

CLAIMS

What is claimed:

1. A targeted active gene editing (TAGE) agent comprising
 - (i) an extracellular cell membrane binding moiety that binds to an extracellular cell membrane-bound molecule, and
a site-directed modifying polypeptide that recognizes a nucleic acid sequence,
wherein the extracellular cell membrane binding moiety and the site-directed modifying polypeptide are stably associated such that the site-directed modifying polypeptide can be internalized into a cell displaying the extracellular cell membrane-bound molecule; and/or
 - (ii) a cell penetrating peptide (CPP) that facilitates uptake of a polypeptide into a cell, and
a site-directed modifying polypeptide that recognizes a nucleic acid sequence,
wherein the CPP and the site-directed modifying polypeptide are stably associated such that the site-directed modifying polypeptide can be internalized into a cell targeted via the CPP.
2. The TAGE agent of claim 1, wherein
 - the extracellular cell membrane binding moiety is an antigen binding polypeptide that specifically binds to an extracellular cell membrane-bound molecule, a cell penetrating peptide (CPP), a ligand that binds to the extracellular cell membrane-bound molecule, or combinations thereof; and/or
 - the TAGE agent comprises at least two extracellular cell membrane binding moieties.
3. The TAGE agent of claim 2, wherein the antigen binding polypeptide is an antibody, an antigen-binding portion of an antibody, or an antibody mimetic.
4. The TAGE agent of claim 2, wherein the at least two extracellular cell membrane binding moieties of the TAGE agent are (i) a CPP and an antigen-binding polypeptide, or (ii) a CPP and a ligand.

5. The TAGE agent of any one of claims 1 to 4, wherein the site-directed modifying polypeptide comprises a nuclease or a nickase.
6. The TAGE agent of claim 5, wherein the nuclease is a DNA endonuclease.
7. The TAGE agent of claim 6, wherein the DNA endonuclease is Cas9 or Cas12.
8. The TAGE agent of claim 2, wherein the ligand is a protein selected from IL-2, CCL2, CCL5, CCL7, CCL8, CCL13, CCL16, IGF2, IL7, IL15, IFN γ , CSF-1, CSF-2, CSF-3, TCR/DC4, or PD-L1.
9. The TAGE agent of claim 1, wherein the site-directed modifying polypeptide comprises an RNA-guided DNA endonuclease that recognizes a CRISPR sequence.
10. The TAGE agent of any one of claims 1-9, wherein the TAGE agent further comprises a guide RNA that specifically hybridizes to a target region of the genome of the cell, wherein the guide RNA and the site-directed modifying polypeptide form a ribonucleoprotein.
11. The TAGE agent of any one of claims 1 to 10, wherein the site-directed modifying polypeptide further comprises at least one nuclear localization signal (NLS).
12. The TAGE agent of any one of claims 2 to 11, wherein
 - the site-directed modifying polypeptide further comprises a conjugation moiety that binds to the antigen binding polypeptide, α ligand, or CPP; or
 - the site-directed modifying polypeptide and the antigen binding polypeptide ligand, or CPP are conjugated via a linker.
13. The TAGE agent of claim 12, wherein:
 - the conjugation moiety is a protein;
 - the linker is cleavable.

14. The TAGE agent of claim 3, wherein:
- the antibody mimetic is an adnectin (i.e., fibronectin based binding molecules), an affilin, an affimer, an affitin, an alphabody, an affibody, a DARPin, an anticalin, an avimer, a fynomer, a Kunitz domain peptide, a monobody, a nanoCLAMP, a unibody, a versabody, an aptamer, or a peptidic molecule;
 - the antigen-binding portion of the antibody is a nanobody, a single domain antibody, an scFv, a Fab, a diabody, a BiTE, a diabody, a DART, a minibody, a F(ab')₂, or an intrabody; or
 - the antibody is an intact antibody or a bispecific antibody.
15. The TAGE agent of claim 11, wherein the at least one NLS comprises an SV40 NLS.
16. The TAGE agent of claim 15, wherein the SV40 NLS comprises the amino acid sequence PKKKRKV (SEQ ID NO: 10).
17. The TAGE agent of any one of claims 1 to 16, wherein the extracellular cell membrane-bound molecule or protein is HLA-DR, CD44, CD11a, CD22, CD3, CD20, CD33, CD32, CD44, CD47, CD59, CD54, CD25, AchR, CD70, CD74, CTLA4, EGFR, HER2, EpCam, OX40, PD-1, PD-L1, GITR, CD52, CD34, CD27, CD30, ICOS, or RSV.
18. A site-directed modifying polypeptide comprising an RNA-guided DNA endonuclease that recognizes a CRISPR sequence and a conjugation moiety that binds to an antibody, an antigen-binding portion of an antibody, or an antibody mimetic that specifically binds to an extracellular cell membrane-bound molecule; or a ligand that facilitates uptake of a polypeptide to which the ligand is conjugated.
19. The site-directed modifying polypeptide of claim 18, wherein
- the ligand is a protein selected from IL-2, CCL2, CCL5, CCL7, CCL8, CCL13, CCL16, IGF2, IL7, IL15, IFN γ , CSF-2, TCR/DC4, or PD-L1; or
 - the extracellular cell membrane-bound molecule is a protein selected from the group consisting of HLA-DR, CD11a, CD44, CD22, CD3, CD20, CD33,

CD32, CD44, CD47, CD59, CD54, CD25, AchR, CD70, CD74, CTLA4, EGFR, HER2, or EpCam.

