

Claims

What is claimed is:

1. An antigen-binding protein or fragment thereof, comprising an antibody heavy chain constant (C_H) domain comprising an engineered reactive amino acid residue at a first position and an engineered reactive amino acid residue at a second position; wherein, according to the numbering of the EU index of Kabat, the first position is position 274 and the second position is selected from the group consisting of: 339, 360, 384, 385, 422, 440, and any combination thereof.
2. An antigen-binding protein or fragment thereof, comprising an antibody heavy chain constant (C_H) domain comprising an engineered reactive amino acid residue at a first position and an engineered reactive amino acid residue at a second position; wherein, according to the numbering of the EU index of Kabat, the first position is position 339 and the second position is selected from the group consisting of: 290, 360, 384, 385, 422, 440, and any combination thereof.
3. An antigen-binding protein or fragment thereof, comprising an antibody heavy chain constant (C_H) domain comprising an engineered reactive amino acid residue at a first position and an engineered reactive amino acid residue at a second position; wherein, according to the numbering of the EU index of Kabat, the first position is position 118 and the second position is selected from the group consisting of: 274, 339, 384, 385, 422, 440, and any combination thereof.
4. An antigen-binding protein or fragment thereof, comprising an antibody heavy chain constant (C_H) domain comprising an engineered reactive amino acid residue at a first position and an engineered reactive amino acid residue at a second position; wherein, according to the numbering of the EU index of Kabat, the first position is position 384 and the second position is selected from the group consisting of: 118, 274, 290, 339, and any combination thereof.
5. The antigen-binding protein or fragment thereof of any one of the preceding claims,

wherein the engineered reactive amino acid residue is selected from the group consisting of: cysteine, lysine, histidine, serine, methionine, tryptophan, tyrosine, aspartate, glutamate, and arginine.

6. The antigen-binding protein or fragment thereof of claim 5, wherein the engineered reactive amino acid residue is cysteine.
7. The antigen-binding protein or fragment thereof of claim 5, wherein the engineered reactive amino acid residue is lysine.
8. The antigen-binding protein or fragment thereof of any one of the preceding claims, wherein the engineered reactive amino acid residue is conjugated to a ligand via a reactive moiety.
9. The antigen-binding protein or fragment thereof of claim 8, further comprising a linker conjugating the engineered reactive amino acid residue to the ligand.
10. The antigen-binding protein or fragment thereof of claim 9, wherein the linker is cleavable.
11. The antigen-binding protein or fragment thereof of claim 9, wherein the linker is non-cleavable.
12. The antigen-binding protein or fragment thereof of any one of claims 8-11, comprising a ligand to antibody ratio (LAR) of at least 3.0.
13. The antigen-binding protein or fragment thereof of any one of claims 8-11, comprising a LAR of at least 3.4.
14. The antigen-binding protein or fragment thereof of any one of claims 8-13, wherein the ligand is a detection probe.
15. The antigen-binding protein or fragment thereof of claim 14, wherein the detection

probe is selected from the group consisting of: a biotin, polyethylene glycol (PEG), fluorescent tag, visualization peptide, and a combination thereof.

16. The antigen-binding protein or fragment thereof of claim 15, wherein the detection probe is PEG.
17. The antigen-binding protein or fragment thereof of any one of claims 8-13, wherein the ligand is a targeting moiety.
18. The antigen-binding protein or fragment thereof of claim 17, wherein the targeting moiety is selected from the group consisting of: a protein, nucleic acid, lipid, carbohydrate, and a combination thereof.
19. The antigen-binding protein or fragment thereof of any one of claims 8-13, wherein the ligand is a drug.
20. The antigen-binding protein or fragment thereof of claim 19, comprising a drug to antibody ratio (DAR) of at least 3.0.
21. The antigen-binding protein or fragment thereof of claim 19, comprising a DAR of at least 3.4.
22. The antigen-binding protein or fragment thereof of any one of claims 19-21, wherein the drug is a prodrug selected from the group consisting of: a phosphate-containing prodrug, amino acid-containing prodrug, thiophosphate-containing prodrug, sulfate-containing prodrug, peptide-containing prodrug, β -lactam-containing prodrug, phenoxyacetamide-containing prodrug, phenylacetamide-containing prodrug, 5-fluorocytosine prodrug, 5-fluorouridine prodrug, and any combination thereof.
23. The antigen-binding protein or fragment thereof of any one of claims 19-21, wherein the drug is selected from the group consisting of: an anti-cancer therapeutic agent, anti-inflammatory therapeutic agent, anti-infective therapeutic agent, anesthetic therapeutic agent, cytotoxic therapeutic agent, radionuclide, immunomodulator, cell

