively.

14. The binding protein or the multispecific binding protein according to any one of claims 1-13, further comprising one or more additional binding domains operably linked to an N- or C-terminus of the binding protein.
15. The binding protein or the multispecific binding protein according to any one of claims 1-14, wherein the one or more additional binding domains are operably linked to N-terminus of the first or second pseudoFab portion.

16. A multispecific binding protein comprising:

a) a first pseudoFab portion comprising:

(1) a first VL domain (VL_a) paired with a first VH domain (VH_a) to form a first antigen binding site that binds target antigen A;

(2) a first stabilized knockout VL domain (VLX) paired with a first stabilized knockout VH domain (VHX) to form a first disulfide stabilized knockout (dsKO) domain;

(3) a first heterodimerization domain (HD1);

wherein the first dsKO domain comprises (i) one or more inactivating mutations which abolish its binding to a target antigen; and (ii) one or more engineered interchain disulfide bonds ;

b) a first Fab portion comprising

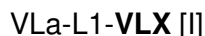
(1) a second VL domain (VL_b) paired with second VH domain (VH_b) to form a second functional antigen binding site that binds target antigen B;

(2) a first CH1 domain paired with a first CL domain; and

(3) a second heterodimerization domain (HD2).

17. The multispecific binding protein of claim 16, wherein the first and second heterodimerization domains comprise first and second Fc domains.
18. The multispecific binding protein of claim 16 or 17, wherein the Fc domains comprise the general structure hinge-CH2 domain-CH3 domain.
19. A multispecific binding protein comprising four polypeptide chains that form at least two antigen-binding sites, wherein

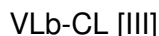
(a) a first polypeptide comprises a structure represented by the formula:



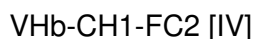
(b) a second polypeptide comprises a structure represented by the formula:



(c) a third polypeptide comprises a structure represented by the formula:



(d) a fourth polypeptide comprises a structure represented by the formula:



wherein:

VL_a is a first immunoglobulin light chain variable domain;

VL_b is a second immunoglobulin light chain variable domain;

VH_a is a first immunoglobulin heavy chain variable domain;

VH_b is a second immunoglobulin heavy chain variable domain;

VLX is a stabilized knockout light chain variable domain;

VHX is a stabilized knockout heavy chain variable domain;

CL is an immunoglobulin light chain constant domain;

CH1 is an immunoglobulin CH1 heavy chain constant domain;

FC1 and FC2 are Fc domains comprising an immunoglobulin hinge region and CH2 and CH3 immunoglobulin heavy chain constant domains; and

L1 and L2 are amino acid linkers, which may independently be the same or different,

wherein

(1) the first VL domain (VL_a) is paired with the first VH domain (VH_a) to form a first functional antigen binding site that binds target antigen A;

(2) the second VL domain (VLb) is paired with the second VH domain (VHb) to form a second functional antigen binding site that binds target antigen B;

(3) the stabilized knockout VL domain (VLX) is paired with the stabilized knockout VH domain (VHX) to form a disulfide stabilized knockout (dsKO) domain;

wherein the dsKO domain comprises (i) one or more inactivating mutations which abolish its binding to a target antigen; and (ii) one or more engineered interchain disulfide bonds.

20. An antigen binding protein comprising six polypeptide chains that form four antigen-binding sites, wherein

(a) a first and a second polypeptide comprises a structure represented by the formula:

VL_a-L1-**VLX** [I] and [II]

(b) a third and a fourth polypeptide comprises a structure represented by the formula:

VL_b-CL [III] and [IV]

(c) a fifth polypeptide comprises a structure represented by the formula:

VH_a-L2-**VHX**-L3-VH_b-CH1-FC1 [V]

(d)a sixth polypeptide comprises a structure represented by the formula:

VH_a-L2-**VHX**-L3-VH_b-CH1-FC2 [VI]

wherein:

VL_a is a first immunoglobulin light chain variable domain;

VL_b is a second immunoglobulin light chain variable domain;

VH_a is a first immunoglobulin heavy chain variable domain;

VH_b is a second immunoglobulin heavy chain variable domain;

VLX is a stabilized knockout light chain variable domain;

VHX is a stabilized knockout heavy chain variable domain;

CL is an immunoglobulin light chain constant domain;

CH1 is an immunoglobulin heavy chain constant domain;

FC1 and FC2 are Fc domains comprising an immunoglobulin hinge region and CH2 and CH3 immunoglobulin heavy chain constant domains; and

L1, L2 and L3 are amino acid linkers,

wherein

(1) the first VL domain (VL_a) is paired with the first VH domain (VH_a) to form a first functional antigen binding site that binds target antigen A;

(2) the second VL domain (VL_b) is paired with the second VH domain (VH_b) to form a second functional antigen binding site that binds target antigen B;

(3) the stabilized knockout VL domain (VLX) is paired with the stabilized knockout VH domain (VHX) to form a disulfide stabilized knockout (dsKO) domain;

wherein the dsKO domains comprise (i) one or more inactivating mutations which abolish its binding to a target antigen; and (ii) one or more engineered interchain disulfide bonds.