20. A nucleoprotein comprising the site-directed modifying polypeptide of any one of claims 18 to 19 and a guide RNA, wherein the guide RNA specifically hybridizes to a target region of the genome of a cell displaying the extracellular cell membrane-bound protein.
21. The TAGE agent of claim 1 or 2, wherein the CPP is Tat, Tat-NLS, His-Tat-NLS (HTN), Tat-HA, S19-Tat, CM18, CM18-Tat, hPH1, L17E, IMT-P8, IMT-P8 (C14S), TDP, TDP-KDEL (SEQ ID NO: 17), penetratin, polyR, Aurein, LAH4-L1, LMWP, Pardaxin, S10, S18, S19, S85, Vectofusin1, or ZF5.3.
22. An isolated nucleic acid encoding the site-directed modifying polypeptide of any one of claims 17 to 19.
23. A vector comprising the nucleic acid of claim 22.
24. A cell comprising the site-directed modifying polypeptide of any one of claims 17 to 19.
25. A method of modifying the genome of a target cell, the method comprising contacting the target cell with a targeted active gene editing (TAGE) agent of any one of claims 1 to 17.
26. The method of claim 25, wherein the target cell is a eukaryotic cell.
27. The method of claim 26, wherein the eukaryotic cell is a mammalian cell.
28. The method of claim 27, wherein the mammalian cell is a mouse cell, a non-human primate cell, or a human cell.

29. The method of any one of claims 25 to 28, wherein the site-directed modifying polypeptide produces a cleavage site at the target region of the genome, thereby modifying the genome.
30. The method of any one of claims 25 to 29, wherein the target region of the genome is a target gene.
31. The method of claim 30, wherein said method is effective to modify expression of the target gene.
32. A method of modifying a nucleic acid sequence within a target cell in a mammalian subject, the method comprising:
 contacting the target cell in the subject with a targeted active gene editing (TAGE) agent comprising an extracellular cell membrane binding moiety, and a site-directed modifying polypeptide that recognizes the nucleic acid sequence within the target cell, such that the nucleic acid sequence of the target cell is modified; or
 locally administering to the subject a targeted active gene editing (TAGE) agent comprising an extracellular cell membrane binding moiety, and a site-directed modifying polypeptide that recognizes the nucleic acid sequence within the target cell, such that the nucleic acid sequence of the target cell is modified.
33. The method of claim 32, wherein the method comprises locally administering the TAGE agent to the subject by intramuscular injection, intraosseous injection, intraocular injection, intratumoral injection, intrathecal delivery, or intradermal injection; and/or
 the method is effective to increase the number of genetically modified target cells in the subject following administration of the TAGE agent; and/or
 the mammalian subject is a human subject; and/or
 the subject has a disease selected from an eye disease, a stem cell disorder, and a cancer, and wherein the method is effective to treat the disease.
34. A method of modifying a nucleic acid sequence within a target mammalian cell, the method comprising contacting the target mammalian cell with a targeted active

gene editing (TAGE) agent under conditions in which the TAGE agent is internalized into the target cell, such that the nucleic acid sequence is modified,

wherein the TAGE agent comprises an extracellular cell membrane binding moiety, and a site-directed modifying polypeptide that recognizes the nucleic acid sequence within the target cell,

wherein the internalization of the TAGE agent is not dependent on electroporation.

35. The method of any one of claims 32 to 34, wherein

the target mammalian cell is (i) a hematopoietic cell (HSC), a neutrophil, a T cell, a B cell, a dendritic cell, a macrophage, or a fibroblast; or (ii) a hematopoietic stem cell (HSC) or a bone marrow cell that is not an HSC; and/or

the target mammalian cell is contacted with the TAGE by co-incubation *ex vivo*; or the target mammalian cell is contacted with the TAGE *in situ* by injection into a tissue of a subject; and/or

the nucleic acid is a gene in the genome of the cell, wherein the expression of said gene is altered following said modification; and/or

the target mammalian cell is a mouse cell, a non-human primate cell, or a human cell.

36. The method of claim 35, wherein the extracellular cell membrane binding moiety specifically binds an extracellular cell membrane-bound molecule on a human HSC.

37. The method of claim 36, wherein the extracellular cell membrane-bound molecule on the HSC is CD34, EMCN, CD59, CD90, ckit, CD45, or CD49F.

39. The method of any one of claims 34 to 37, wherein the TAGE is administered to the subject by intramuscular injection, intraosseous injection, intraocular injection, intratumoral injection, or intradermal injection.