signaling peptide, growth factor, enzyme, oligonucleotide, photoactive therapeutic agent, and any combination thereof.

24. The antigen-binding protein or fragment thereof of claim 23, wherein the anti-cancer therapeutic agent is selected from the group consisting of: a cytostatic, cytotoxic nucleoside, tubulin binding agent, hormone and hormone antagonist, anti-angiogenesis agent, enzyme inhibitor, gene regulator, proteasome inhibitor, pteridine, diynene, podophyllotoxin, auristatin, geldanamycin, calicheamicin, gramicidin D, maytansanoids, neocarzinostatin, topotecan, taxanes, cytochalasin B, ethidium bromide, emetine, tenoposide, colchicin, dihydroxy anthracindione, mitoxantrone, procaine, tetracaine, lidocaine, propranolol, puromycin, maytansine derivative, anthracycline derivative, bisphosphonate derivative, leptomycin derivative, streptonigrin derivative, auristatine derivative, duocarmycin derivative, and any combination thereof.
25. The antigen-binding protein or fragment thereof of claim 24, wherein the cytostatic is selected from the group consisting of: an anthracine, DNA synthesis inhibitor, DNA-intercalator, DNA-RNA transcription regulator, ansamycin benzoquinone, quinonoid derivative, busulfan, ifosfamide, mechlorethamine, triaziquone, diaziquone, carbazilquinone, indoloquinone E09, diaziridiny1-benzoquinone methyl DZQ, triethylenephosphoramide, nitrosourea compound, and any combination thereof.
26. The antigen-binding protein or fragment thereof of claim 24, wherein the cytotoxic nucleoside is selected from the group consisting of: adenosine arabinoside, cytarabine, cytosine arabinoside, 5-fluorouracil, fludarabine, floxuridine, fltorafur, 6-mercaptopurine, and any combination thereof.
27. The antigen-binding protein or fragment thereof of claim 24, wherein the tubulin binding agent is selected from the group consisting of: taxoid, nocodazole, rhizoxin, dolastatin, colchicine, colchicinoid, combretastatin, vinca alkaloid, and any combination thereof.
28. The antigen-binding protein or fragment thereof of claim 24, wherein the hormone and

hormone antagonist is selected from the group consisting of: corticosteroid, progestin, estrogen, antiestrogen, androgen, aromatase inhibitor, 17-(allylamino)-17-demethoxygeldanamycin, 4-amino-I, 8-naphthalimide, apigenin, brefeldin A, cimetidine, dichloromethylene-diphosphonic acid, leuprolide, luteinizing hormone-releasing hormone, pifithrin-a, rapamycin, sex hormone-binding globulin, thapsigargin, and any combination thereof.

