

CLAIMS

What is claimed is:

1. A CRISPR guide molecule, comprising:

a targeting region capable of binding a target nucleic acid sequence and

an activating region comprising the RNA sequence

UAAUUUCUACUCUUGUAGAU (SEQ ID NO: 6) including at least one

deoxyribonucleotide in place of a ribonucleotide, wherein the activating region is

capable of forming a nucleoprotein complex with a Cas12 protein.
2. The CRISPR guide molecule of claim 1, wherein one or more of positions 1, 3, 7, 10, 12, 14, 15, 16, 17, 18, and 19 in the activating region comprise a deoxyribonucleotide base.
3. The CRISPR guide molecule of claim 1, wherein ~~ten or less of positions 1, 3, 7, 10, 12, 14, 15, 16, 17, 18, and 19 in the activating region comprises~~ sequence selected from SEQ ID NOs.: 459-465 ~~deoxyribonucleotide bases.~~
4. The CRISPR guide molecule of claim 1, comprising one or more phosphorothioate ~~chemical modifications selected from the group consisting of base modifications including inosine, deoxy inosine, deoxy uracil, xanthosine, C3 spacer, 5-methyl dC, 5-hydroxybutynl 2'-deoxyuridine, 5-nitroindole, 5-methyl iso-deoxycytosine, iso-deoxyguanosine, deoxyuridine, iso-deoxycytidine, and an abasic site, and backbone modification, including a phosphorothioate modification.~~
56. The CRISPR guide molecule of claim 1, wherein the targeting region targets the B2M gene and comprises the RNA sequence AGUGGGGGUGAAUUCAGUGU (SEQ ID NO: 211), ~~wherein optionally, at least one of the bases in the sequence is replaced with a base analog or an abasic site.~~

- ~~6. The CRISPR guide molecule of claim 5, wherein one or more of positions 1, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, and 20 in the targeting region comprise a deoxyribonucleotide base.~~
- ~~7. The CRISPR guide molecule of claim 5, wherein five or less of positions 1, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, and 20 in the targeting region comprise deoxyribonucleotide bases.~~
- ~~8. The CRISPR guide molecule of claim 5, wherein the targeting region is capable of hybridizing to a sequence selected from SEQ ID NOs: 51-133.~~
97. The CRISPR guide molecule of claim ~~5-6~~ comprising a sequence selected from SEQ ID NOs: 212-231, 275-~~294~~, ~~296~~-315, and 331-~~334~~, and 346-350.
- ~~108.~~ The CRISPR guide molecule of claim ~~5-6~~ comprising the sequence of SEQ ID NO: 416.
- ~~119.~~ The CRISPR guide molecule of claim 1, wherein the targeting region targets the TRAC gene and comprises the RNA sequence GAGUCUCUCAGCUGGUACAC (SEQ ID NO: 232), wherein optionally, at least one of the bases in the sequence is replaced with a base analog or an abasic site.
- ~~12. The CRISPR guide molecule of claim 11, wherein one or more of positions 1, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, and 20 in the targeting region comprise a deoxyribonucleotide base.~~

- ~~13.~~ The CRISPR guide molecule of claim 11, wherein five or less of positions 1, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, and 20 in the targeting region comprise deoxyribonucleotide bases.
- ~~14.~~ The CRISPR guide molecule of claim 11, wherein the targeting region is capable of hybridizing to a sequence selected from SEQ ID NOs: 15-20.
- ~~15~~10. The CRISPR guide molecule of claim ~~11~~9 comprising a sequence selected from SEQ ID NOs: 233-252, 317-329, 331-334, 491-492, and 508.
- ~~16~~5. The CRISPR guide molecule of claim ~~11~~4, ~~further comprising a chemical modification, wherein the CRISPR guide molecule comprises~~ing a sequence selected from SEQ ID Nos: 512-517.
- ~~17~~11. The CRISPR guide molecule of claim ~~11~~9 comprising the sequence of SEQ ID NO: 415.
- ~~18~~12. The CRISPR guide molecule of claim 1, wherein the targeting region targets the CISH gene and comprises the RNA sequence~~is~~ capable of hybridizing to a human genomic sequence selected from SEQ ID NOs: 1567-165, wherein one or more of positions 1, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, and 20 in the targeting region comprise a deoxyribonucleotide base.
- ~~19.~~ The CRISPR guide molecule of claim 18, comprising the sequence selected from SEQ ID NO: 509, and ~~519-529~~.
- ~~20~~13. The CRISPR guide molecule of claim 1, wherein the targeting region targets the PDCD1 gene and comprises the RNA sequence~~is~~ capable of hybridizing to a human genomic sequence selected from SEQ ID NOs: ~~135~~134-155, wherein one or more of positions 1, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, and 20 in the targeting region comprise a deoxyribonucleotide base.