21. An antigen binding protein comprising six polypeptide chains that form four antigen-binding sites, wherein

(a) a first and a second polypeptide comprises a structure represented by the formula:

VL_a-L1-**VLX** [I] and [II]

(b) a third and a fourth polypeptide comprises a structure represented by the formula:

VL_b-CL [III] and [IV]

(c) a fifth polypeptide comprises a structure represented by the formula:

VH_b-CH1-L3-VH_a-L2-**VHX**-FC1 [V]

(d) a sixth polypeptide comprises a structure represented by the formula:

VH_b-CH1-L3-VH_a-L2-**VHX**-FC2 [VI]

wherein:

VL_a is a first immunoglobulin light chain variable domain;

VL_b is a second immunoglobulin light chain variable domain;

VH_a is a first immunoglobulin heavy chain variable domain;

VH_b is a second immunoglobulin heavy chain variable domain;

VLX is a stabilized knockout light chain variable domain;

VHX is a stabilized knockout heavy chain variable domain;

CL is an immunoglobulin light chain constant domain;

CH1 is an immunoglobulin heavy chain constant domain;

FC1 and FC2 are Fc domains comprising an immunoglobulin hinge region and CH2 and CH3 immunoglobulin heavy chain constant domains; and

L1, L2 and L3 are amino acid linkers,

wherein

(1) the first VL domain (VL_a) is paired with the first VH domain (VH_a) to form a first functional antigen binding site that binds target antigen A;

(2) the second VL domain (VL_b) is paired with the second VH domain (VH_b) to form a second functional antigen binding site that binds target antigen B;

(3) the stabilized knockout VL domain (VLX) is paired with the stabilized knockout VH domain (VHX) to form a disulfide stabilized knockout (dsKO) domain;

wherein the dsKO domains comprise (i) one or more inactivating mutations which abolish its binding to a target antigen; and (ii) one or more engineered interchain disulfide bonds.

22. An antigen binding protein comprising six polypeptide chains that form four antigen-binding sites, wherein

(a) a first and a second polypeptide comprises a structure represented by the formula:

VL_a-L1-**VLX** [I] and [II]

(b) a third and a fourth polypeptide comprises a structure represented by the formula:

VL_b-CL [III] and [IV]

(c) a fifth polypeptide comprises a structure represented by the formula:

VH_a-L2-**VHX**-L3-VH_a-L4-**VHX**-FC1 [V]

(d) a sixth polypeptide comprises a structure represented by the formula:

VH_b-CH1-L5-VH_b-CH1-FC2 [VI]

wherein:

VL_a is a first immunoglobulin light chain variable domain;

VL_b is a second immunoglobulin light chain variable domain;

VH_a is a first immunoglobulin heavy chain variable domain;

VH_b is a second immunoglobulin heavy chain variable domain;

VLX is a stabilized knockout light chain variable domain;

VHX is a stabilized knockout heavy chain variable domain;

CL is an immunoglobulin light chain constant domain;

CH1 is an immunoglobulin heavy chain constant domain;

FC1 and FC2 are Fc domains comprising an immunoglobulin hinge region and CH2 and CH3 immunoglobulin heavy chain constant domains; and

L1, L2, L3, L4 and L5 are amino acid linkers,

wherein

(1) the first VL domain (VL_a) is paired with the first VH domain (VH_a) to form a first functional antigen binding site that binds target antigen A;

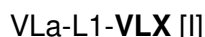
(2) the second VL domain (VL_b) is paired with the second VH domain (VH_b) to form a second functional antigen binding site that binds target antigen B;

(3) the stabilized knockout VL domain (VL_X) is paired with the stabilized knockout VH domain (VH_X) to form a disulfide stabilized knockout (dsKO) domain;

wherein the dsKO domains comprise (i) one or more inactivating mutations which abolish its binding to a target antigen; (ii) one or more engineered interchain disulfide bonds.