29. The antigen-binding protein or fragment thereof of claim 24, wherein the anti-angiogenesis agent is selected from the group consisting of: Angiostatin K1 -3, DL-a-difluoromethyl-ornithine, endostatin, fumagillin, genistein, minocycline, staurosporine, (+)-thalidomide, and any combination thereof.
30. The antigen-binding protein or fragment thereof of claim 24, wherein the enzyme inhibitor is selected from the group consisting of: S(+)- camptothecin, curcumin, (-)-deguelin, 5,6-diChlorobenz-imidazole I- β -D- ribofuranoside, etoposide, formestane, fostriecin, hispidin, 2-imino-l- imidazolidineacetic acid, mevinolin, trichostatin A, tyrphostin AG 34, tyrphostin AG 879, and any combination thereof.
31. The antigen-binding protein or fragment thereof of claim 24, wherein the gene regulator is selected from the group consisting of: 5-aza-2'- deoxycytidine, 5-azacytidine, cholecalciferol, 4-hydroxytamoxifen, melatonin, mifepristone, raloxifene, trans-retinal, retinoic acid, vitamin A acid, 9-cis-retinoic acid, 13-cis-retinoic acid, retinol, tamoxifen, troglitazone, and any combination thereof.
32. The antigen-binding protein or fragment thereof of any one of the preceding claims, further comprising an antibody heavy chain variable (V_H) domain.
33. The antigen-binding protein or fragment thereof of any one of the preceding claims, further comprising an antibody light chain variable (V_L) domain.
34. The antigen-binding protein or fragment thereof of any one of the preceding claims, wherein the antigen-binding protein is a chimeric or humanized antibody.

35. The antigen-binding protein or fragment thereof of any one of claims 1-33, wherein the antigen-binding protein is a human antibody.
36. The antigen-binding protein or fragment thereof of any one of the preceding claims, wherein the antigen-binding protein is a monoclonal antibody.
37. The antigen-binding protein or fragment thereof of any one of the preceding claims, wherein the antigen-binding protein comprises one or more full-length antibody heavy chains comprising an Fc region.
38. The antigen-binding protein or fragment thereof of claim 37, wherein the Fc region is a human IgG1 Fc region.
39. A method of producing an antigen-binding protein or fragment thereof, comprising an engineered reactive amino acid residue at a first position and an engineered reactive amino acid residue at a second position, the method comprising:
- (a) producing a first library of engineered parental antigen-binding proteins or fragments thereof, wherein each parental antigen-binding protein or fragment thereof comprises a first engineered reactive amino acid residue;
 - (b) producing a second library of ligand-conjugated engineered parental antigen-binding proteins or fragments thereof by conjugating a ligand to the first engineered reactive amino acid residue of each engineered parental antigen-binding protein or fragment thereof in the first library;
 - (c) producing a third library of engineered positions by screening the second library for a ligand to antibody ratio (LAR) above 1.7, wherein the positions at which the engineered parental antigen-binding proteins or fragments thereof with an LAR above 1.7 have an engineered reactive amino acid residue comprise the third library of engineered positions;
 - (d) producing a fourth library of antigen-binding proteins or fragments thereof, wherein each antigen-binding protein or fragment thereof comprises an engineered reactive amino acid residue at a first position selected from the third library of engineered positions and an engineered reactive amino acid residue at a second position selected from the third library of engineered positions;

- (e) producing a fifth library of ligand-conjugated double-engineered antigen-binding proteins or fragments thereof by conjugating a ligand to the engineered reactive amino acid residue at the first position and the engineered reactive amino acid residue at the second position; and
 - (f) producing a sixth library of double-engineered antigen-binding proteins or fragments thereof by screening the fifth library for an LAR above 3.4.
40. The method of claim 39, further comprising producing a third library of engineered positions by screening the second library for conjugation of 60% or above of one ligand per single-engineered parental antigen-binding protein or fragment thereof, conjugation of 20% or below of multiple ligands per single-engineered parental antigen-binding protein or fragment thereof, and conjugation of 20% or below of no ligand per single-engineered parental antigen-binding protein or fragment thereof.
41. The method of claim 39 or 40, further comprising producing a sixth library of double-engineered antigen-binding proteins or fragments thereof by screening the fifth library for conjugation of 80% or above of one or two ligands per double-engineered antigen-binding protein or fragment thereof, conjugation of 10% or below of multiple ligands per double-engineered antigen-binding protein or fragment thereof, and conjugation of 5% or below of no ligand per double-engineered antigen-binding protein or fragment thereof.
42. The method of any one of claims 39-41, further comprising conjugating a ligand to the engineered reactive amino acid residues of the double-engineered antigen-binding proteins or fragments thereof comprising the sixth library.
43. The method of any one of claims 39-42, wherein the engineered reactive amino acid residue is selected from the group consisting of: cysteine, lysine, histidine, serine, methionine, tryptophan, tyrosine, aspartate, glutamate, and arginine.
44. The method of claim 43, wherein the engineered reactive amino acid residue is cysteine.