- ~~21~~14. The CRISPR guide molecule of claim 1, wherein the targeting region targets the CBLB gene and comprises the RNA sequence~~is~~ capable of hybridizing to a human genomic sequence selected from SEQ ID NOs: ~~467~~166-189, wherein one or more of positions 1, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, and 20 in the targeting region comprise a deoxyribonucleotide base.
- ~~22.~~ The CRISPR guide molecule of claim 21, comprising the sequence of SEQ ID NO: ~~510.~~
- ~~23~~15. A CRISPR nucleic acid/protein composition, comprising the CRISPR guide molecule of claim 1 and a Cas12 protein.
- ~~24~~16. The CRISPR nucleic acid/protein composition of claim ~~23~~15, wherein the Cas12 protein is a Cas12a protein comprising at the C-terminus, a linker- and a nuclear localization signal (NLS)-containing sequence having at least 80% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 479-490.
17. The CRISPR nucleic acid/protein composition of claim 16, wherein the Cas12a protein comprises an amino acid sequence of SEQ ID NOs: 1.
- ~~25~~21. A CAR-expressing cell produced by the method of claim 18, ~~comprising the CRISPR nucleic acid/protein composition of claim 1,~~ wherein the cell is selected from a lymphocyte, a ~~chimeric antigen receptor (CAR)-T cell, a T-cell receptor (TCR) cell, a TCR-engineered CAR-T cell, a tumor-infiltrating lymphocyte (TIL), a CAR TIL, a dendritic cell (DC), a CAR-DC, a macrophage, a CAR-macrophage (CAR-M), a natural killer (NK) cell, an induced pluripotent stem cell (iPSC), a cell differentiated from an iPSC cell,~~ an NK cell, or a CAR-NK cell.

2618. An *in vitro* method for producing a chimeric antigen receptor (CAR)-expressing cell, said method comprising:
- a) contacting a first target nucleic acid comprising a TRAC sequence in a cell with a first nucleoprotein complex of claim 15, wherein ~~comprising a catalytically active Cas12 protein and a first CRISPR guide molecule having a targeting region capable of binding the first target nucleic acid sequence; and an activating region capable of forming a nucleoprotein complex with the Cas12 protein, w~~ said CRISPR guide molecule comprises ribonucleotide bases and at least one deoxyribonucleotide base in the activating region, the targeting region, or both, ~~and the~~ first nucleoprotein complex is capable of cleaving the first target nucleic acid sequence;
 - b) contacting a second target nucleic acid sequence comprising a B2M sequence in the same cell with a second nucleoprotein complex of claim 15, wherein ~~comprising a catalytically active Cas12 protein and a second CRISPR guide molecule having a targeting region capable of binding the second target nucleic acid sequence; and an activating region capable of forming a nucleoprotein complex with the Cas12 protein, wherein said CRISPR guide molecule~~ comprises ribonucleotide bases and at least one deoxyribonucleotide base in the activating region, the targeting region, or both, ~~and the~~ second nucleoprotein complex is capable of cleaving the second target nucleic acid sequence;
 - c) providing a first donor polynucleotide encoding a CAR comprising an scFv, a transmembrane domain, a co-stimulatory domain, and an activating domain, wherein the CAR is capable of being inserted into the cleavage site ~~in~~ of the first target nucleic acid sequence;
 - d) providing a second donor polynucleotide encoding a B2M-HLA-E fusion construct comprising a B2M secretion signal, a HLA-G peptide signal sequence,

a first linker sequence, a B2M sequence, a second linker sequence, and a HLA-E sequence, wherein the B2M-HLA-E fusion construct is capable of being inserted into the cleavage site ~~in~~of the second target nucleic acid sequence;

- e) cleaving the first target nucleic acid sequence and inserting at least a portion of the first donor polynucleotide into the cleavage site; and
- f) cleaving the second target nucleic acid sequence and inserting at least a portion of the second donor polynucleotide into the cleavage site.

~~27. The method of claim 26, wherein the second donor polynucleotide further comprises a P2A sequence at the 5' end of the B2M-HLA-E fusion construct.~~

~~28. The method of claim 26, wherein the first donor polynucleotide comprises the SEQ ID NO: 413.~~

~~29~~19. The method of claim ~~26~~18, wherein the second donor polynucleotide comprises the SEQ ID NO: 414.