40. The method of any one of claims 32 to 39, wherein

the extracellular cell membrane binding moiety of the TAGE agent is a cell penetrating peptide, a ligand, an antigen binding polypeptide that specifically binds to an extracellular cell membrane-bound molecule, or combinations thereof, and/or

the extracellular cell membrane binding moiety of the TAGE agent comprises one or more cell penetrating peptides (CPPs); and/or

the extracellular cell membrane binding moiety of the TAGE agent comprises an antigen binding polypeptide that specifically binds to an extracellular cell membrane-bound molecule; and/or

the extracellular cell membrane binding moiety of the TAGE agent comprises a ligand that specifically binds to an extracellular cell membrane-bound molecule; and/or

the TAGE agent further comprises at least one nuclear localization signal (NLS); and/or

the TAGE agent comprises at least two extracellular cell membrane binding moieties.

41. The method of claim 40, wherein

the one or more CPPs is NLS, Tat, Tat-NLS, His-Tat-NLS (HTN), Tat-HA, S19-Tat, CM18, CM18-Tat, hPH1, L17E, IMT-P8, IMT-P8 (C14S), TDP, TDP-KDEL (SEQ ID NO: 17), penetratin, polyR, Aurein, LAH4-L1, LMWP, Pardaxin, S10, S18, S19, S85, Vectofusin1, ZF5.3, or a combination thereof; or

the extracellular cell membrane-bound molecule bound by the antigen binding polypeptide is HLA-DR, CD44, CD11a, CD22, CD3, CD20, CD33, CD32, CD44, CD47, CD59, CD54, CD25, AchR, CD70, CD74, CTLA4, EGFR, HER2, EpCam, OX40, PD-1, PD-L1, GITR, CD52, CD34, CD27, CD30, ICOS, or RSV; or

the ligand is selected from IL-2, CCL2, CCL5, CCL7, CCL8, CCL13, CCL16, IGF2, IL7, IL15, IFN γ , CSF-1, TCR/DC4, or PD-L1.

42. The method of claim 40, wherein the antigen binding polypeptide is an antibody, an antigen-binding portion of an antibody, or an antibody-mimetic.

43. The method of claim 42, wherein:

the antibody mimetic is an adnectin (i.e., fibronectin based binding molecules), an affilin, an affimer, an affitin, an alphabody, an affibody, a DARPin, an anticalin, an avimer, a fynomer, a Kunitz domain peptide, a monobody, a nanoCLAMP, a unibody, a versabody, an aptamer, or a peptidic molecule;

the antigen-binding portion of the antibody is a nanobody, a domain antibody, an scFv, a Fab, a diabody, a BiTE, a diabody, a DART, a mnibody, a F(ab')₂, or an intrabody; or

the antibody is an intact antibody or a bispecific antibody.

44. The method of any one of claims 40-43, wherein the NLS comprises an SV40 NLS.

45. The method of claim 44, wherein the SV40 NLS comprises the amino acid sequence PKKKRKV (SEQ ID NO: 10).

46. The method of claim 45, wherein the at least two extracellular cell membrane binding moieties of the TAGE agent are (i) a CPP and an antigen-binding polypeptide; or (ii) a CPP and a ligand.

47. The method of any one of claims 32 to 46, wherein

the target mammalian cell is a population of target mammalian cells, optionally wherein the method is effective to increase the number of genetically modified target mammalian cells; and/or

the site-directed modifying polypeptide of the TAGE has increased cellular internalization in the target mammalian cell; and/or

the site-directed modifying polypeptide of the TAGE has increased nuclear internalization in the target mammalian cell; and/or

the site-directed modifying polypeptide comprises a nuclease or a nickase; and/or

the site-directed modifying polypeptide is a nucleic acid-guided nuclease, and the TAGE agent further comprises a guide nucleic acid that specifically hybridizes to a target region of the nucleic acid sequence of the target mammalian cell, wherein the guide nucleic acid and the nucleic acid-guided nuclease form a nucleoprotein; and/or

the site-directed modifying polypeptide further comprises a conjugation moiety that binds to the extracellular cell membrane binding moiety or a complementary binding moiety attached thereto and/or

the site-directed modifying polypeptide and the extracellular cell membrane binding moiety are conjugated via a linker; and/or

the TAGE agent further comprises an endosomal escape agent.

48. The method of claim 47, wherein the site-directed modifying polypeptide is a RNA-guided nuclease, and the TAGE agent further comprises a guide RNA that specifically hybridizes to a target region of the nucleic acid sequence of the target mammalian cell, wherein the guide RNA and the RNA-guided nuclease form a ribonucleoprotein.
49. The method of claim 48, wherein the guide RNA is a single guide RNA (sgRNA) or a cr:trRNA.
50. The method of claim 48, wherein the RNA-guided nuclease is a Class 2 Cas polypeptide.
51. The method of claim 50, wherein the Class 2 Cas polypeptide is a Type II Cas polypeptide or a Type V Cas polypeptide.
52. The method of claim 51, wherein the Type II Cas polypeptide is Cas9 or the Type V Cas polypeptide is Cas12.
53. The method of claim 47, wherein:
 - the conjugation moiety is a protein;
 - the linker is a cleavable linker;
 - the endosomal escape agent is TDP or TDP-KDEL.