45. The method of claim 43, wherein the engineered reactive amino acid residue is lysine.
46. The method of any one of claims 39-45, wherein the engineered reactive amino acid residue is conjugated to a ligand via a reactive moiety.
47. The method of claim 46, further comprising a linker conjugating the engineered reactive amino acid residue to the ligand.
48. The method of claim 47, wherein the linker is cleavable.
49. The method of claim 47, wherein the linker is non-cleavable.
50. The method of any one of claims 46-49, comprising a LAR of at least 3.0.
51. The method of any one of claims 46-49, comprising a LAR of at least 3.4.
52. The method of any one of claims 46-51, wherein the ligand is a detection probe.
53. The method of claim 52, wherein the detection probe is selected from the group consisting of: a biotin, polyethylene glycol (PEG), fluorescent tag, visualization peptide, and a combination thereof.
54. The method of claim 53, wherein the detection probe is PEG.
55. The method of any one of claims 46-51, wherein the ligand is a targeting moiety.
56. The method of claim 55, wherein the targeting moiety is selected from the group consisting of: a protein, nucleic acid, lipid, carbohydrate, and a combination thereof.
57. The method of any one of claims 46-51, wherein the ligand is a drug.
58. The method of claim 57, comprising a drug to antibody ratio (DAR) of at least 3.0.

59. The method of claim 57, comprising a DAR of at least 3.4.
60. The method of any one of claims 57-59, wherein the drug is a prodrug selected from the group consisting of: a phosphate-containing prodrug, amino acid-containing prodrug, thiophosphate-containing prodrug, sulfate-containing prodrug, peptide-containing prodrug, β -lactam-containing prodrug, phenoxyacetamide-containing prodrug, phenylacetamide-containing prodrug, 5-fluorocytosine prodrug, 5-fluorouridine prodrug, and a combination thereof.
61. The method of any one of claims 57-59, wherein the drug is selected from the group consisting of: an anti-cancer therapeutic agent, anti-inflammatory therapeutic agent, anti-infective therapeutic agent, anesthetic therapeutic agent, cytotoxic therapeutic agent, radionuclide, immunomodulator, cell signaling peptide, growth factor, enzyme, oligonucleotide, photoactive therapeutic agent, and a combination thereof.
62. The method of claim 61, wherein the anti-cancer therapeutic agent is selected from the group consisting of: a cytostatic, cytotoxic nucleoside, tubulin binding agent, hormone and hormone antagonist, anti-angiogenesis agent, enzyme inhibitor, gene regulator, proteasome inhibitor, pteridine, diynene, podophyllotoxin, auristatin, geldanamycin, calicheamicin, gramicidin D, maytansanoids, neocarzinostatin, topotecan, taxanes, cytochalasin B, ethidium bromide, emetine, tenoposide, colchicin, dihydroxy anthracindione, mitoxantrone, procaine, tetracaine, lidocaine, propranolol, puromycin, maytansine derivative, anthracycline derivative, bisphosphonate derivative, leptomycin derivative, streptonigrin derivative, auristatine derivative, duocarmycin derivative, and any combination thereof.
63. The method of claim 62, wherein the cytostatic is selected from the group consisting of: an anthracine, DNA synthesis inhibitor, DNA-intercalator, DNA-RNA transcription regulator, ansamycin benzoquinone, quinonoid derivative, busulfan, ifosfamide, mechlorethamine, triaziquone, diaziquone, carbazilquinone, indoloquinone E09, diaziridinyl-benzoquinone methyl DZQ, triethylenephosphoramidate, nitrosourea compound, and any combination thereof.