~~30. The method of claim 26, wherein the scFv in the CAR is capable of binding to a cellular target selected from the group consisting of a CD37, a CD38, a CD47, a CD73, a CD4, a CS1, a PD-L1, a NGFR, a ENPP3, a PSCA, a CD79B, a TACI, a VEGFR2, a B7-H3, a B7-H6, a B-cell maturation antigen (BCMA), a CD123, a CD138, a CD171/L1CAM, a CD19, a CD20, a CD22, a CD30, a CD33, a CD70, a CD371, a CEA, a Claudin 18.1, a Claudin 18.2, a CSPG4, a EGFRvIII, a EpCAM, a EphA2, a Epidermal growth factor receptor, a ErbB, a ErbB2 (HER2), a FAP, a)5I^Δa GD2, a GD3, a Glypican 3, a IL-11R α , a IL-13R α 2, a IL13 receptor alpha, a LewisY/LeY, a Mesothelin, a MUC1, a MUC16, a NKG2D ligands, a PD1, a PSMA, a ROR-1, a SLAMF7, a TAG72, a ULBP and a MICA/B proteins, a VEGF2, and a WT1.~~

~~31. The method of claim 26, wherein the scFv is capable of binding BCMA and comprises a first variable region comprising the amino acid sequence of SEQ ID NO: 474, a~~

- second variable region comprising the amino acid sequence of SEQ ID NO: 475, and a linker between the first and second variable regions comprising the amino acid sequence of SEQ ID NO: 476.
32. The method of claim 26, wherein the scFv comprises the amino acid sequence of SEQ ID NO: 477.
33. The method of claim 26, wherein the transmembrane domain of the CAR is derived from a T cell receptor α chain, a T cell receptor β chain, a CD3 ζ chain, a CD28, a CD3 ϵ , a CD45, a CD4, a CD5, a CD8, a CD9, a CD16, a CD22, a CD33, a CD37, a CD64, a CD80, a CD86, a CD134, a CD137, an ICOS, a CD154, or a GITR.
34. The method of claim 26, wherein the co-stimulatory domain of the CAR is derived from a CD28, a 4-1BB, a GITR, an ICOS-1, a CD27, an OX-40, or a DAP-10.
35. The method of claim 26, wherein the CAR comprises a transmembrane domain derived from a CD8, a 4-1BB co-stimulatory domain, or a CD28 co-stimulatory domain, and a CD3 ζ activating domain.
36. The method of claim 26, wherein the vector containing the CAR sequence comprises a leader sequence having the nucleic acid sequence of SEQ ID NO: 478.
37. The method of claim 26, wherein the catalytically active Cas12 protein comprises, at the C-terminus, a linker, and a nuclear localization signal (NLS)-containing sequence having at least 80% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 479-490.
38. The method of claim 26, further comprising:
- a. contacting a third target nucleic acid sequence in the same cell with a nucleoprotein complex comprising a catalytically active Cas12 protein and a third CRISPR guide molecule having a targeting region capable of binding the third target nucleic acid sequence; and an activating region capable of forming a nucleoprotein complex with the Cas12 protein, wherein said CRISPR guide

~~molecule comprises ribonucleotide bases and at least one deoxyribonucleotide base in the activating region, the targeting region, or both, and the nucleoprotein complex is capable of cleaving the third target nucleic acid sequence;~~

~~b. — cleaving the third target nucleic acid sequence and deleting one or more nucleotides from the third target nucleic acid sequence at the cleavage site, wherein the third target nucleic acid sequence is selected from a PDCD gene, a CISH gene, and a CBLB gene.~~

~~39. — The method of claim 26, wherein the CAR-expressing cell is an allogeneic or autologous CAR T cell produced from a T lymphocyte.~~

~~40. — A CAR-expressing cell produced by the method of claim 26, wherein the cell is selected from a lymphocyte, a CAR T cell, a TCR cell, a TCR engineered CAR T cell, a THL, a CAR THL, a dendritic cell, a CAR DC, a macrophage, a CAR M, an iPSC cell, a cell differentiated from an iPSC cell, an NK cell, or a CAR NK cell~~

~~41. — Use of the CAR-expressing cell of claim 21 in the preparation of a medicament for use in a method of adaptive-adoptive cell therapy, comprising administering to a subject in need thereof the CAR-expressing cell of claim 40.~~

~~42. — The method of claim 41, wherein the adaptive cell therapy comprises killing BCMA-positive cancer cells.~~

~~43. — The method of claim 42, wherein the BCMA-positive cancer cells comprise multiple myeloma cancer cells.~~