23. An antigen-binding protein comprising four polypeptide chains that form three antigen-binding sites, wherein:

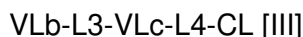
(a) a first polypeptide comprises a structure represented by the formula:



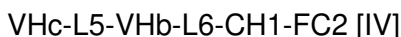
(b) a second polypeptide comprises a structure represented by the formula:



(c) a third polypeptide comprises a structure represented by the formula:



(d) a fourth polypeptide comprises a structure represented by the formula:



wherein:

VL_a is a first immunoglobulin light chain variable domain;

VL_b is a second immunoglobulin light chain variable domain;

VL_c is a third immunoglobulin light chain variable domain;

VH_a is a first immunoglobulin heavy chain variable domain;

VHb is a second immunoglobulin heavy chain variable domain;

VHc is a third immunoglobulin heavy chain variable domain;

CL is an immunoglobulin light chain constant domain;

CH1 is an immunoglobulin CH1 heavy chain constant domain;

VLX is a stabilized knockout light chain variable domain;

VHX is a stabilized knockout heavy chain variable domain;

FC1 and FC2 are Fc domains comprising an immunoglobulin hinge region and CH2 and CH3 immunoglobulin heavy chain constant domains; and

L1, L2, L3, L4, L5 and L6 are amino acid linkers,

wherein

(1) the first VL domain (VL_a) is paired with the first VH domain (VH_a) to form a first functional antigen binding site that binds target antigen A;

(2) the second VL domain (VL_b) is paired with the second VH domain (VH_b) to form a second functional antigen binding site that binds target antigen B;

(3) the third VL domain (VL_c) is paired with the third VH domain (VH_c) to form a third functional antigen binding site that binds target antigen C;

(4) the polypeptide of formula III and the polypeptide of formula IV form a cross-over light chain-heavy chain pair (CODV);

(5) the stabilized knockout VL domain (VLX) is paired with the stabilized knockout VH domain (VHX) to form a disulfide stabilized knockout (dsKO2) domain;

wherein the dsKO domain comprises (i) one or more inactivating mutations which abolish its binding to a target antigen; and (ii) one or more engineered interchain disulfide bonds.

24. The multispecific binding protein according to any one of claims 17-19 or the antigen binding protein according to any one of claims 20-23, wherein the Fc domains comprise one or more knob-in-hole (KIH) mutations.
25. The multispecific binding protein according to any one of claims 17-19 or the antigen binding protein according to any one of claims 20-24, wherein the melting temperature (T_m) of the pseudoFab portion is at least 4 degrees Celsius higher than a reference Fab molecule.
26. The multispecific binding protein according to any one of claims 17-19 or the antigen binding molecule according to any one of claims 20-25, wherein at least one of the one or more engineered interchain disulfide bonds is VH44C-VL100C.

27. The multispecific binding protein according to any one of claims 17-19 or the antigen binding protein according to any one of claims 20-26, wherein target antigen A and target antigen B are different epitopes of the same antigen.
28. The multispecific binding protein according to any one of claims 17-19 or the antigen binding protein according to any one of claims 20-27, wherein the VLX/VHX pair is selected from the group consisting of:
 - (i) VLX comprising an amino acid sequence of SEQ ID NO: 76 and
VHX comprising an amino acid sequence of SEQ ID NO: 77;
 - ii) VLX comprising an amino acid sequence of SEQ ID NO: 76 and
VHX comprising an amino acid sequence of SEQ ID NO: 78; and
 - iii) VLX comprising an amino acid sequence of SEQ ID NO: 76 and
VHX comprising an amino acid sequence of SEQ ID NO: 79.
29. Use of a stabilized knockout domain to reduce heavy chain- light chain mispairing in a multispecific binding protein wherein the stabilized knockout domain comprises VHX and VLX domains comprising (1) one or more inactivating mutations which abolish its binding to a target antigen relative to wild type domains and (2) one or more engineered interchain disulfide bonds which confer enhanced thermal stability (T_m) of the pseudoFab relative to a reference Fab molecule, wherein the reference Fab molecule is identical to the pseudoFab molecule except that, in a pseudoFab reference molecule, CH1 and CL domains of the reference Fab molecule are replaced with VHX and VLX domains.
30. Use according to claim 29, wherein the VLX/VHX pair is selected from the group consisting of:
 - (i) VLX comprising an amino acid sequence of SEQ ID NO: 76 and
VHX comprising an amino acid sequence of SEQ ID NO: 77;
 - ii) VLX comprising an amino acid sequence of SEQ ID NO: 76 and
VHX comprising an amino acid sequence of SEQ ID NO: 78; and
 - iii) VLX comprising an amino acid sequence of SEQ ID NO: 76 and
VHX comprising an amino acid sequence of SEQ ID NO: 79.
31. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the binding protein according to any one of claims 1-28.
32. An expression vector comprising the isolated nucleic acid molecule of claim 31.
33. An isolated host cell comprising the isolated nucleic acid molecule of claim 31 or the expression vector of claim 32.
34. A method of producing the multispecific binding protein according to any one of claims 17-19 or the antigen binding protein according to any one of claims 20-28 comprising culturing the isolated host cell of claim 33 under conditions such that the binding protein is expressed; and purifying the binding protein from the host cell.

35. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of the multispecific binding protein according to any one of claims 17—19 or the antigen binding protein according to any one of claims 20-28.
36. The pharmaceutical compositions according to any one of claims 1-35 for use in the treatment of a disorder in which antigen activity is detrimental.