64. The method of claim 62, wherein the cytotoxic nucleoside is selected from the group consisting of: adenosine arabinoside, cytarabine, cytosine arabinoside, 5-fluorouracil, fludarabine, floxuridine, florafur, 6-mercaptopurine, and any combination thereof.
65. The method of claim 62, wherein the tubulin binding agent is selected from the group consisting of: taxoid, nocodazole, rhizoxin, dolastatin, colchicine, colchicinoid, combretastatin, vinca alkaloid, and any combination thereof.
66. The method of claim 62, wherein the hormone and hormone antagonist is selected from the group consisting of: corticosteroid, progestin, estrogen, antiestrogen, androgen, aromatase inhibitor, 17-(allylamino)-17-demethoxygeldanamycin, 4-amino-I, 8-naphthalimide, apigenin, brefeldin A, cimetidine, dichloromethylene-diphosphonic acid, leuprolide, luteinizing hormone-releasing hormone, pifithrin-a, rapamycin, sex hormone-binding globulin, thapsigargin, and any combination thereof.
67. The method of claim 62, wherein the anti-angiogenesis agent is selected from the group consisting of: Angiostatin K1 -3, DL-a-difluoromethyl-ornithine, endostatin, fumagillin, genistein, minocycline, staurosporine, (+)-thalidomide, and any combination thereof.
68. The method of claim 62, wherein the enzyme inhibitor is selected from the group consisting of: S(+)- camptothecin, curcumin, (-)-deguelin, 5,6-diChlorobenzimidazole I- β -D- ribofuranoside, etoposide, formestane, fostriecin, hispidin, 2-imino-1- imidazolidineacetic acid, mevinolin, trichostatin A, tyrphostin AG 34, tyrphostin AG 879, and any combination thereof.
69. The method of claim 62, wherein the gene regulator is selected from the group consisting of: 5-aza-2'- deoxycytidine, 5-azacytidine, cholecalciferol, 4-hydroxytamoxifen, melatonin, mifepristone, raloxifene, trans-retinal, retinoic acid, vitamin A acid, 9-cis-retinoic acid, 13-cis-retinoic acid, retinol, tamoxifen, troglitazone, and any combination thereof.

70. The method of any one of claims 39-69, further comprising an antibody heavy chain variable (V_H) domain.
71. The method of any one of claims 39-70, further comprising an antibody light chain variable (V_L) domain.
72. The method of any one of claims 39-71, wherein the antigen-binding protein is a chimeric or humanized antibody.
73. The method of any one of claims 39-71, wherein the antigen-binding protein is a human antibody.
74. The method of any one of claims 39-73, wherein the antigen-binding protein is a monoclonal antibody.
75. The method of any one of claims 39-74, wherein the antigen-binding protein comprises one or more full-length antibody heavy chains comprising an Fc region.
76. The method of claim 75, wherein the Fc region is a human IgG1 Fc region.
77. The method of any one of claims 39-76, wherein the first library of single-engineered parental antigen-binding proteins or fragments thereof is screened for thermostability comparable to the thermostability of an un-engineered parental antigen-binding protein or fragment thereof.
78. A pharmaceutical composition comprising the antigen-binding protein or fragment thereof of any one of the preceding claims, further comprising a pharmaceutically acceptable carrier.
79. A method of treating a disease or disorder in a subject, comprising administering to a subject in need thereof the antigen-binding protein or fragment thereof of any one of claims 1-77 or the pharmaceutical composition of claim 78.

- 80. An isolated nucleic acid molecule encoding the antigen-binding protein or fragment thereof of any one of the preceding claims.
- 81. An expression vector comprising the nucleic acid molecule of claim 80.
- 82. A host cell comprising the expression vector of claim 81.